Selective activation of tumour necrosis factor receptor-mediated intracellular signalling pathways

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Summary

Tumour necrosis factor (TNF) is a pleiotropic cytokine that has been shown to play a major role in defence against infections and malignancy, and regulation of the innate and adaptive immune responses. Despite its beneficial role, the cytokine has been implicated in the pathophysiology of a range of diseases including sepsis, cerebral malaria and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. While blocking the activity of excessive TNF has become a therapeutic approach to managing patients with these diseases, there are concerns since this also decreases resistance against infection and cancer. Attempts to target intracellular signalling pathways used by TNF, such as the p38 mitogen activated protein kinase (MAPK) have also met with limitations and studies have been discontinued due to toxicity. Since most proteins exert their biological activity through the interaction between very small regions of their folded surfaces to their cognate receptors, smaller peptides which mimic the shape of the proteins at these points of contact with the receptors can be used to mimic and/or block the actions of these proteins. We have previously demonstrated that the TNF mimetic peptides TNF_{70-80} and $TNF_{132-150}$ exhibited distinct biological activities, which in combination represented the spectrum of biological activities displayed by TNF. Research in this thesis sought to use these properties to develop new targets for development of anti-inflammatory agents. The mimetic TNF_{70-80} was shown to bind and act as a ligand for the TNF receptor I (TNFRI) and selectively activated the p38 MAPK pathway, and not the c-Jun NH_2-terminal kinase (JNK) and extracellular-signal-regulated kinase 1 and 2 (ERK1/ERK2) pathways. In contrast $TNF_{132-150}$ selectively activated the JNK and ERK1/ERK2 pathways. This is consistent with the biological properties of

these peptides. The basis for the activation of a restricted signalling pathway by TNF_{70-80} was related to a reduced capability to recruit adapter proteins. The peptide mimetic ligated TNFR was able to functionally couple TNF receptor associated factor 2 (TRAF2) to the p38 and NF-κB pathway but was unable to effect the coupling of germinal centre kinase (GCK) and apoptosis signal-regulating kinase (ASK1) to TRAF2, probably explaining the lack of activation of JNK and ERK1/ERK2 pathways. Using the ability of TNF_{70-80} to activate p38, we identified the region to which TNF_{70-80} binds to the TNFRI. Synthetic peptides representing the 206-211 amino acid residues of the TNFRI were made and examined for anti-TNF effects *in vitro* and *in vivo*. These TNFR mimetic peptides were found to selectively block TNF induced p38 activation and associated functions of neutrophil superoxide production, CD11b upregulation and cytokine production. Similar results were found with the monocytic cell line, Mono Mac 6. These TNFRI-derived peptides were found to inhibit leukocyte infiltration into inflammatory sites in acute and chronic inflammation models. Our findings open new opportunities for the development of therapeutics which selectively target the TNFR-p38 signalling pathway in chronic inflammatory diseases.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Violet R.S. Mukaro Date

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Publications, presentations and awards

Publications

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2. Marantos, C., V. Mukaro, J. Ferrante, C. Hii, and A. Ferrante. 2008. Inhibition of the Lipopolysaccharide-Induced Stimulation of the Members of the MAPK family in Human Monocytes/Macrophages by 4-Hydroxynonenal, a Product of Oxidized Omega-6 Fatty Acids. Am J Pathol. 173(4):1057-66.

3. Mukaro, V., X. Gao, C. Haddad, G. Mayne, H. Sundqvist, R. Flower, Z. H Huang, C. S. T. Hii, and A. Ferrante. 2008. Selective signaling via p38 MAP kinase by the TNF peptide mimetic TNF_{70-80} involving the TNF receptor. In preparation.

4. Yeh, M, V. Mukaro, C. Marantos, C. Hii and A. Ferrante. 2008. Regulation of neutrophil chemotaxis and phagocytosis by c-jun NH2 terminal kinases. Submitted.

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Abbreviations

ACTH	adenocortiotropic hormone
ADAM	a disintegrin and matrix metalloprotease domains
APC	antigen presenting cells
AREs	adenosine-uridine rich elements
ASK1	apoptosis signal-regulating kinase
A-SMase	acid sphingomyelinase
ATF	activation transcription factors
BAL	bronchoalveolar lavage
CAMS	cell adhesion molecules
CAPK	ceramide-activated protein kinase
cIAP	cellular inhibitor of apoptosis protein
CINC	cytokine-induced neutrophil chemoattractant
CM	cerebral malaria
COPD	chronic obstructive pulmonary disease
COX	cyclo-oxygenases
CR	complement receptor
CRD	cysteine rich domains
DC	dendritic cells
DC-SIGN	DC specific-ICAM-3-grabbing non-integrin
DD	death domain
DED	death effector domain
DISC	death-inducing signal complex
DMEM	Dulbecco's Modified Eagle's Medium

DTH	delayed-type hypersensitivity
EPO	erythropoietin
ERK	extracellular-signal-regulated kinases
FAD	flavin adenine nucleotide
FADD	Fas-associated death domain
FAN	factor associated with N-SMase activation
FCS	foetal calf serum
FLICE	FADD-like ICE
fMLP	formyl-methionine-leucine-phenylalanine
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCK	germinal centre kinase
GH	growth hormone
GM-CSF	granulocyte-macrophage colony stimulating factor
GROα	growth-related gene product
HBSS	Hanks' Balanced salt solution
HF	heart failure
HSP	heat shock proteins
HUVECs	human umbilical vein endothelial cells
ICAM	intracellular-adhesion-molecule
IFNγ	interferon gamma
IgG	immunoglobulins
IκB	inhibitor kappa B
IKK	IκB kinase

IL	interleukin
IP	interferon-γ-inducible protein
ITAMS	immunoreceptor tyrosine-based activation motifs
JNK	c-Jun NH_2-terminal kinase
KC	keratinocyte chemoattractant
LIF	leukaemia inhibitory factor
LOX	lipoxygenases
LPS	lipopolysaccharide
LT	lymphotoxin
LTBI	latent tuberculosis infection
MAPK	mitogen activated protein kinase
MCP	monocyte chemotactic protein
M-CSF	macrophage CSF
MEF2C	myocyte enhancing factor 2C
MEKK1	MAPK kinase kinase
MHC	major histocompatability complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MNL	mononuclear cells
MPO	myeloperoxidase
MPS	mononuclear phagocyte system
mTNF	membrane bound TNF
mTOR	mammalian target of rapamycin
NADPH	nicotinamide adenine nucleotide phosphate

NEMO	NF-κB essential modulator
NF-κB	nuclear factor kappa B
NIK	NF-κB inducing kinase
NK	natural killer cells
NOS	nitric oxide synthase
NSD	neutral sphingomyelinase domain
N-SMase	neutral sphingomyelinase
PAMPs	pathogen-associated molecular patterns
PDK1	phosphoinositide dependent kinase
PECAM-1	platelet endothelial adhesion molecule-1
PI	3'-phosphoinositide
PI3K	phosphatidylinositol-3-kinase
PLAD	pre-ligand assembly domain
PRR	pattern recognition receptors
RA	rheumatoid arthritis
RIP	receptor interacting protein
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
S1P	sphingosine-1-phosphate
SLE	systemic lupus erythematosus
SMase	sphingomyelinase
SODD	silencer of death domain
SphK	sphingosine kinases
SRBC	sheep red blood cell

STAT	signal transduction and activators of transcription
T regs	regulatory T cells
TACE	TNFα converting enzyme
TANK	TRAF-associated NF-κB activator
TGFα	transforming growth factor alpha
TLR	toll-like receptors
TMB	3',3',5',5'-tetramethylbenzidine
TNF	tumour necrosis factor
TNF-RM	TNF rich medium
TNFRI	TNF receptor I
TNFRII	TNF receptor II
TPO	thrombopoietin
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor associated factor 2
TSH	thyroid stimulating hormone
VEGF	vascular endothelial growth factor

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1.0 CHAPTER ONE: INTRODUCTION

1.1 GENERAL INTRODUCTION

The immune system operates as a network of organs, tissues, cells and molecules strategically positioned or deployed throughout the body, to protect against infection and cancer. Its intricate properties enable discrimination between self and non-self antigenic structures. The cellular components of the immune system communicate in this network through direct cell-to-cell interaction and by synthesising a variety of molecules, including immunoglobulins, complement proteins, cytokines, growth factors and lipid mediators which act on cellular receptors to orchestrate the immune response and inflammation required to eliminate foreign matter from body tissues.

These immunological networks operate as innate immunity and adaptive immunity. In innate immunity, the local tissue cells are perturbed by exogenous mediators of bacterial origin as well as endogenous mediators resulting from tissue damage and cell activation (Figure 1.1). The innate immune reaction may be precipitated through the activation of complement, pattern recognition systems and toll-like receptors (Gasque, 2004, Salaun *et al.*, 2007). Interaction with this recognition system leads to cell activation and the release of chemokines for attracting and activating neutrophils and monocytes, leading to the release of a more complex and intense cytokine networks, as well as oxygen derived radicals and tissue damaging enzymes (Figure 1.1) (Brown and Gordon, 2005, Ferrante, 2005).

The innate immune response provides an opportunity to the immune system to become sensitised as part of the adaptive immune response. As neutrophils engulf and degrade microbial pathogens and altered tissues the cells become a source of antigens for antigen presenting cells (APC) to process and present to lymphocytes. Neutrophils themselves also generate chemokines and leukocyte activators, promoting infiltration and activation of monocytes and dendritic cells (DC) (Chertov *et al.*, 1997, Bennouna *et al.*, 2003, Nathan, 2006) (Figure 1.1). As antigens are processed and expressed on major histocompatability complex (MHC) class II by APC they engage the T cell receptors on CD4+ helper T lymphocytes. Other surface molecules on APC provide co-stimulatory signals to T cells. Consequently, the T cells are activated resulting in their expansion which, amongst many properties have the ability to help in B cell responses and antibody production. The adaptive immune response has a high level of specificity, with key regulating arms and a more complex and intricate cellular and molecular network (Brown and Gordon, 2005).

The types of leukocytes that predominate in the reactions, to some extent, classifies the inflammatory reactions and may be based on the content of T cells, B cells, macrophages, natural killer cells, neutrophils, eosinophils or basophils/mast cells of varying proportions. Endogenous mediators such as cytokines and chemokines, which form a network of intercellular signalling molecules, regulate the migration of these cells into inflammatory sites and their function. These molecules thereby control the characteristics of such inflammatory reactions. An acute inflammatory reaction is generally of a rapid onset and short lived (minutes, several hours or a few days). Its main

features are the exudation of fluid and the infiltration of leukocytes, predominantly neutrophils.

In contrast, when an acute inflammation is not resolved, a persistent and prolonged inflammation ensues, chronic inflammation. It is characterised by the presence of lymphocytes and macrophages, the proliferation of blood vessels, fibrosis and tissue necrosis. The reaction persists for weeks, and years (Chaplin, 2003). The body can also be subjected to an autoimmune inflammatory response in which the defence mechanisms break down and the immune system cannot distinguish self from non-self, resulting in major inflammatory conditions such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), and manifested by the destruction of key organs/tissues as in type I diabetes (Kurien and Scofield, 2008).

The intercellular mediator interactions stimulate a variety of intracellular signalling pathways. The pattern of signals activated may also be considered as elements of the type of inflammation operating. Their activation and importance is also likely to be a reflection of the cell type infiltrating the infection site. For example, of the mitogen-activated protein kinase (MAPK) family, JNK, ERK1/ERK2 and p38, only the latter is activated in neutrophils under the influence of inflammatory mediators (Waterman *et al.*, 1996, Guo *et al.*, 1998, Zu *et al.*, 1998).

Innate and chronic inflammation are characterised by quantitative and qualitative differing cytokine networks. However, at the “heart” of the inflammatory reaction there are some key mediators and pathways which dictate the events, and in some cases in both

the innate and adaptive immune responses. Tumour necrosis factor (TNF) is one of these molecules of such interest. The effects of TNF are mediated by specific cell surface receptors, TNF receptor I (TNFRI) and TNFRII, with the former being responsible for the majority of its biological actions (Tartaglia *et al.,* 1993). TNF has been shown to play a major role in the body's defence against infections due to a wide range of microbial pathogens of viral, bacterial or parasitic origin and malignancy (Haranaka *et al.*, 1986, Mestan *et al.*, 1986, Nakane *et al.*, 1988). At present, the ability to exploit the biological properties of TNF for therapeutic purposes is impeded by its highly toxic nature and multiple biological actions. Despite its beneficial role, the cytokine has been implicated in the pathogenesis of sepsis, cerebral malaria and autoimmune diseases such as RA and multiple sclerosis (Tracey *et al.*, 1988, Grau *et al.*, 1989, Kwiatkowski *et al.*, 1993, Hohlfeld, 1996). Thus blocking the activity of excessive TNF has become a therapeutic goal in managing patients with some of these diseases (Feldmann and Maini, 2001, Taylor, 2003). However, studies have shown potential risk for worsening heart failure and increased risk of skin infections, lymphoma and reactivation of latent tuberculosis and other infections caused by intracellular microbes in patients undergoing anti-TNF therapy (Anker and Coats, 2002, Coletta *et al.*, 2002, Mann *et al.*, 2004, Dixon *et al.*, 2006).

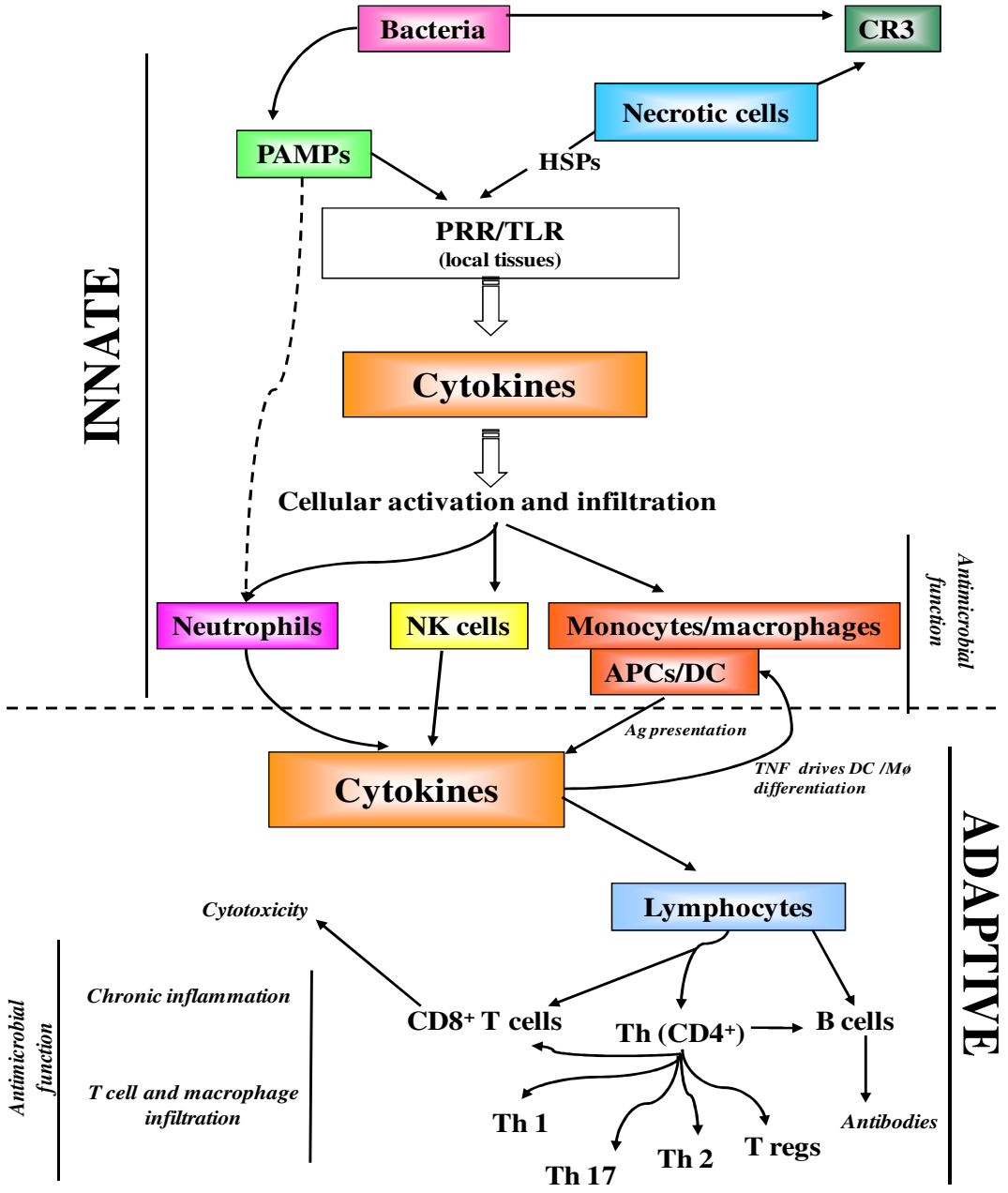

Figure 1.1 Relationship between innate and adaptive immunity. Abbreviations: APC, antigen presenting cells; CR3, complement receptor 3; DC, dendritic cells; HSP, heat shock proteins; NK, natural killer cells; PAMPs, pathogen-associated molecular patterns; PRR, pattern recognition receptors; T regs, regulatory T cells; TLR, toll-like receptors.

1.2 NEUTROPHILS AND INFLAMMATION

The neutrophil plays a pivotal role as the body's "first line of defence", phagocytosing bacteria, fungi, protozoa, viruses, virally infected cells and tumour cells (Smith, 1994, Witko-Sarsat *et al.*, 2000, Ferrante, 2005). Critical in the objective of containing infection early is the rapid response of the neutrophil to the infectious environment. Using a regulated set of functions of adhesion, extravasation from blood vessels, chemotaxis, phagocytosis, oxygen radical generation and degranulation the cell can effectively deal with such microbial pathogens. A defect in these key functions can ultimately lead to a failure in resolving infections and risk of death (Ferrante, 2005).

The neutrophil responds to infection by expressing a co-ordinated array of biophysical and biochemical responses. Products of microbial origin such as the lipopolysaccharide (LPS) of the bacterial cell wall and bacterial peptides act either directly or indirectly to stimulate production of pro-inflammatory cytokines including TNF, interleukin-1 (IL-1), IL-6 and IL-8 by local tissues, endothelial cells and leukocytes (Witko-Sarsat *et al.*, 2000). These mediators promote the local adherence of neutrophils to the endothelium, diapedesis and migration of cells via electrostatic linkage of L-selectin molecules with endothelial carbohydrate ligands (Ley, 2002).

As neutrophils migrate into the sites of infections, they become primed as a result of their interaction with mediators such as TNF making these cells respond to bacteria much more efficiently (Ferrante *et al.*, 1993). Neutrophils exposed to adhesion cell molecules

on the endothelium and chemotactic factors, generated locally, cause the rolling neutrophils to become stationary. These induce the expression of neutrophil β2-integrins which have high-affinity interactions with intracellular adhesion molecule-1 (ICAM-1) expressed on endothelial cells (Gahmberg, 1997, Witko-Sarsat *et al.*, 2000). The diffusion of chemoattractants (IL-8) from infection sites induces the expression of platelet endothelial adhesion molecule-1 (PECAM-1) or CD31 on the surfaces of both the neutrophil and endothelial cell junctions (Carlos and Harlan, 1990, Carlos and Harlan, 1994). The neutrophils are signalled to increase their CD11/CD18 binding capacity, to manoeuvre through the endothelial layer by diapedesis (Carlos and Harlan, 1994), and then migrate towards the site of infection via a gradient of chemoattractants. These chemoattractants include the complement component, C5a, TNF, IL-8 and the bacterial tripeptide, formyl-methionine-leucine-phenylalanine (fMLP) (Burg and Pillinger, 2001), which interact with cell surface receptors to signal contractile microfilaments that orient and direct the neutrophils through tissues. Eventually, they adhere to extracellular matrix components such as laminin and fibronectin and accumulate at the infection site (Vaday and Lider, 2000).

Lack of neutrophils is not compatible with life. In severe neutropenia, life-threatening infections are experienced. There have been several lines of evidence, which show that neutrophils are important in resistance to microbial pathogens. Depletion of neutrophils in animals leads to increased susceptibility to bacterial, fungal and parasitic infections. Using a granulocyte-specific antibody RB6-8C5, studies have shown that neutrophils are essential in the clearance of both extracellular microbial pathogens such as *Escherichia coli* (Haraoka *et al.,* 1999) and intracellular bacteria such as *Listeria monocytogenes*

(Czuprynski *et al.,* 1994). There is also evidence that in a number of inflammatory diseases neutrophils play a key role in the pathogenesis of these conditions. Thus, depletion of neutrophils leads to protection against diseases such as collagen induced arthritis and atherogenesis in mice (Eliason *et al.*, 2005, Tanaka *et al.*, 2006, Zernecke *et al.*, 2008).

1.2.1 Functional cell surface receptors and phagocytosis

Recognition of foreign matter and altered tissues by neutrophils occurs through well defined receptors (Table 1.1) (Ferrante, 2005). These include immunoglobulin G (IgG) Fcγ receptors: FcγRI (CD64) FcγRIIA (CD32) and FcγRIIIB (CD16) which function to recognise the Fc domain of IgG (Ferrante, 2005). The importance of FcγRI is unclear as it is only expressed following pre-exposure to interferon-γ (IFNγ), which suggests a role in activation and priming as is evident in macrophages (Ferrante, 1992). Neutrophils also have an FcαR receptor which recognises the Fc domain on IgA and considered to be important for phagocytosis and activation of the neutrophil respiratory burst (Ferrante, 2005). The complement receptors: CR1 (CD35), CR3 (CD11b/CD18) and CR4 (Cd11c/CD18) contribute to the phagocytic process by binding to complement components that are released during inflammation and act as opsonins (Witko-Sarsat *et al.*, 2000, Ferrante, 2005). Neutrophils also express numerous pattern recognition receptors (PRR) that facilitate identification of invading microbial pathogens by binding/recognising highly conserved germline encoded patterns known as pathogen-

associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997, Moller *et al.*, 2005). The type 1 transmembrane toll-like receptors (TLR) are PRRs that play an important role in innate immune recognition of microbial pathogens (Hayashi *et al.,* 2003). Neutrophils have been shown to express TLR1, 2, 4, 5, 6, 7, 8, 9, and 10 i.e. all the TLRs except TLR3 (Hayashi *et al.*, 2003). Neutrophils also express various cytokine receptors such as GM-CSF and TNF receptors that serve to modulate neutrophil responses. Lastly, neutrophils express two main chemokine receptors on their surfaces: CXCR1 and CXCR2. CXCR2 is thought to be predominantly responsible for neutrophil recruitment in response to its many ligands such as IL-8, while CXCR1 is thought to be involved in activation (Sabroe *et al.*, 2005, Tarlowe *et al.*, 2005)

Phagocytosis of complement and IgG opsonised particles occurs via two distinct processes. The ingestion of IgG coated targets is promoted by FcγRII receptors and leads to the phosphorylation of their cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMS) via the activation of Src-tyrosine kinases (Greenberg *et al.,* 1996). The phosphorylation of ITAMS triggers several downstream signalling pathways, including to the activation of the small G-protein, Rho. This results in membrane protrusions extending over the surface of the opsonised particle to form a "phagocytic cup" which engulfs the particle (Greenberg *et al.*, 1996, Massol *et al.*, 1998). Phagocytosis of complement opsonised targets involves a different process, whereby targets "sink" into the neutrophil, producing very little protrusions (Greenberg and Grinstein, 2002). However, in both processes the neutrophil plasma membrane invaginates into a phagosome, trapping the organism and facilitating the subsequent release of anti-microbial substances into phagolysosome, preventing host cell damage.

The formation of a phagolysosome during phagocytosis creates a microenvironment in which neutrophils can release their highly toxic granules against pathogens. This prevents release of the contents into the proximal extracellular environment. However, it is also evident that this process is not properly regulated during some inflammatory conditions such as the inflamed joints of patients with RA. Tissue damage occurs as a result of frustrated phagocytosis of neutrophils binding to surfaces coated with immune complexes and activated complement components (Witko-Sarsat *et al.*, 2000).

1.2.2 Neutrophil microbicidal mechanisms

When neutrophils re-localise to sites of infection, the cells are stimulated to release a wide range of anti-microbial substances; which can be divided into oxidative or non-oxidative mechanisms (Ferrante, 2005). These responses are coordinated with the event of phagocytosis of microbial pathogens enabling the release of toxic substances within the phagosome.

Table 1.1 Neutrophil functional receptors

Receptors	*Function/responses*
FcR (FcγRI, FcγRIIA FcγRIIIB)	Bind to exposed Fc domains of Ig on opsonised microbial pathogens
CR (CR1, CR3, CR4)	Bind to complement components on opsonised microbial pathogens
Chemokine receptors (CXCR1, CXCR2)	Allow chemokines to bind and recruitment of neutrophils to inflammatory sites
Cytokine receptors (TNFRI, IL-1R)	Allow cytokines such as TNF, IL-1β to bind resulting in degranulation and release of antimicrobial and inflammatory mediators
PRR	Facilitate identification of invading microbial pathogens and ultimately promote resolution of disease
TLR (TLR1, 2, 4, 5, 6, 7, 8, 9, 10)	Detect the presence of a pathogen by recognising microbe-associated molecular patterns

Adapted from (Witko-Sarsat *et al.*, 2000, Hayashi *et al.*, 2003, Ferrante, 2005). Abbreviations: CR, complement receptors; IL-1R, interleukin 1 receptor; PRR, pattern recognition receptor; TLR, toll-like receptor; TNFRI, tumour necrosis factor receptor I.

1.2.2.1 Non-oxidative mechanisms

In non-oxidative microbicidal mechanisms, lysosomes (azurophilic or primary granules) fuse with the plasma membrane of the phagosome and degranulate, releasing an array of cytotoxic peptides and proteolytic enzymes into the phagosome (Smith, 1994). The process begins with the release of the peroxidase-negative lactoferrin, lipocalin, lysozyme, matrix metalloproteinase (MMP) 8, 9 and 25 as they degranulate (Table 1.2). MMP are known to play an important role in facilitating neutrophil recruitment and tissue breakdown (Faurschou and Borregaard, 2003, Nathan, 2006). This release is accompanied by the degranulation of the peroxidase-positive (azurophilic) primary granules. These azurophilic granules contain defensins and proteases such as elastase and cathespin G (Smith, 1994, Witko-Sarsat *et al.*, 2000, Nathan, 2006) which contribute to the microbial killing. Defensins, which account for 30-50 % of the granule protein are small potent antimicrobial peptides that are cytotoxic to a broad range of bacteria, fungi and viruses (Smith, 1994). They act in synergy with bactericidal permeability-increasing protein (BPI), which renders bacterial cell membranes more permeable, particularly gram-negative bacteria (Burg and Pillinger, 2001).

1.2.3 Oxygen dependent mechanisms

In association with degranulation, the oxidative microbicidal mechanism is also initiated by the consumption of molecular oxygen (O_2) and the production of toxic oxygen-

derived radicals, which oxidise the lipid, protein and nucleic acid components of phagocytosed organisms (Heyworth *et al.,* 1998). The consumption of oxygen and generation of oxygen reactive species (ROS) is referred to as the respiratory burst. It is a key event during neutrophil activation and is mediated and catalysed by the action of a plasma membrane enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Figure 1.2). This enzyme is composed of a number of cytoplasmic proteins and a plasma membrane localised cytochrome b_{558} protein, which are assembled on the plasma membrane. Cytochrome b_{558} itself consists of two subunits: $p22^{phox}$ (p, protein; phox, phagocyte oxidase) and $gp91^{phox}$ (gp-glycoprotein) in association with rap1A. The $gp91^{phox}$ contains prosthetic haeme groups and flavin adenine nucleotide (FAD) binding sites as well as NADPH binding sites (Cross and Segal, 2004). Activation of the oxidase occurs when phagocyte receptors are engaged by opsonised particles (Goldblatt and Thrasher, 2000). It is only on activation that $p22^{phox}$ and $gp91^{phox}$ fuse with the plasma membrane- resulting in the formation of the cytochrome b_{558} complex.

Table 1.2 Neutrophil granules and constituents

Azurophilic/primary granules	Specific/secondary granules	Tertiary granules	Secretory vesicles
Myeloperoxidase	Lysozyme	Gelatinase	CR1
β-Glucoronidase	Lactoferrin		Albumin
Elastase	Vitamin B_{12}-binding protein		
Cathespin G	Receptors- CR3		
Defensins	Components of the NADPH oxidase		
BPI	Collagenase		
Lysozyme	Gelatinase		

Adapted from (Witko-Sarsat *et al.*, 2000, Ferrante, 2005). Abbreviations: BPI, bactericidal/permeability increasing protein; CR, complement receptor; fMLP, formyl-methionine-leucine-phenylalanine; NADPH, nicotinamide adenine dinucleotide phosphate.

The NADPH oxidase also contains cytosolic proteins- $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$, which are translocated to the plasma membrane and bind to the cytochrome b_{558} at the plasma membrane, thus forming the active oxidase (Figure 1.2) (Nathan, 1987, Heyworth *et al.*, 1998). Another cytoplasmic component rac2, a small G-protein, is also required for the assembly of the active NADPH oxidase. Although the majority of the $p22^{phox}$ and $gp91^{phox}$ is localised in the membrane of specific granules and of secretory vesicles, due to constant cell turnover, formylated mitochondrial proteins from damaged tissue can act as a source of endogenous chemoattractants (Le *et al.,* 2002). This may result in some $p22^{phox}$ and $gp91^{phox}$ being expressed on the plasma membrane without phagocytosis.

The activation of NADPH enzymatic complex allows it to generate superoxide anion (O_2^-) following the phagocytic process. The O_2^- produced is then used as a substrate for a series of enzymes such as superoxide dismutase which result in the generation of H_2O_2 and hydroxyl radicals (OH•) during the reduction of O_2 to H_2O_2 (Britigan *et al.,* 1986). The generation of O_2^- also acts as a starting point for the production of other reactive oxidants including halogenated oxidants through the myeloperoxidase (MPO) pathway. The major product of this pathway is hypochlorous acid (HOCl) (Witko-Sarsat *et al.*, 2000).

Recently, a major mechanism of microbial killing has been described which highlighted the importance of H_2O_2, involving the transport of H^+ and K^+ into the cell to promote the activity of elastase which then destroys the bacteria (Reeves *et al.,* 2002). Their studies suggest that the antimicrobial action inside the phagolysosome is not due to the high concentration of ROS only but rather due to the influx of H^+ and K^+ to counteract the

anions (ROS), this increase in ionic strength causes the release of cationic proteins, elastase and cathespin G which consequently destroy the bacteria. Using mice lacking elastase and cathespin G, they showed that these animals were ineffective at clearing *Staphylococcus. aureus* and *Candida albicans* (Reeves *et al.*, 2002). A commentary by Bokoch, 2002, suggests that MPO might play a role in the buffering of proteases to protect them from oxidation from H_2O_2 as opposed to a direct role in the killing of microbial pathogens (Bokoch, 2002).

1.2.4 Neutrophils as a source of cytokines and chemokines

Recent interest has also focussed on the finding that neutrophils are capable of producing a variety of cytokines (IL-1β, IL-1Ra, IL-12 and TNF), (Table 1.3) and chemokines such as IL-8, growth-related gene product, macrophage inflammatory protein (MIP)-1α, MIP1β and interferon-γ-inducible protein (IP-10) (Table 1.3) (Cassatella, 1999, Ethuin, 2005). These chemokines primarily act as chemotactic factors for neutrophils, monocytes, immature dendritic cells (DC), and T-lymphocyte subsets (Ludwig *et al.,* 2006). The cytokines such as IL-1β and TNF are primarily inflammatory and cell activating. In this manner neutrophils have the ability to contribute to cytokine levels during physiologic and pathophysiologic processes. This may play an important role in innate immunity and acute inflammatory conditions (Feldmann *et al.*, 1996, Kmiec, 1998, Taylor, 2003).

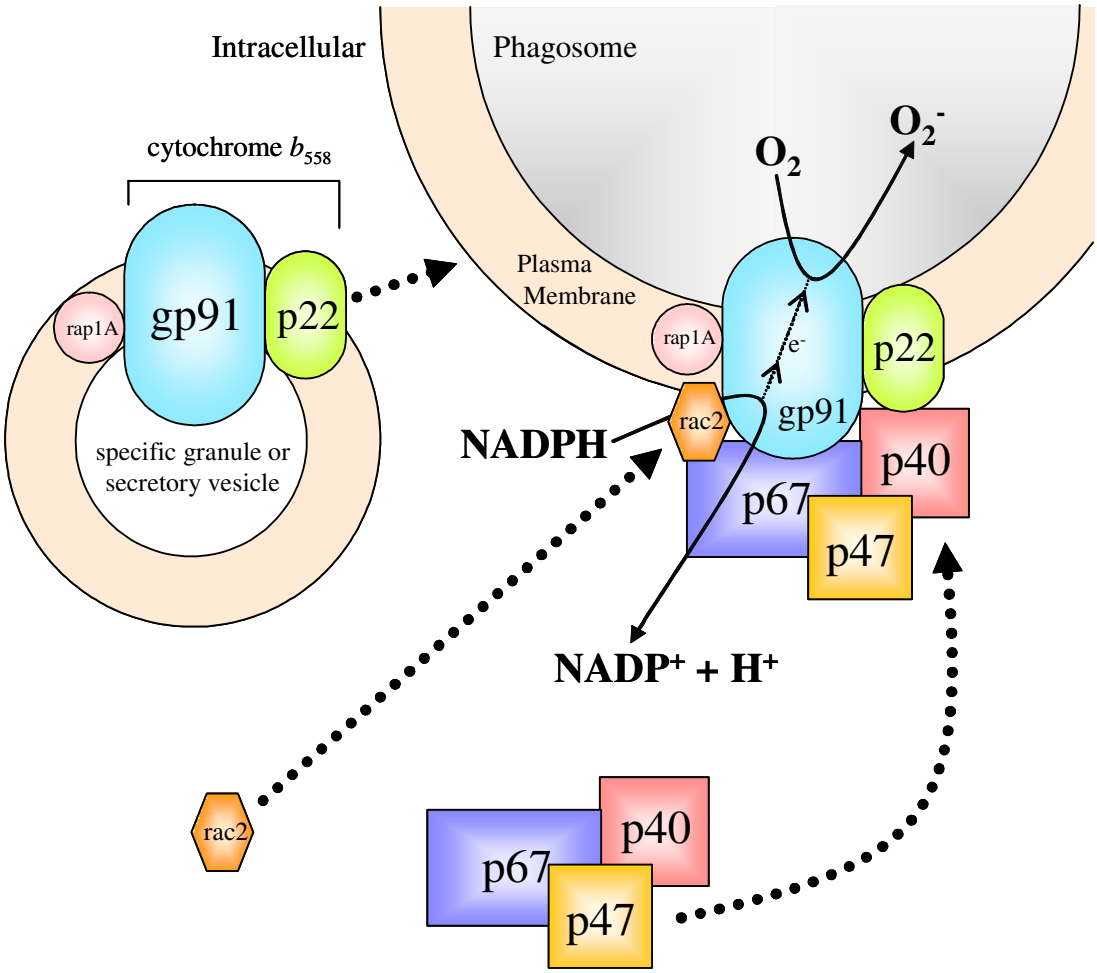

Figure 1.2 The components and activation of the NADPH oxidase. In resting neutrophils, the cytochrome b_{558} subunits $p22^{phox}$ and $gp91^{phox}$, in association with rap1A, are located in the membranes of specific granules and secretory vesicles. Phagocytosis leads to the formation of a phagosome and fusion of these subunits in the plasma membrane. The p40-p47-$p67^{phox}$ cytosolic complex translocates to the plasma membrane and forms a complex with cytochrome b_{558} to complete the NADPH oxidase. The release of electrons into the cytosol ultimately results in it attaching to molecular oxygen present in the phagosome, reducing it to the superoxide anion (O_2^-) (adapted from (Roos *et al.*, 1996, Heyworth *et al.*, 2003).

Cytokine release by neutrophils occurs via secretion of pre-synthesised stores of cytokines e.g. vascular endothelial growth factor (VEGF) and shedding of membrane-bound cytokine such as the release of TNF under the action of TNFα converting enzyme (TACE) (Ethuin, 2005). In addition, *de novo* protein synthesis such as the production of IL-8 mRNA.

In the first instance the rapid release of cytokines via degranulation may be a significant contribution in the first line of defence in cellular activation for microbial pathogen elimination (Kasama *et al.,* 2005), as well as influencing the processing and presentation of antigen to lymphocytes and thus contributing to the nature of adaptive immune response (Megiovanni *et al.,* 2006). In chronic inflammation, neutrophils become prominent in the exacerbation phases of diseases such as RA and atherosclerosis (Gelderman *et al.*, 1998, Lefkowitz and Lefkowitz, 2001). The neutrophils may hence be a source of the pathogenesis-mediated processes in these chronic illnesses (Nambi, 2005, Bathoorn *et al.*, 2008)

1.2.5 Role in adaptive immunity

Although neutrophils are primarily recognised for their role in innate immunity, in recent years there has been increasing evidence for their role in adaptive immunity. Currently, there are three mechanisms by which neutrophils form a bridge between innate and adaptive immunity. Firstly, neutrophils have been shown to produce chemokines that

attract DC and T cells to sites of inflammation and mediators that promote the cells' adaptive immune responses (Ludwig *et al.*, 2006). Neutrophils can also migrate to local lymph nodes where they undergo apoptosis allowing DC to present neutrophil-derived antigens to T cells. Secondly neutrophils have been shown to acquire antigen-presenting function (Ferrante *et al.,* 2007); and have even been reported to express T cell receptors based on the variable immunoreceptor (Puellmann *et al.,* 2006). The interaction of neutrophils and DC during infection results in neutrophils inducing maturation of DC. This process is mediated by TNF and the cellular contact is regulated by receptors such as the neutrophil CD11b/CD18 and the C-type lectin receptor such as DC specific intracellular-adhesion-molecule 3-grabbing non-integrin (DC-SIGN) (Ludwig *et al.*, 2006). Megiovanni et al. (2006) demonstrated through co-culture of neutrophils and immature DC, that neutrophils were able to directly transfer *Candida albicans* antigens to DC allowing them to stimulate sensitised T cells to produce IL-2 and IFNγ (Megiovanni *et al.*, 2006).

Table 1.3 Cytokines produced by neutrophils

NOTE:
This table is included on page 21
of the print copy of the thesis held in
the University of Adelaide Library.

Adapted from (Cassatella, 1999). Abbreviations: CINC, cytokine-induced neutrophil chemoattractant; G-CSF, granulocyte colony-stimulating-factor; GROα, growth-related gene product; IP, interferon-γ-inducible protein; KC, keratinocyte chemoattractant (murine equivalent of GROα MCP, monocyte chemotactic protein; M-CSF, macrophage CSF; TGFα, transforming growth factor; VEGF, vascular endothelial growth factor.

1.3 MONOCYTES/MACROPHAGES

Macrophages belong to the mononuclear phagocyte system (MPS) which consists of committed myeloid progenitor cells from the bone marrow that differentiate to form blood monocytes, circulate in the blood and consequently enter tissues to become resident tissue macrophages (Hume *et al.,* 2002) (Figure 1.3). Proliferation and differentiation into monocytes is dependent on the presence of lineage determining cytokines such as colony-stimulating 1 (CSF-1 also known as macrophage colony-stimulating factor), and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as on interactions with stroma in haematopoietic organs.

During inflammation the first wave of neutrophil migration into tissues is followed by a second wave of monocytes, typically occurring several hours after the initial inflammatory insult, but persists in chronic inflammation when neutrophils are no longer present (Schmid-Schonbein, 2006). Monocytes are more closely related to neutrophils in regards to their anti-microbial systems. As such, monocytes respond to stimuli with a brisk respiratory burst as in neutrophils, though its magnitude is much less than that of neutrophils, which is further diminished in resident macrophages (Kumaratilake and Ferrante, 1988). Recently it has been shown that the transcriptomes of macrophages and neutrophils are clearly very similar. *In vitro* experiments have shown that in response to various stimuli, granulocytes can be induced to adopt macrophage and DC-like phenotypes (Araki *et al.*, 2004, Lindemann *et al.*, 2004). This inter-conversion while

likely, opposes the accepted dogma of neutrophils rapidly infiltrating, then dying/removed and replaced by monocytes (Hume, 2006).

1.3.1 Origin and activation of macrophages

Macrophages have three major functions, which can be broadly classified as phagocytosis, antigen presentation, and immunomodulation. These functions have been further classified and attributed to the different subsets of macrophages, which have developed as a result of different local stimuli (Figure 1.3).

a) Classically activated macrophages (M1) develop as a result of stimulation with either IFN-γ or TNF or upon recognition of PPAR such as LPS, bacterial dsRNA and other microbial products. This activation leads to increased production of NO and ROS, thus facilitating their microbicidal and tumoricidal activities (Gordon, 2003). As this subset of macrophages contains ingested microbial products they have an important function as APC inducing Th1 adaptive immune responses by releasing cytokines such as, IL-1, IL-6, TNF, type I IFNs (IFNα/β), IL-10, IL-12 and IL-18 (Fujiwara and Kobayashi, 2005, Van Ginderachter *et al.*, 2008).
b) Alternatively activated macrophages (M2a) develop from stimulation with IL-4 and/or IL-13 and tend to have an increased expression of mannose and scavenger receptors and decreased inflammatory cytokine production (Gordon, 2003). These macrophages have low NO production and are poor APC, but the presence of receptors allows them to phagocytose debris, aid in wound healing and promote

Th2 responses by a high production of IL-10 and low production of IL-2 (Gordon, 2003, Fujiwara and Kobayashi, 2005, Van Ginderachter *et al.*, 2008).

c) Type II activated macrophages (M2b) differentiate from immature macrophages subsequent to the ligation to Fcγ receptors, TLR, CD40 or CD44 (van der Bij *et al.*, 2005, Van Ginderachter *et al.*, 2008). M2b macrophages are less cytotoxic and tend to promote the production of Th2 cytokine and antibody production (Anderson and Mosser, 2002).

d) Deactivated macrophages (M2c) develop following stimulation with IL-10, TGF-β, and glucocorticosteroids or after phagocytosis of apoptotic cells. MHC class I and II expression is downregulated, while cytokine production is skewed towards and anti-inflammatory profile thus, downregulating inflammation (Gordon, 2003).

NOTE:
This figure is included on page 25 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.3 Origin and activation of macrophages. Myeloid progenitor cells undergo differentiation into monocytes/macrophages after stimulation with GM-CSF, CSF-1 and IL-3. The presence of diverse environmental stimuli leads to differentiation into different macrophage subsets with dissimilar functional characteristics. Adapted from (van der Bij *et al.*, 2005). Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; CSF, colony-stimulating factor; IL-13, interleukin 13.

1.3.2 Functional surface receptors on mononuclear phagocytes

Mononuclear phagocytes like neutrophils express similar receptors that aid in the recognition of microbial pathogens. Macrophages express Fc receptors that are known to be important for mediating endocytosis, antibody-dependent cellular cytotoxicity and triggering the release of inflammatory mediators (Mellman *et al.,* 1988). Like neutrophils, macrophages similarly express PRRs that recognise conserved microbial structures such as PAMP, LPS of gram-negative bacteria and β-glucan of fungi. The TLRs in macrophages unlike the PRRs that are involved in the binding and internalisation of microbes are able to generate appropriate responses by activating transcription factors and leading to the generation of inflammatory mediators (Brown and Gordon, 2005, Moller *et al.*, 2005). Phagocytosis of microbial pathogens is the primary function of macrophages. This function is aided by the complement receptors CR1, CR2, CR3, CR4 and a recently described Ig superfamily member, complement receptor Ig (CRIg) (Helmy *et al.*, 2006, van Lookeren Campagne *et al.*, 2007). Macrophages also possess various other receptors that play important roles in their response to infection but are not involved in recognition of microbial pathogens. These include chemokines and cytokine receptors that modulate a wide range of functions such as migration, adhesion and antigen presentation (Brown and Gordon, 2005).

1.3.3 Antimicrobial function

Macrophages contain a spectrum of systems which play roles in the inactivation and elimination of microbial pathogens. Many of these have similarities to those of neutrophils (described above). Recognition through appropriate cell surface receptors enables macrophages to contain microbial pathogens within phagocytic vacuoles. The release of toxic metabolites, peptides and enzymes in the phagolysosome leads to microbial killing. These processes of microbial recognition and killing are most effective when the pathogen is coated with antibody and complement.

However, for some bacteria and parasites these recognition systems are not adequate. Several pathogens can invade and multiply within the macrophages. This includes *Listeria* (Cossart and Mengaud, 1989), *Salmonella* (Ohl and Miller, 2001), *Mycobacteria* (Pai *et al.,* 2003) *Toxoplasma* (Yarovinsky, 2008), Trypansoma cruzi (Maganto-Garcia *et al.,* 2008) and *Leishmania* (Alexander and Russell, 1985). To prevent the survival and multiplication of these pathogens, macrophages need to undergo activation. This is best achieved through the induction of cell-mediated immunity, involving the stimulation of Th1 cells and the production of IFNγ, which is a recognised classical activator of macrophages (Gordon, 2003, Fujiwara and Kobayashi, 2005).

Infiltration of macrophages into tissue sites can also be regulated by altered self-molecules, autoantibodies, deposited antigen-antibody complexes and complement. The very same processes which macrophages use to kill microbial pathogens are unfortunately responsible for the tissue damage and long term loss of tissue and organ function (Fujiwara and Kobayashi, 2005). The accumulation and activation of macrophages in tissues is the hallmark of chronic inflammatory diseases such as RA,

atherosclerosis and granuloma formation in diseases such as tuberculosis (Brown and Gordon, 2005).

1.4 INFLAMMATORY MEDIATORS

The propagation and resolution of inflammation is orchestrated by a network of a host of soluble mediators such as, chemokines, complement, clotting factors, leukotrienes, protease inhibitors and cytokines. These mediators act in unison to either stimulate or downregulate cellular responses. In recent years, these have been intensively investigated to identify some opportunities for targeting to prevent disease.

1.4.1 Arachidonic acid-derived mediators

The arachidonic acid cascade is activated by a number of inflammatory stimuli that result in activation of phospholipase A_2, cyclo-oxygenases (COX), and lipoxygenases (LOX). Arachidonic acid metabolism results in the production of eicosanoids such as thromboxanes, leukotrienes and prostaglandins (Kozik and Tweddell, 2006). Eicosanoids are rudimentary hormones or mediators in their own right, regulating processes and conditions such as pain, platelet aggregation, blood clotting, smooth muscle contraction, leukocyte chemotaxis, inflammatory cytokine production (IL-1, IL-6 and TNF) and

immune function, in paracrine and autocrine manner (Tilley *et al.*, 2001, Harris *et al.*, 2002).

1.4.2 Complement

The major effector of innate immunity is the complement system. The complement system is made up of soluble proteins as well as complement receptors and regulatory proteins. The soluble proteins constitute 5 % of the plasma; their major role is to recognise and promote the clearance by phagocytosis of invading organisms or damaged/altered host cells (Morgan and Harris, 2003). In innate immunity, complement is activated via the alternative pathway by molecules such as techoic acid, LPS and polysaccharide released during infection (Gasque, 2004, Nauta *et al.*, 2004). This type of immunity is also dependent on the lectin (mannose-binding) pathway for complement activation by microbes with terminal mannose groups. Once the adaptive immune response is set in motion, the antibody produced initiates the classical pathway to activate complement. As a result of complement activation by all three systems, several biological fragments are generated in a cascade manner (Sim and Tsiftsoglou, 2004). Apart from components with anaphylatoxin and microbial membrane attack complex, activities relevant to chemotaxis, C5a and opsonisation and phagocytosis, C3b, iC3b are generated. Neutrophils and monocytes use receptors to these components to infiltrate infection site, recognise and engulf bacteria (Gasque, 2004, Nauta *et al.*, 2004, Sim and Tsiftsoglou, 2004).

1.4.3 Nitric oxide (NO)

NO• is derived from the conversion of the amino acid L-arginine to L-citrulline by nitric oxide synthase (NOS). NOS isoforms generally fall into two categories: (i) constitutive NO synthases (NOS1 and NOS3) that are dependent on Ca^{2+}/calmodulin and (ii) inducible NOS (NOS2 or iNOS), the expression of which is increased by cytokines such as TNF, and other inflammatory stimuli. The latter, is responsible for the role of NO in inflammatory processes and is expressed by leukocytes, including macrophages and neutrophils, and its activity is independent of [Ca^{2+}]. NO• may affect diverse cellular responses and can have both pro- and anti-inflammatory effects (Kubes *et al.,* 1994). Similar to ROS, unregulated NO• synthesis may have detrimental effects, as has been suggested in sepsis and autoimmune diseases, where increased NO levels are related increased pro-inflammatory cytokines (TNF, IL-6, IL-8) (Zamora *et al.*, 2000, Bateman *et al.*, 2003). Again similar to ROS, NO• participates in diverse cellular signalling, including regulation of plasma membrane receptors, endocytic pathways, GTP-binding proteins, ion channels, transcription factors, and tyrosine kinases (Fialkow *et al.,* 2007). It is noteworthy that there are physiologically important interactions between NO• and ROS such as $O_2^{\bullet-}$. NO• can interact with $O_2^{\bullet-}$, thus acting as a biologic scavenger or inactivator of ROS (Fialkow *et al.*, 2007).

1.4.4 Cytokines

Cytokines are a large group of soluble proteins and peptides that act as intercellular signalling molecules. They are inducible molecules produced in response to infection, to become involved in regulation of inflammation, immunity, differentiation, proliferation and many other functions. Although cytokines share common properties with hormones they can be differentiated by the fact that hormones are made by specialised cells and glands whilst cytokines are produced by a number of different cell types. Another important distinction is the fact that cytokines often act locally while hormones act systemically (Ethuin, 2005, Ferrante, 2005, Romagnani, 2005, Via, 2007). Many cytokines are pleiotropic and in addition many have redundant activity. Cytokines can be tentatively divided according to their functions (Table 1.4). Colony stimulating factors (CSF) are cytokines that promote growth and development of haematopoietic cells and are secreted by a variety of cell types which include fibroblasts, B cells and bone marrow cells. Erythropoietin and thrombopoietin are also included with growth factors (Table 1.4).

Growth and differentiation factors for lymphoid cells include a number of ILs, molecules capable of acting between leukocytes (Cameron and Kelvin 2003). These cytokines regulate functions such as maturation, differentiation and growth factors of T cells, B cells and NK cells (Table 1.4).

Chemokines are a group of chemotactic cytokines that have structural homology and overlapping functions with cytokines. They are the only group that act on the

superfamily of 7-transmembrane G-protein-coupled receptors (Cameron and Kelvin 2003). While their main role is to guide leukocytes to inflammatory sites, they also perform other functions such as T cell differentiation, wound healing, embryonic development (Cameron and Kelvin, 2003, Via, 2007)

IL-1β, an inflammatory cytokine is mainly produced by monocytes and macrophages, and to lesser amounts by cells such as neutrophils and endothelial cells. IL-1β is also known as a pyrogenic cytokine, as very small doses of this cytokine induce fever in mice and humans. IL-1 induces production of inflammatory cytokines and chemokines such as IL-6 and neutrophilia (Romagnani, 2005). The other inflammatory cytokines including TNF, IFNγ, IL-6, IL-12 and IL-16 are summarised and presented in (Table 1.4). TNF will be the focus of this thesis and is described in greater detail below.

Anti-inflammatory and regulatory cytokines include IL-1 receptor I antagonist (IL-1Ra) which binds competitively to IL-1Ra thus inhibiting IL-1α and IL-1β's actions. It is mainly produced by monocytes and macrophages and is a natural control mechanism for IL-1's pro-inflammatory activities. IL-4, when induced by microbial pathogens such as *Plasmodium falciparum*, was shown to inhibit the anti-parasitic function of macrophages, thereby allowing survival of pathogen (Kumaratilake and Ferrante, 1992). IL-10, a powerful cytokine produced by regulatory T cells is a powerful suppressor of production of IFNγ, IL-2, IL-3, TNF and GM-CSF by macrophages, neutrophils and Th1 cells (Cools *et al.*, 2007, Via, 2007). IL-10 is also known to down regulate the expression of activating and co-stimulatory molecules on macrophages and DC; and upregulate the

expression of neutralising IL-1Ra and TNF receptor 2 (Romagnani, 2005). IL-13 is another important regulatory cytokine which is known to have anti-inflammatory effects by inhibiting production of IL-1, IL-6, IL-8 and chemokines MIP-1 and MCP. It is mainly produced by activated T cells. IL-13 is also known to induce B cells to produce IgE (Via, 2007).

TNF plays a key role in this cytokine network as all of the cytokine producing leukocytes express TNF receptors. Thus neutrophils, macrophages, T cells, NK cells, B cells, mast cells, eosinophils will all come under the influence of this cytokine. The rapid production of TNF at inflammatory sites means that it is likely to be critical in both the initiation of the innate and adaptive immune response (Getz, 2005, Ludwig *et al.*, 2006)).

Table 1.4 Classification of cytokines based on major functional activities

Cytokines	Predominant producer	Comments
	Growth factors for haemopoietic precursors, myeloid cells and thrombocytes	
SCF, CSF	B cells, epithelial cells, fibroblasts, bone marrow cells	Promote growth and development
IL-3	Activated T cells, NK cells, endothelial cells, mast cells	Supports growth and development of hematopoietic cells
G-CSF, M-CSF; GM-CSF	B cells, epithelial cells, fibroblasts, macrophages, bone marrow stromal cells	Development of colonies from granulocyte and monocyte-macrophage precursors
EPO, TPO	Kidney, liver	Regulate production of erythrocytes and thrombocytes
IL-5	Activated T cells	Promotes growth and differentiation for eosinophils and granulocytes
IL-11	Bone marrow stromal cells and mesenchymal cells	Growth factor for thromobocytes
	Growth and differentiation factors for lymphoid cells	
IL-2	Activated T cells, NK cells, endothelial cells, mast cells	Stimulates T and B cell growth
IL-4	Activated T cells, NK cells, endothelial cells, mast cells	Proliferation and differentiation of activated B cells
IL-7	Bone marrow stromal cells, intestinal epithelial cells	Critical in maturation of immune system, proliferation of T cells
IL-9	Activated Th2 cells	Promotes the growth of mast cells, B cells and other T cells
IL-15	Monocytes, fibroblasts, endothelial cells	Acts as a T cell growth factor
IL-21	Activated CD4+ T cells	Regulates NK and CD8+ T cell function
IL-27	Macrophages and dendritic cells	Synergises with IL-2 to induce IFNγ production by NK cells

Cytokines	Predominant producer	Comments
	Anti-inflammatory and regulatory cytokines	
IL-Ra	Monocytes, activated macrophages	Dampens the effects of IL-1
IL-10	Monocytes, Th2 cells, T regs, B cells, DC	Suppresses inflammation by inhibiting synthesis of TNF, IL-2, IL-3, IFNγ
IL-13	Activated T cells, Th2 cells	Induces B cells to produce IgE, inhibits pro-inflammatory cytokine production
TGFβ	Chondrocytes, osteocytes, monocytes, T regs	Regulates inflammation, immune tolerance, cell growth, differentiation, wound healing
	Inflammatory cytokines	
IL-1α and IL-1β	Monocytes and activated macrophages	Induces inflammatory reaction in response to infection
IL-6	Stimulated monocytes, fibroblasts	Induces fever, hormones and acute phase proteins in response to injury and infection
TNF	Monocytes, macrophages, neutrophils, Th1 cells	Mediate inflammation, recruit granulocytes to areas of inflammation
LTα and LTβ	T cells, B cells, fibroblasts	Mediate inflammation, recruit granulocytes to areas of inflammation
IFNγ IFNα1/α2, IFNβ	T cell, NK cells, macrophages	Anti-viral effects, stimulates antigen presentation, stimulates NK cytotoxic activity
IL-12	Monocytes, macrophages	Activates and regulates NK cells, induces IFNγ production by T cells
IL-16	CD4+ and CD8+ T cells	Chemotactic for CD4+ lymphocytes, eosinophils and monocytes
IL-17 family	Activated T cells	Activates macrophages, fibroblasts
IL-18	Macrophages, dendritic cells	Induces IFNγ secretion and enhances NK cell activity
IL-23	Activated macrophages and dendritic cells	Enhances proliferation of Th1 cells
IL-25	Activated Th2 cells and mast cells	Induces IL-4, IL-5 and IL-13 production

Adapted from (Ferrante, 2005, Via, 2007)

1.5 TUMOUR NECROSIS FACTOR (TNF)

TNF, a pro-inflammatory cytokine with pleiotropic effects, was initially described in mice in the 1970s as a macrophage-derived product that caused haemorrhagic necrosis of solid tumours (Carswell *et al.*, 1975, Haranaka *et al.*, 1986). Its cytotoxic effect towards tumour cells gave rise to its name. Two functionally related species of tumour necrosis factor exist, TNF and LT (TNFβ). The two forms have approximately 30 % sequence homology. LT can exist either as a secreted LTα homotrimer that binds to the two TNFR, or as a membrane-associated LTβ heterodimer composed of LTα and LTβ chains. LTαβ binds to a unique LTβ receptor (Schneider *et al.*, 2004, Schottelius *et al.*, 2004). LT is produced by stimulated T-lymphocytes while TNF is mainly produced by stimulated macrophages. Newly synthesised TNF is initially displayed on the plasma membrane, and is then cleaved in the extracellular domain by a transmembrane metalloprotease enzyme- TNF converting enzyme (TACE) into a 157 amino acid, 17.3-kDa soluble protein which oligomerises to form the active homotrimer (Moss *et al.,* 1997). Membrane bound TNF (mTNF) is biologically active and is presumed to mediate the cytotoxic and inflammatory effects of TNF through cell-to-cell contact (Schottelius *et al.*, 2004). Recently mTNF has been shown to be essential in host defence mechanisms against mycobaterical infection (Allie *et al.,* 2008). The human TNF gene is located on chromosome 6 and is translated as a 233 amino acid, 26-kDa precursor protein (Kriegler *et al.,* 1988). TACE is an adamlysin, which belongs to a class of membrane-associated enzymes that contain both a disintegrin and matrix metalloprotease domains (ADAM) (Kriegler *et al.*, 1988). TNF production by macrophages is stimulated by a variety of

agents that includes bacterial LPS, bacterial toxins, viruses and fungi (Ferrante *et al.*, 1990, Beutler, 1992). TNF is secreted by a variety of cell types: immune cells (T-lymphocytes, NK-cells, B-lymphocytes, macrophages, monocytes, mast cells, neutrophils, basophils, eosinophils), non-immune cells (astrocytes, fibroblasts, glial cells, granuloma cells, keratinocytes, neurons, osteoblasts, smooth muscle cells and many kinds of tumour cells (Bazzoni and Beutler, 1996). However, macrophages and monocytes serve as primary sources of TNF, especially during the inflammatory responses (Beutler *et al.*, 1985, Clark, 2007).

1.5.1 Regulation of TNF expression

Given that TNF plays a key role in both physiologic and pathophysiologic processes it is not surprising that the expression and activity of TNF are tightly regulated at many different levels. TNF gene expression is stimulated by a wide variety of agents. In macrophages, expression is induced by stimuli that include viruses, bacterial and parasitic products, tumour cells, complement, cytokines [such as IL-1β, IL-2, IFNγ and GM-CSF], ischemia, trauma and irradiation (Bazzoni and Beutler, 1996). In other cell types, other stimuli such as LPS, the cross-linking of immunoglobulins, ultraviolet light and phorbol esters are effective in inducing TNF gene expression (Bazzoni and Beutler, 1996). Under resting conditions the amount of TNF in cells other than macrophages is barely detectable. However, following stimulation, levels of TNF mRNA increase rapidly, resulting in large quantities of soluble TNF being released. TNF expression is

also controlled at the post transcriptional level by adenosine-uridine rich elements (AREs) (Chen and Shyu, 1995). AREs have been shown to destabilise heterologous transcripts into which they are inserted and thus determine their translational efficiency. Such sequences are usually common on the mRNA of pro-inflammatory cytokines (Han and Beutler, 1990, Beutler and Brown, 1993, Chen and Shyu, 1995).

Recently, Ferrante and Ferrante (2005), have shown that the product of the 15-lipoxygenase of the metabolism of arachidonic acid, 15-hydroperoxyeicosatetraenoic acid inhibits the LPS-induced TNF production by macrophages occurs by decreasing the stability of TNF mRNA (Ferrante and Ferrante, 2005). Downregulation of TNF production has also been described to be due to cytokines such as IL-4 (Hart *et al.,* 1991), IL-10 (Rajasingh *et al.,* 2006) and IL-13 (Manna and Aggarwal, 1998). Inhibition of cytokine production including TNF occurs through the consumption of n-3 polyunsaturated fatty acids (Trebble *et al.,* 2003).

1.5.2 Biology of TNF

The wide spectrum of biological effects of TNF has been appreciated for some time (Table 1.5) (Schottelius *et al.*, 2004, Rahman and McFadden, 2006). Its anti-cancer activity is mediated through a direct effect on the malignant cells, inducing apoptosis or indirectly by upregulating the immune system (Younes and Kadin, 2003). However, its wider activity has been appreciated in defence against microbial pathogens on the one

hand and microbial pathogenesis in the other (Roilides *et al.*, 1998, Ehlers, 2003). Even more concerning has been the finding that the cytokine is a key player in the pathogenesis of several chronic inflammatory diseases (Feldmann *et al.*, 1996, Sekut and Connolly, 1998) and indeed TNF has become a target for therapeutics (LaDuca and Gaspari, 2001, Taylor, 2003).

The biological characteristics of TNF clearly support its role in physiologic and pathophysiologic processes. Apart from its action on all leukocyte types, it stimulates local tissue cells at the foci of infection or autoimmune reaction and the vascular endothelium. The cytokine through its receptor stimulates transcription of key players of the inflammatory reaction. The cytokine promotes extravasation of leukocytes by upregulation/inducing the expression of cell adhesion molecules (CAMS) on endothelial cells. These include ICAM-1, E-selectin and VCAM-1 (Jersmann *et al.,* 2001). TNF will also induce pro-coagulant activity and cytokine production in endothelial cells (Bevilacqua *et al.,* 1986). The stimulation of chemokines production in endothelial cells and local tissue cells by TNF is a further mechanism by which it contributes to cellular accumulation at inflammatory sites (Clauss *et al.*, 2001, Varfolomeev and Ashkenazi, 2004).

Leukocyte stimulation by TNF can lead to major functional changes in these cells. The cells show upregulation of receptors, production of cytokines, lipid mediator generation and release of a host of products which promote microbial killing, inflammation and tissue damage. Neutrophils are primed by TNF to exhibit enhanced antimicrobial activity against bacteria, fungi and parasites (Ferrante, 1992) as well as tissue damage (Kowanko

and Ferrante, 1996, Witko-Sarsat *et al.*, 2000). Pre-treatment of neutrophils with TNF leads to migration inhibition, enhancement of FcγR and CR3 expression and associated interaction with ligands for enhanced release of oxygen derived reactive species and degranulation (Ferrante *et al.*, 1988, Ferrante, 1992). Apart from stimulating cytokine release from macrophages, TNF promotes the adaptive immune response by increasing expression of MHC class II antigens and presentation of antigen by APC to T cells (Varfolomeev and Ashkenazi, 2004).

At low concentrations, it acts in a paracrine and autocrine manner, upregulating vascular adhesion molecules, activating neutrophils, and stimulating monocytes to secrete IL-1, IL-6 and more TNF (Figari *et al.*, 1987, Ferrante *et al.*, 1988, Jupin *et al.*, 1989). The importance of TNF in host defence against pathogens was demonstrated through the use of TNF neutralising antibodies (Skerrett *et al.,* 1997). Similarly, a lack of TNF was shown to impair host defence mechanisms severely (Nakane *et al.*, 1988, Hauser *et al.*, 1990). Mice lacking the TNFRI gene (p55) while resistant to lethal doses of LPS or enterotoxins, readily succumb to infections by *Listeria monocytogenes*, an intracellular bacterial pathogen, despite having fully functional anti-microbial systems that generate reactive oxygen radicals and reactive nitrogen intermediates (Pfeffer *et al.,* 1993). Interestingly, the re-expression of TNFRI on bone marrow derived cells was able to control infection in these mice, showing that the expression of TNFRI is essential for the killing of intracellular bacterial infections such as *L. monocytogenes* (Pfeffer *et al.*, 1993, Endres *et al.*, 1997). Similarly, mice that have had their TNFRI gene disrupted show increased susceptibility to infection (Rothe *et al.,* 1993).

TNF has been demonstrated to regulate and/or interfere with adipocyte metabolism via a number of mechanisms. These include transcriptional regulation, glucose and fatty acid metabolism and hormone receptor signalling (Sethi and Hotamisligil, 1999). It has been shown that TNF deficient mice exhibit lower circulating levels of free fatty acids and triglycerides than the wild-types (Uysal *et al.,* 1997). This reduction is thought to be attributed to inhibition of lipoprotein lipase activity and expression of free fatty acid transporters in adipose tissue by TNF (Cornelius *et al.*, 1988, Memon *et al.*, 1998). TNF is therefore likely to contribute to the hyperlipidemia that is observed during infections and obesity (Sethi and Hotamisligil, 1999).

1.5.3 Pathophysiologic actions of TNF

The role of TNF in the pathogenesis of a range of diseases is highly appreciated and as such has been the focus of intense research. While some of the actions of TNF are physiologically beneficial to the human body, especially in assisting in the defence against microbial pathogens, prolonged or excessive production often becomes harmful and impedes normal physiological processes. Localised action of TNF is clearly important but production in excess leads to extensive diffusion into the circulation and acts like an endocrine hormone, so displaying its pyrogenic properties, stimulating other cytokines, activating the coagulation system, and suppressing bone marrow stem cell maturation.

Table 1.5 Summary of the biological effects of TNF

	Effects on immune cells
Monocytes/macrophages	Activate/autoinduce TNF
	Induce cytokines and prostaglandins
	Inhibit chemotaxis and migration
	Stimulates metabolism
	Inhibits differentiation and suppresses proliferation
Neutrophils	Stimulates respiratory burst
	Primes integrin response and increases adherence to extracellular matrix
	Increases phagocytic capacity
Lymphocytes	Involved in T-cell co-activation
	Induces apoptosis in mature T cells
	Activates cytotoxic T cell invasiveness
	Effects on non-immune cells
Endothelial cells	Suppresses anticoagulant properties
	Induces cytokine production
	Upregulates cell adhesion molecules
	Rearranges cytoskeleton
	Induces nitric oxide synthase
Fibroblasts	Induces proliferation
	Increase cytokine production (IL-1,6 and LIF)
	Induces MMPs
	Inhibits collagen synthesis
Adipocytes	Inhibits lipoprotein lipase and lipid storage
	Systemic non-immune effects
Cardiovascular: shock, capillary leakage, anti-thrombosis	
CNS: fever, anorexia, altered pituitary hormone secretion	
GIT: ischemia, colitis, hepatic necrosis, inhibits albumin expression, decreases hepatic catalase	
Metabolic: net lipid catabolism, net protein catabolism, release stress hormone, insulin resistance	
Endocrine: stimulate ACTH and prolactin, inhibits TSH, FSH, GH	

Adapted from (Schottelius *et al.*, 2004). Abbreviations: MMPs metalloproteases, ACTH, adenocortiotropic hormone; TSH, thyroid stimulating hormone; FSH, follicle stimulating Hormone; GH, growth hormone; LIF, leukaemia inhibitory factor.

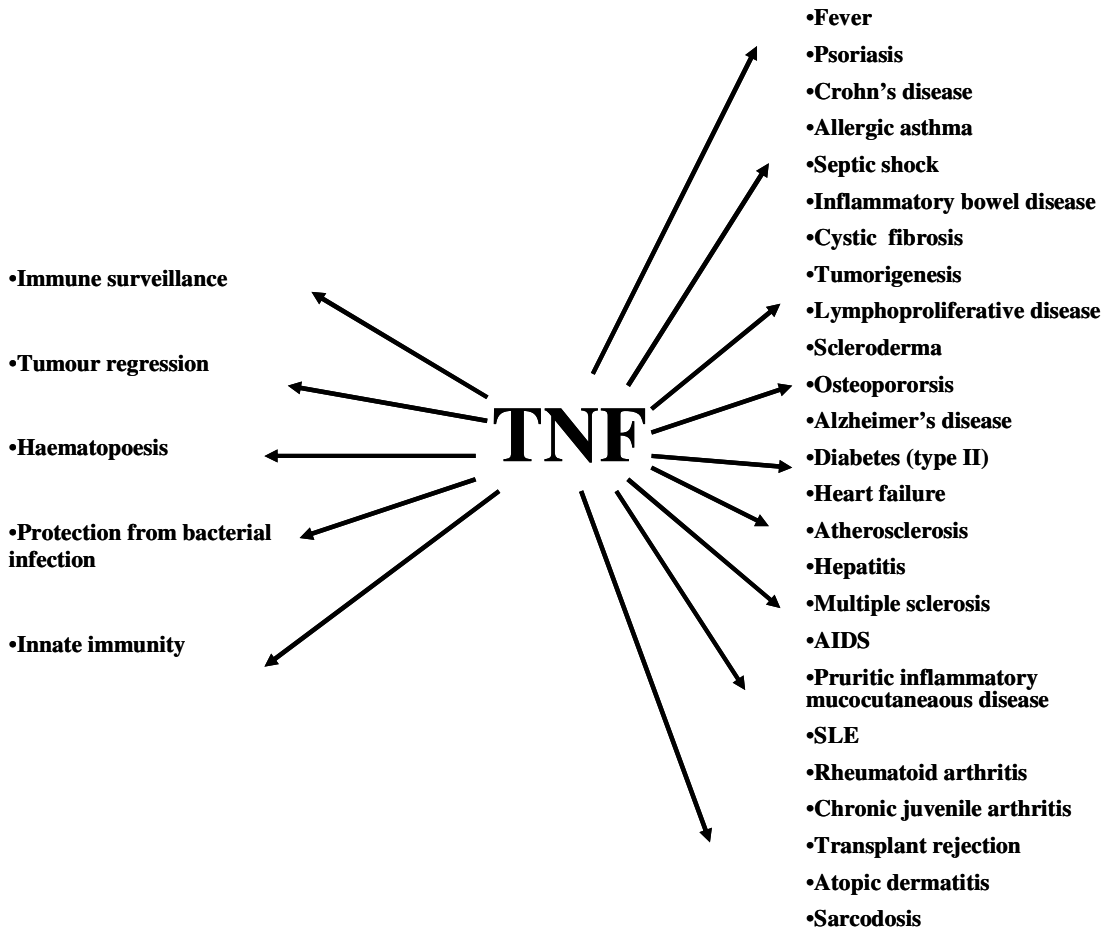

Figure 1.4 "Good versus Evil": TNF, a critical component of effective immune surveillance and immunity also promotes a wide range of inflammatory diseases. Adapted from (Aggarwal *et al.,* 2006).

Other events seen with further increases in TNF include myocardial depression, hypotension (probably through induction of NO synthesis), and induction of disseminated intravascular coagulation. During invasive bacterial infections, bacterial LPS stimulates macrophages to produce excessive levels of IL-1 and TNF. Bacterial septic shock is then triggered resulting in diffuse capillary leakage, hypotension, myocardial suppression, and haemorrhagic necrosis of tissues, multiple organ failure and death (Beutler, 1992).

Excessive production of TNF as seen in chronic disease states or major injury such as burns, greatly alters homeostasis and results in cachexia, characterised by anorexia, accelerated catabolism, extreme weight loss, anaemia and depletion of body tissues (Beutler, 1992). Although TNF itself can produce cachexia in experimental animals, other cytokines such as IL-1β (induced by TNF) are thought to contribute to the cachectic state accompanying some diseases such as cancer (Beutler and Cerami, 1986).

The pathological actions of TNF are also manifested by upregulation in expression of adhesion molecules on the endothelium. These adhesion molecules include E-selectin, VCAM-1 and ICAM-1 (Pohlman and Harlan, 1989). Upregulation of these molecules promotes the adhesion of cells to the endothelium (Carlos and Harlan, 1990) and can result in disorders such as atherosclerosis (Ross, 1993).

While TNF is known to cause the death of tumour cells, the cytokine can also cause apoptosis of normal tissues (Ferrante, 1992). In physiological terms, these cytotoxic

effects of TNF can result in extensive tissue damage, such as that seen in chronic inflammatory disorders.

1.6 TNF RECEPTORS

TNF exerts its biological activity through its receptors, the 55-kDa TNFRI/CD120a and the 75-kDa TNFRII/120b, both of which belong to the TNF receptor super-family (Tartaglia and Goeddel, 1992). These receptors are classified as type I transmembrane glycoprotein receptors and are characterised by the presence of multiple cysteine-rich repeats of about 40 amino acids in their extracellular amino-terminal domain (Herbein and O'Brien, 2000). TNFRI and TNFRII are present virtually on all cell types except for red blood cells. TNFRI is more ubiquitously expressed, while TNFRII is found in abundance on endothelial cells and haematopoietic cell lineage (Hohmann *et al.,* 1989) (Brockhaus *et al.,* 1990).

TNF receptors can be shed to yield the soluble form which inhibit the effects of TNF by reducing the number of binding sites on cells. Serum levels of TNF receptors are increased in a large number of inflammatory diseases such as AIDS, rheumatoid arthritis, heart failure and cancer (Gabay *et al.*, 1997, Lee *et al.*, 1997, Nozaki *et al.*, 1997, Goetz *et al.*, 2002, Hunt and Grau, 2003, Herbein and Khan, 2008). Abnormalities in TNF receptor shedding has been linked to autoimmune diseases such as multiple sclerosis (Jurewicz *et al.,* 1999).

1.6.1 TNFRI and TNFRII mediated signalling

TNFR is responsible for the majority of this cytokine's biological actions. While both TNFRI and TNFRII have cysteine-rich repeats in their extracellular domains, only TNFRI has a death domain (DD) in its cytoplasmic tail. TNFRI mediates both cell survival and death signals due to its DD, as opposed to TNFRII which primarily mediates cell survival signals (Gupta, 2002).

It is widely accepted that TNFRI mediates the majority of TNF's inflammatory properties. The kinetics of association between TNF and its ligands and receptors show that TNF associates more rapidly with TNFRI than with TNFRII. Many studies have been carried out in order to define the exact role of TNFRII in mediating the effects of TNF. Some investigators have proposed that TNFRII potentiates TNF-induced apoptosis. (Tartaglia *et al.*, 1993) suggested that TNFRII acts as a high-affinity trap of TNF that delivers TNF to TNFRI i.e. serves as a "ligand passer" by delivering/passing TNF to TNFRI for signalling when TNF concentrations are low (Figure 1.5). When TNF has access to both receptors on the same cell the presence of TNFRII greatly enhances the rate of association of TNF with TNFRI, thus *in vivo* TNFRII contributes to cytotoxicity of TNF via TNFRI (Van Ostade *et al.,* 1993). Weiss et al. proposed that the cytoplasmic domain of TNFRII may be responsible for the potentiation of apoptosis (Weiss *et al.,* 1998).

Using TNFRII knockout mice Peschon *et al.*, (1998), showed that in some cases TNFRII acts as a decoy receptor since these knockout (KO) mice manifested an exacerbated inflammatory response, with dramatically increased serum TNF levels, suggesting that TNFRII may play a down-regulatory role in pulmonary responses. Similarly, mice lacking TNFRI are more susceptible whereas TNFRII KO mice are resistant to infection by L. monocytogenes. This resistance is conferred by activated macrophages, a function which is dependent upon TNF and IFNγ (Pfeffer *et al.*, 1993, Rothe *et al.*, 1993). TNFRII seems to have no intrinsic inflammatory properties of its own but potentiates the actions of TNFRI (Erickson *et al.*, 1994, Peschon *et al.*, 1998), as discussed above.

The differences in signalling properties between TNFRI and TNFRII can also be related structurally i.e. the intracellular domains of TNFRI shares greater homology with the domains of the Fas rather than TNFRII, coupled with the lack of the death domain on TNFRII. Recently it has been shown that due to the lack of a cytoplasmic death domain (DD) on TNFRII, binding of TNF to TNFRII results in the recruitment of TNFR associated factor 1 (TRAF1) and TRAF2 to the cytoplasmic portion of TNFRII independently of TNFR associated death domain (TRADD). This consequently leads to the recruitment of cellular inhibitor of apoptosis proteins (cIAP)-1 and cIAP-2 (Deveraux *et al.,* 1999). These proteins are known to directly bind to caspases 3 and 7 hence preventing the activation of caspase 9 (Deveraux *et al.*, 1999). Pimentel-Muinos and Seed (1999) demonstrated that in the Jurkat T-cell line, in the presence of receptor interacting protein (RIP), TNFRII triggers apoptosis, whereas in the absence of RIP, TNFRII activates nuclear factor kappa B (NF-κB) (Pimentel-Muinos and Seed, 1999).

However, the requirement of RIP seems to be distinct among different cell types. In fibroblasts, RIP is essential for TNFRI mediated NF-κB activation but not for apoptosis. In contrast, in T cells, RIP is required for TNFRI dependent NF-κB activation and apoptosis and for TNFRII mediated apoptosis but not TNFRII mediated NF-κB activation (Kelliher *et al.*, 1998, Pimentel-Muinos and Seed, 1999). These studies support the role of TNFRII as a more potent regulator of apoptosis, rather than serving an accessory role as originally proposed.

1.6.2 TNFR adaptor proteins

The cytoplasmic domain of TNFRI and TNFRII do not have any intrinsic signalling capability. Hence, they recruit cytoplasmic molecules known as "adaptor proteins" to the cytoplasmic domain of the receptors to bind to downstream signalling molecules. The binding of TNF to TNFRI leads to the release of the inhibitory protein, silencer of death domain (SODD) from the receptor's intracellular domain, and this leads to the recruitment of the important adaptor protein, TRADD. This facilitates the recruitment of additional adaptor proteins which include RIP, TRAF-2 and Fas-associated death domain (FADD) (Chen and Goeddel, 2002) (Figure 1.5). As above, TNFRII recruits TRAF2 independently of TRADD. These adaptor proteins in turn recruit additional key pathway-specific enzymes to the TNFRI such as procaspase-8 [also called FADD-like IL-1β converting enzyme (FLICE)] by interaction via homologous death effector domain (DED) to form a death-inducing signal complex (DISC) (Gupta, 2002).

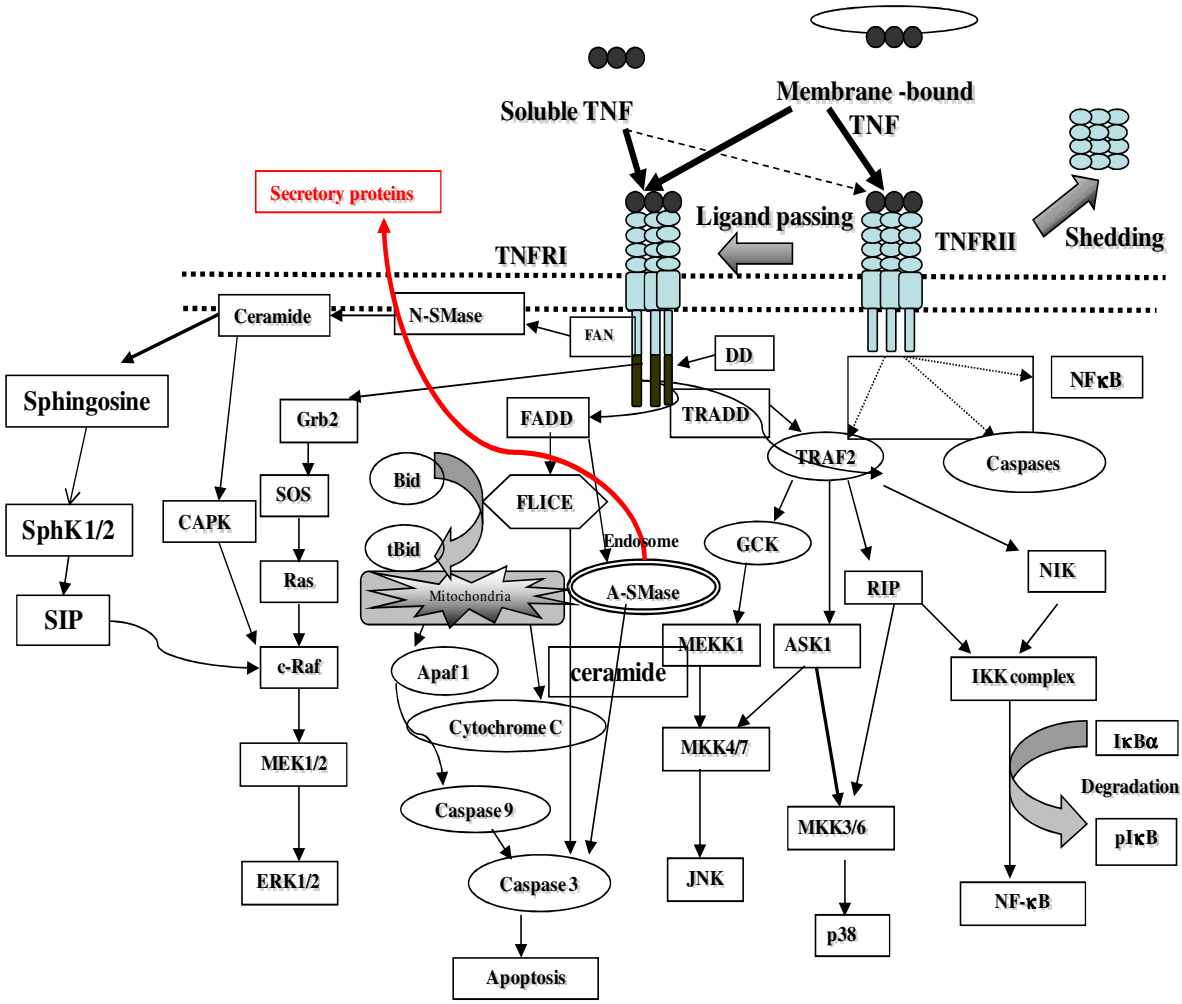

Figure 1.5 Signalling pathways activated by TNFRI and TNFRII. Adapted from (Kronke, 1999, Gupta, 2002, MacEwan, 2002, Aggarwal *et al.*, 2006). The highlighted pathways emanating from TNFRII are not fully understood yet, and the above represents possible mechanisms of biological responses. Not every signalling pathway depicted is activated in all cell types by TNF. Abbreviations: A-SMase, acid sphingomyelinase; ASK1, apoptosis signal-regulating kinase; CAPK, ceramide activated protein kinase; DD, death domain; ERK1/2, extracellular-signal-regulated kinases; FAN, factor associated with N-SMase activation; FADD, Fas-associated DD; FLICE, FADD-like IL-1β converting enzyme; MEK1/2, MAPK/ERK kinase, GCK, germinal centre kinase; IKK, IκB kinase; MEKK1, MAPK kinase kinase; NFκB, nuclear factor kappa B; NIK, NF-κB inducing kinase; IκB, inhibitor kappa B; N-SMase, neutral sphingomyelinase; RIP, receptor interacting protein; TRADD, TNFR associated DD; TRAF2, TNFR associated factor 2.

During DISC formation, procaspase-8 is activated by self-cleavage and initiates a protease cascade that leads to apoptosis (Chen and Goeddel, 2002, Gupta, 2002). The complex of adaptor molecules is also responsible for coupling TNFRs to signalling pathways such as the MAPKs, the inhibitor of kappa B (IκB) kinase-nuclear factor kappa B (NF-κB) pathway and the sphingomyelinase-ceramide pathway, which are responsible for mediating the actions of TNF (Gupta, 2002).

1.7 SIGNALLING PATHWAYS ACTIVATED BY TNF

1.7.1 Activation of NF-κB

The activation of transcription factor NF-κB plays an important role in the regulation of the immune response and inflammation (Baeuerle and Henkel, 1994). Recent studies have increased our knowledge and understanding of this component of the TNF signalling network (Locksley *et al.*, 2001, Ghosh and Karin, 2002). TNF can activate the NF-κB pathway via the classical or non-canonical mechanism. The classical pathway requires the activation of IKK/NEMO (see below), whereas the non-canonical pathway can be activated independently (Karin, 2006, Niederberger and Geisslinger, 2008). Although the enzymatic activity of RIP is not required for TNF-induced activation of NF-κB, knockout studies ($Rip^{-/-}$ mice) have shown that RIP is essential for the activation of this transcription factor in fibroblasts (Kelliher *et al.*, 1998). In the resting cell, NF-κB resides in the cytoplasm through an interaction with the inhibitory protein, inhibitor of

kappa B (IκB). Identification of the multiprotein, IκB kinase (IKK) complex which mediates the phosphorylation of IκB in a TNF-dependent manner, was a significant step in the understanding of NF-κB signalling (Ghosh and Karin, 2002). The IKK complex contains two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NF-κB essential modulator (NEMO or IKKγ) (Karin and Lin, 2002). The IKK complex also contains a kinase-specific chaperone consisting of Cdc37 and Hs90 which play a role in the shuttling of the complex from the cytoplasm to the membrane (Chen and Goeddel, 2002). The phosphorylation of two sites at the activation loop of IKKβ mediates NF-κB activation by TNF, while the IKKα subunit mediates activation by LT (Delhase *et al.,* 1999). The NF-κB inducing kinase (NIK) has been reported to stimulate the activation of IKKβ. Upon phosphorylation, IκB is proteolytically degraded by 26S proteasome, resulting in the release of NF-κB, which translocates to the nucleus to regulate gene transcription (Garrington and Johnson, 1999).

It has also been recently reported that Akt, a serine-threonine kinase is similarly capable of phosphorylating IκB (Ozes *et al.,* 1999). Akt is a kinase which is downstream of phosphatidylinositol-3-kinase (PI3K) which is also activated by TNF (Vanhaesebroeck and Alessi, 2000). The PI3-kinase/Akt pathway is thought to be important in protecting cells from nonapoptotic necrotic cell death (Shaik *et al.*, 2007, Zhou *et al.*, 2008). PI3K phosphorylates the 3'-OH position of the inositol ring in inositol phospholipids, generating 3'-phosphoinositides (PI). Inside cells, this produces two lipid products: PI3P, and PI (3,4,5) P3 which subsequently breaks down to PI (3,4) P2 (Vanhaesebroeck and Alessi, 2000). PI (3,4) P2 and PI(3,4,5)P3 are crucial for activation of Akt

(Vanhaesebroeck and Alessi, 2000). Accumulation of the 3' phosphorylated PIs results in the activation of phosphoinositide dependent kinase (PDK1), which phosphorylates and activates Akt on tyrosine 308. Full activation of Akt requires phosphorylation on serine 473 by another kinase, most likely mammalian target of rapamycin (mTOR) (Wang *et al.*, 2008) thereby allowing Akt to activate IκB in turn (Ozes *et al.*, 1999).

1.7.2 Activation of sphingomyelinase-ceramide by TNFRI

Ceramide is a neutral sphingosine-based lipid signalling molecule that regulates cellular differentiation, proliferation and apoptosis (Pushkareva *et al.*, 1995, Spiegel *et al.*, 1996). Due to its neutral lipid nature ceramide does not distribute in the cytosol but rather remains within the membrane bilayer (Kronke, 1999). Ceramide is produced by the hydrolysis of the phosphodiester bond of sphingomyelin (by sphingomyelinases (SMases), which results in the formation of ceramide and phosphorycholine. There are two distinct forms SMases, a membrane-associated and soluble neutral (N-) SMase and an acidic form (Wiegmann *et al.*, 1994, Liu and Anderson, 1995).

Studies have shown that through the binding to TNFRI, TNF rapidly activates both forms of SMases resulting in the generation of ceramide. By using mutant TNFRI, (Wiegmann *et al.*, 1994) showed that the activation of A-SMase is signalled through the carboxyl terminus containing the DD, whereas, N-SMase is activated through a cytoplasmic portion of the TNFRI separate from the DD. This portion contains a motif of 11 amino

acids known as neutral sphingomyelinase domain (NSD) (Adam *et al.,* 1996). TNF causes the rapid, within seconds, activation of N-SMase in most cells. This activation has been demonstrated to occur through the WD-repeat protein, Factor Associated with N-SMase activation (FAN), which specifically binds to the NSD region of TNFRI which results in the coupling of NSD to N-SMase (Adam-Klages *et al.,* 1996). The release of ceramide results in the activation of ceramide-activated protein kinase (CAPK) (Mathias *et al.,* 1991). CAPK then phosphorylates Raf-1 kinase, which stimulates a dual specificity MAPK/extracellular-signal-regulated kinases (ERK) kinase (MEK)-1 and MEK-2, leading to the downstream activation of ERK1/ERK2 (Yao *et al.*, 1995, Zhang *et al.*, 1997).

The activation of A-SMase was identified to occur through the association of TNFRI DD with adaptor proteins TRADD and FADD (Schwandner *et al.,* 1998). This group showed that neither TRAF2 nor RIP were required for A-SMase activation while overexpression of TRADD and FADD in HEK293 cells enhanced TNF-induced A-SMase activation. It is believed that these adaptor proteins link the TNF-TNFRI complex to the A-SMase in the endosomal-lysosomal compartment. A-SMase is thought to couple the TNFRI to the secretory pathway and to apoptosis via protein-protein interaction known as the caspase cascade and thus plays an active role in cytokine-induced apoptosis (Deiss *et al.*, 1996, Kronke, 1999, Carpinteiro *et al.*, 2008). Unlike most cell types TNF does not stimulate the activity of the SMases in neutrophils (Robinson *et al.,* 1996).

1.7.3 Activation of sphingosine kinase by TNF

As discussed above, TNF stimulates the breakdown of sphingomyelin to produce ceramide, which is subsequently metabolised to the single sphingoid chain, sphingosine (Melendez, 2008). Sphingosine can be phosphorylated by sphingosine kinases (SphKs) to generate sphingosine-1-phosphate (S1P). Two mammalian SphKs have been characterised thus far, SphK1 and SphK2 which phosphorylated erytho-sphingosine, dihydrosphingosine and phytosphingosine. There is increasing evidence that this bioactive phospholipid, S1P, acts as either an extracellular or intracellular second messenger (Melendez, 2008). While its role as an extracellular mediator has been reported during its release by activated platelets (English *et al.,* 2000), its role as intracellular mediator is more defined. S1P, like other intracellular messengers increases cytosolic Ca^{2+} in immune cell activation mediating a wide range of biological effects such as proliferation, differentiation, chemotaxis, cytokine/chemokines generation and apoptosis (Niwa *et al.*, 2000, MacKinnon *et al.*, 2002, Melendez, 2008). Specifically, increases in S1P levels have been shown to be increased by agents that activate MAPK pathways and increased expression of adhesion molecules. Recently using a SphK inhibitor, it was shown that fMLP and TNF-stimulated superoxide production in neutrophils can be regulated by SphK (Niwa *et al.*, 2000). SphK1 activity has also been implicated in macrophage properties of wound healing (English *et al.,* 2001), mobilisation of Ca^{2+} for the monocytic NADPH oxidase activity (Melendez *et al.*, 1998), and adhesion molecule expression by endothelial cells (Kee *et al.,* 2005). However, recently two independent studies using either SphK1 or both SphK1 and SphK2 KO mice, reported normal inflammatory cell recruitment during thioglycollate, KC, MIP-2 or

LPS-induced peritonitis, collagen-induced arthritis and normal responses to fMLP by neutrophils (SphK KO mice), while a SphK2 KO showed an accelerated bacterial lung inflammation (Michaud *et al.*, 2006, Zemann *et al.*, 2007). These two studies highlight the need for further research into the exact role of S1P in inflammation as the *in vitro* observations do not mirror the KO studies, suggesting SphK's role inflammation may not be as essential as previously thought, perhaps due to redundancy in the system.

1.7.4 Activation of MAPK

In mammalian cells at least three MAPK have been identified, the classical MAPKs, ERK1 and ERK2, and the more recently described c-Jun NH_2-terminal kinase JNK 1/JNK2 and p38 MAPK pathways. MAPK have been implicated in regulating a range of biological functions, including cell proliferation, differentiation and death, expression of adhesion molecules, and cytokine production (Seger and Krebs, 1995, Kyriakis and Avruch, 2001). Studies have shown that TNF activates all three MAPKs (Karin, 1998, Davis, 1999).

1.7.4.1 Extracellular-signal-regulated kinase (ERK)

The ERK pathway comprises the greatest assembly of proto-oncogenes in any known signalling pathway (Kolch, 2002). This includes ligands such as sis and TGFα, tyrosine

kinase receptors (neu/erbB2, fms, kit), G-proteins (Ha-ras, Ki-ras, N-ras), kinases (raf, mos and tpl2/cot) and nuclear transcription factors (fos, ets, myc). Tyrosine kinases such as ErbB2 is often overexpressed in human tumours and B-raf mutated in 60% of melanomas (Davis, 1999). Hence ERKs are primarily involved in mediating responses to mitogenic signals (Boldt and Kolch, 2004). In addition, they are involved in proliferation, differentiation, apoptosis and metabolism (Dhillon and Kolch, 2002). Several isoforms of ERK have been described e.g. ERK1, ERK2, ERK3, ERK4, ERK5 and ERK7 (Pearson *et al.,* 2001). ERK1 and ERK2 are proteins of 43 and 41 kDa respectively that are nearly 85 % identical overall, with much greater identity in the core regions involved in binding substrates (Boulton *et al.*, 1990, Boulton *et al.*, 1991). Both are ubiquitously expressed, although their relative abundance in tissues is variable. In immune cells for example ERK2 is the predominant species, while in cells of neuroendocrine origin these are equally expressed (Pearson *et al.*, 2001). They are activated by serum, growth factors, certain stresses, cytokines, and ligands for G-protein-coupled receptors, adhesion signals and transforming agents (Pearson *et al.*, 2001, Boldt and Kolch, 2004). Receptor mediated stimulation of ERK1/ERK2 activity requires the activation of ras, protein kinase C (PKC) or PI3K. These act on raf-1, a serine/threonine kinase which phosphorylates and activates the dual specificity of MAPK/ERK kinase (MEK) 1 and MEK2 (Pearson *et al.*, 2001). The MEKs phosphorylate the tyrosine and threonine residues on the TEY activation motif of ERK1/ERK2, resulting in the activation of ERK1/ERK2. Activated ERK1/ERK2 phosphorylate their substrates e.g. Elk1, Ets2 on serine or threonine residues in the PXS/TP consensus phosphorylation motif where P = proline, X = any amino acid and S/T = serine/threonine.

1.7.4.2 c-Jun NH_2-terminal kinases (JNK)

JNKs are a family of serine/threonine kinases which phosphorylate c-Jun in its N-terminal transactivation domain at serine residues 63 and 73 (Liu and Lin, 2005). JNK activation is controlled by MEKK1 which phosphorylates MKK4 and MKK7. The activated MKK4/MKK7 subsequently phosphorylate JNK isoforms on the TPY motif resulting in their activation (Yan *et al.*, 1994, Weston and Davis, 2002). JNK consists of at least 10 isoforms created from the alternative splicing of three distinct genes: JNK1, JNK2 and JNK3. JNK1 and 2 are ubiquitously expressed, whilst JNK3 is predominantly expressed in the brain (Gupta *et al.*, 1996, Dreskin *et al.*, 2001, Cui *et al.*, 2005). Two alternative splice sites present in JNK1 and JNK2 give rise to the p46 isoforms, JNK1α1, JNK1β1, JNK2α1 and JNK2β1 and the p54 isoforms, JNK1α2, JNK1β2, JNK2α2, and JNK2β2 (Dreskin *et al.*, 2001). JNK substrates include transcription factors c-Jun and ATF2 (Kyriakis and Avruch, 2001). Depending on the cell type and agonist involved, JNK may regulate gene expression, the stability of target proteins, cell transformation (Antonyak *et al.,* 1998), T-cell function (Dong *et al.,* 2000), apoptosis (Hirata *et al.,* 1998), cell proliferation (Wisdom *et al.,* 1999) and tissue regeneration (Brenner, 1998).

Scaffold proteins regulate the specificity in the targets and cellular function of JNK signalling. There are four groups of JNK scaffolds known to date namely: JNK-interacting protein (JIP), JNK/SAPK associated proteins (JSAP), filamin, p130CAS and β-arrestin (Manning and Davis, 2003). These scaffolds then assemble different external

stimuli e.g. filamin mediates JNK activation by the TNFR while β-arrestin links JNK to G-protein couples receptors (Boldt and Kolch, 2004).

JNK is mainly known to promote apoptosis through its phosphorylation of proapoptic proteins such as BAD or BH3 domain proteins Bim and Baf (Donovan *et al.*, 2002, Lei and Davis, 2003). However, observations such as the increase in liver cell death apoptosis seen in MKK4 knockout mice, clearly suggests a protective role of JNK (Nishina *et al.,* 1999). JNK activation is also believed to promote insulin resistance in diabetes by blocking the phosphorylation of serine 307 on insulin-receptor substrate 1 (Aguirre *et al.,* 2000).

1.7.4.3 p38 kinase

The p38 MAPK family are serine-threonine kinases, that are activated by environmental stresses such as UV irradiation, heat or osmotic stress but which can also be triggered by products of microorganisms such as endotoxins and pro-inflammatory cytokines such as IL-1 or TNF (Lee *et al.*, 1994). Hence it has a role in innate immunity (Guo *et al.*, 2003). p38 is not only involved in cytokine induction but its activation is induced by inflammatory cytokines. The role of p38 in innate immunity was reinforced by the discovery that SB203580 inhibited the release of TNF or IL-1 from endotoxin-stimulated monocytes (Lee *et al.*, 1994). Subsequently, p38 was shown to have four isoforms: p38α, p38β, p38γ and p38δ (Lee *et al.*, 1994, Stein *et al.*, 1997, Wang *et al.*, 1997, Young *et*

al., 1997). The p38α and p38β are expressed in most tissues; p38γ is mainly expressed in skeletal muscle and in normal and diseased cardiomyocytes, p38δfound in several adult tissues and during development (Kumar *et al.,* 2003). Activation of p38 occurs through the dual phosphorylation of the threonine and tyrosine residues in the TGY motif by the upstream MAPK kinases, MKK3 and MKK6, which subsequently phosphorylate a small heat shock protein 27 (Freshney *et al.*, 1994, Hii *et al.*, 1998).

From an inflammatory aspect, p38α is most commonly expressed in the different inflammatory cell lineages i.e. monocytes/macrophages, neutrophils and CD4+ T cells. The p38 inhibitor SB203580, which has been shown to inhibit the production of inflammatory cytokines such as TNF and IL-1β, inhibits the activation of the p38α and p38β isoforms (Lee *et al.*, 1994, Lee *et al.*, 2000, Kumar *et al.*, 2003). p38 regulates gene expression by four different general mechanisms:

a) via the phosphorylation of transcription factors e.g. activation transcription factors 2 and 6 (ATF2/6) and myocyte enhancing factor 2C (MEF2C) (Lee *et al.*, 2000)
b) by regulating mRNA stability via the action of downstream kinases such as the MAPK-activated protein kinase 2 (MAPKAP K2). Messages for IL-2, cyclooxygenase-2 (COX-2) and other inflammatory mediators is short lived due to the presence of AU-rich elements in the 3' untranslated region of the message that associate with AU-binding proteins that destabilise the message (Caput *et al.*,

1986, Shaw and Kamen, 1986). Phosphorylation of these proteins by p38 results in increased message stability (Winzen *et al.*, 1999, Ming *et al.*, 2001).

c) regulate mRNA translation into protein via MAPKAP K2 which also control translation and production of mediators e.g. TNF by phosphorylating AU-binding proteins (Kumar *et al.*, 2003).

d) promote the phosphorylation of histone H3 in chromatin at NF-κB binding sites of certain genes. The activity of this histone is required for successful transcriptional induction of IL-8 and monocyte chemotactic protein 1 (MCP-1) by NF-κB (Saccani *et al.,* 2002).

The roles of p38 MAPKs in physiological responses are multifaceted, and these include processes such as cellular differentiation, myogenesis and cellular infiltration into inflammatory sites.

1.7.4.4 Summary

Although TNF-induced activation of MAPKs has been studied intensively in terms of biological effects, the molecular mechanisms that regulate the activation of these MAPKs are still incompletely understood. Studies have shown that TRAF2 is essential for TNF-induced activation of JNK, ERK1/ERK2 and p38 (Liu *et al.*, 1996, Reinhard *et al.*, 1997, Yeh *et al.*, 1997, Liu and Han, 2001). Binding of TNF to TNFRI activates the MAPK kinase kinase kinase kinase (MKKKK) known as the germinal centre kinase (GCK)

through TRAF2 and TRAF-associated NF-κB activator (TANK). TANK is thought to increase the affinity of TRAF2 for GCK, thus increasing MAPK activation by TNF. This ultimately leads to the activation of MAPK kinase 1 (MEKK) 1 (Chin *et al.*, 1999, Chadee *et al.*, 2002). Although both GCK and MEKK1 interact with TRAF2, and GCK is required for MEKK1 activation by TNF, GCK kinase activity does not appear to be required for MEKK1 activation (Chadee *et al.*, 2002). It has been shown that GCK activates MEKK1 by causing MEKK1 oligomerisation and autophosphorylation (Chadee *et al.*, 2002). Once activated, MEKK1 activates the downstream MAPK kinase kinase (MKK) 4 and MKK7, leading to JNK activation with downstream effects on growth, differentiation, survival and apoptosis (Garrington and Johnson, 1999) (Figure 1.5). Recently, using (*Rip*$^{-}$/$^{-}$) fibroblasts, it was shown that RIP is required for TNF-induced activation of all three MAPKs, while the enzymatic activity of the kinase is only required for the activation of ERK, but not JNK and p38 (Devin *et al.,* 2003). Using SB203580 an inhibitor of the enzymatic activity of p38, it was shown that TNF stimulated IL-8 production and superoxide generation from human neutrophils were completely abolished (Zu *et al.*, 1998). fMLP induced chemotaxis and superoxide production were also markedly suppressed by the p38 inhibitor.

1.8 TARGETING TNF-TNFR IN PATHOGENESIS

Owing to its ability to kill tumour cells, TNF held promise as a potent anti-tumour agent. Extensive studies have demonstrated direct cytostatic and cytotoxic effects of TNF

against subcutaneous human xenografts and lymph node metastases in nude mice, as well as immunomodulatory effects to promote tumour cell killing by various cell types, including neutrophils, macrophages and T-cells (Ohnuma *et al.,* 1993). On the other hand, TNF has also been shown to protect haematopoietic progenitors against irradiation and cytotoxic agents, a property which could be applied to aplasia induced by chemotherapy or bone marrow transplantation (Warren *et al.,* 1990).

It is interesting that TNF can initiate two opposing actions, proliferation versus apoptosis. This is likely to be due to the signalling pathways which are activated via the TNF receptors. The IKK-NFκB pathway is believed to promote cell survival whereas the Fas-like IL1-converting enzyme (FLICE -caspase 8), sphingomyelinase-ceramide and JNK pathways are likely to mediate apoptosis. Thus, it has been shown that in the absence of NF-κB activity, susceptibility to TNF-induced apoptosis increases (Beg *et al.*, 1995, Li *et al.*, 1999) whereas enforced activation of NF-κB protects against apoptosis (Chen and Goeddel, 2002). The type of response is very much dependent on the cell type since it has been argued that not only do different cell types express different levels of the various signalling molecules but, the signalling pathways to which receptors are coupled to are also dependent on the cell-types (Nutchey *et al.,* 2005). For example, TNF has been widely demonstrated to activate the sphingomyelinase-ceramide pathway in cell-types such as HL-60 and U937 cells but this effect is not seen in neutrophils (Robinson *et al.*, 1996), as discussed above.

The ability of TNF to enhance the killing of micro-organisms by neutrophils suggests that it could be useful as a natural, non-specific immune enhancer especially in

immunocompromised patients (Ferrante *et al.*, 1995). During the late 1980's, there were many phase 1 trials of recombinant TNF in patients with advanced cancer, in particular in the regional treatment of sarcomas and melanomas (in conjunction with anti-neoplastic agents) (Spriggs *et al.,* 1988). Despite the large number of trials being conducted, these failed to demonstrate significant improvements in cancer treatment, with TNF resistance and TNF-induced systemic toxicity being two major limitations for their use. The most common systemic side effects included fever, rigors, headache, fatigue, myalgia, nausea and vomiting (Schottelius *et al.*, 2004).

Due to its protein-nature TNF has to be administered subcutaneously, intramuscularly or intravenously at doses ranging from 0.04 to 13 mg/kg, with skin ulceration and necrosis at the injection site not uncommon at the higher doses (Zamkoff *et al.,* 1989). Thus, the many pathophysiological properties associated with a pleiotropic molecule such as TNF make it highly unsuitable for use in this manner unless the beneficial properties of the molecule can be harnessed, eliminating the risk of pathological damage.

1.8.1 Anti-TNF antibodies and soluble TNF receptors

The use of TNF inhibitors in inflammatory diseases, such as rheumatoid arthritis, where the cytokine is found in copious amounts in the synovial fluid of patients, has been largely successful. At present, the only drugs that are in clinical practice or in clinical trials to block TNF are biologicals, protein-based drugs, either antibody to TNF or based

on TNF receptors (e.g. linked to Fc dimmers) (Table 1.6). Table 1.7 shows a list of diseases which have been treated with this anti-TNF approach. Although these inhibitors have the major advantage of specificity they also have significant disadvantages such as the need for repeated injection and their relative high cost compared to small organic chemical drugs (Breedveld, 2000, Tugwell, 2000).

The rationale behind the use of anti-TNF therapy in RA is evident in the following:

a) In the joints of the inflamed synovium and the destructive pannus-cartilage junction TNF/TNFR expression is upregulated (Chu *et al.*, 1991, Deleuran *et al.*, 1992, Gabay *et al.*, 1997)
b) When anti-TNF antibodies were used to neutralise inflammatory cytokines in rheumatoid synovial cultures, the results were striking. IL-1 mRNA levels were reduced and within 3 days, IL-1 bioactivity had virtually disappeared. Thus it has been concluded that TNF was the major stimulus inducing IL-1 synthesis in RA synovial cultures (Brennan *et al.*, 1989, Feldmann *et al.*, 1996).
c) Anti-TNF antibodies ameliorate inflammation and joint destruction in murine collagen-induced arthritis (Piguet *et al.*, 1992, Wooley *et al.*, 1993).

Infliximab (Table 1.6) is a chimeric human IgG1 constant region/mouse V region anti-TNF monoclonal antibody which neutralises human TNF but not LT. The ability of infliximab to neutralise TNF both *in vitro* and *in vivo* has been well established (Elliott *et al.*, 1994a, Elliott *et al.*, 1994b, Sekut and Connolly, 1998). Infliximab works by binding to soluble TNF monomers, trimers and membrane-bound TNF to form a stable complex which prevents TNF from binding to its receptor thus blocking its action (Geletka and St

Clair, 2005). The *in vivo* TNF-neutralising action of infliximab may be enhanced due to its ability to activate complement, probably via its gamma-1 heavy chain, and thus to kill cells expressing membrane-bound TNF (Gardnerova *et al.,* 2000). Infliximab has also been reported to have therapeutic effects on patients with psoriasis, Crohn's disease, juvenile chronic arthritis as well as RA (Table 1.7) (Antoni and Kalden, 1999, Chaudhari *et al.*, 2001, Ogilvie *et al.*, 2001). Other anti-TNF monoclonal antibodies include CDP571, adalimumab and PEG Fab (Table 1.6).

An alternative approach has been to use TNFR based agents. Examples of these are: etanercept (Table 1.6), a soluble TNFRII (p75) construct fused to the Fc portion of human IgG1, lenercept, a soluble TNFRI (p55) construct fused to the Fc portion of human IgG1, that binds to and neutralises circulating TNF (Kapadia *et al.*, 1995, Kubota *et al.*, 2000) and onercept, the recombinant unmodified fully human soluble TNFRI, which has shown good efficacy in the treatment of psoriasis and psoriatic arthritis.

1.8.2 Anti-TNF therapy: shortcomings and failures

The use of anti-TNF therapy (infliximab, adalimumab and etanercept) in RA has dramatically improved the outcome of the disease. However, as TNF is also important in host defence mechanisms, especially against intracellular microbial pathogens, the blockade of this cytokine has the potential to lead to an increased rate of infection.

Table 1.6 Inhibitors of TNF in current clinical use

Name	*Composition*
	Monoclonal antibodies
Infliximab (Remicade®)	Chimeric (mouse x human) mAb
CDP571	Humanised murine CDR3 engrafted mAb
D2E7, Adalimumab	Human mAb
PEG-linked Fab	Human mAb
	Soluble TNF receptor: Fc (IgG) fusion proteins
Etanercept (Enbrel®)	p75 TNFR :Fc
Lenercept ®	p55 TNFR: Fc
	PEGp55-TNFR

Modified from (Feldmann and Maini, 2001, Via, 2007).

Table 1.7 Anti-TNF based therapies in various diseases

Disease	Agent
Rheumatoid Arthritis	Etanercept; pegsunercept infliximab; adalimumab; CDP870
Psoriatic arthritis	Etanercept; infliximab; oncercept
Psoriasis	Etanercept; infliximab; oncercept; adalimumab
Sarcoidosis	Infliximab
Adult's Still's disease	Infliximab
Severe acute ulcerative colitis	Infliximab
Spondyloarthropathies	Etanercept; infliximab
Anklosing spondylitis	Etanercept; infliximab
Behcet's syndrome	Infliximab
Crohn's disease	Infliximab; onercept; adalimumab; CDP870
Orofacial Crohn's disease	Infliximab
Uveitis	Etanercept; infliximab
HIV-1 associated psoriatic arthritis	Etanercept
Graft-vs-host disease	Etanercept
Advanced heart failure	Etanercept
CVID	Etanercept
Wegener's granulomatosis	Etanercept

Some studies are reports of non-controlled observations in small number of selected patients (Schottelius *et al.*, 2004). Abbreviations: CVID, common variable immunodeficiency

Recently it has been reported that in patients with active RA, anti-TNF therapy namely infliximab, adalimumab and etanercept, was associated with an increased rate of incidence and seriousness of soft skin infections (Dixon *et al.*, 2006). However, it must be noted that a simple cause and effect relationship between anti-TNF therapy and infections has not been proven, since ageing patients with severe RA who have never been exposed to TNF antagonists have an increased risk of serious infections and cancer (especially lymphoma).

Recently, the elevated levels of TNF in patients with heart failure (HF) triggered interest in investigating TNF's role in the pathogenesis of the condition (Kapadia *et al.*, 1997, Torre-Amione *et al.*, 1999). Experimental and clinical evidence has shown that the high levels of TNF lead to progression of left ventricular dysfunction (Sarzi-Puttini *et al.,* 2005). This has led to anti-TNF clinical trials of etanercept and infliximab in patients with HF. A case report (Kwon *et al.,* 2003), showed that etanercept and infliximab induced a 'new' onset of HF in patients suffering from arthritis and Crohn's disease. Further evidence from the large multi-centre trials of etanercept, RENAISSANCE- Randomised Etanercept North American Strategy to Study Antagonism of Cytokines, RECOVER- Research into Etanercept Cytokine antagonism in Ventricular dysfunction and RENEWAL- Randomised Etanercept Worldwide evaluation, showed that in moderate to severe HF, etanercept did not have any clinical benefits and even suggested that it in fact worsens the disease (Anker and Coats, 2002, Coletta *et al.*, 2002, Mann *et al.*, 2004).

Similarly with infliximab, a clinical trial ATTACH (Anti-TNF Therapy Against Chronic Heart failure) concluded that TNF antagonism did not improve HF and high doses adversely affected the clinical condition of patients with moderate to severe HF (Chung *et al.,* 2003). Failure of anti-TNF therapies on HF and development of HF in RA and recent studies have further highlighted the beneficial role of TNF. Recently, studies have shown that anti-TNF therapy will be more beneficial against RA and HF when there is no concomitant therapy with glucocorticoids or COX-2 inhibitors (Listing *et al.,* 2008). However, it should be noted that this study could not conclusively eliminate the risk of worsening of HF or *de novo* HF as a result of anti-TNF therapy. As the risk of developing HF could be reduced by 30-35 % but that the risk could be increased by 84 % (Gabriel, 2008). At low physiologic levels TNF has been shown to have a cytoprotective effect in the heart during ischemic injury (Nakano *et al.*, 1998, Kurrelmeyer *et al.*, 2000). It is thought that TNF is vital in tissue remodelling and repair (Mann, 2003), as well as enhancing the production of vascular nitric oxide, thus maintaining peripheral blood flow in patients with HF (Katz *et al.*, 1994, Sugamori *et al.*, 2002).

Another major drawback has been the reactivation of latent tuberculosis infection (LTBI) in patients undergoing anti-TNF therapy. The importance of TNF in immunity against *M. tuberculosis* is well documented. Using TNFRI KO and TNF-deficient mice, studies have shown that the formation of granulomas is delayed and not well organised in these animals, resulting in poorly contained foci of infection, due to the lack of recruitment of inflammatory cells by TNF (Roach *et al.*, 2002, Ehlers, 2005). TNF also mediates the bactericidal activity of macrophages against intracellular pathogens, such as *M. tuberculosis* by synergising with IFNγ to reduce bacterial replication and to induce

synthesis of bactericidal NO (Briscoe *et al.*, 2000, Ehlers, 2005). Thus, it is not surprising that the clinical use of anti-TNF therapy is associated with an increased risk of reactivation of LTBI, as animal models have shown regression of formed granulomas and consequently reactivation under such conditions (Keane, 2005, Theis and Rhodes, 2008). Therefore, it is evident that anti-TNF therapies may decrease TNF below physiological levels required for repair of the myocardium in the case of HF or levels required to clear intracellular pathogens in the case of infection.

1.9 TARGETING INTRACELLULAR SIGNALLING MOLECULES IN INFLAMMATORY DISEASES

The well-accepted finding that inflammatory diseases are a consequence of intercellular signalling by cytokines and the appropriate activation of intracellular signalling pathways leading to non-genomic and genomic changes which promote tissue damage, has made the targeting of intracellular signalling molecules an attractive approach in the development of therapeutics for these diseases. This includes inflammatory disorders in which TNF plays a major role. The MAPK family of intracellular signalling molecules has been one of these target focus, namely JNK, ERK and p38. Targeting p38 has been a particularly active area (Table.1.8) (Saklatvala, 2004, Cuenda and Rousseau, 2007, Schindler *et al.*, 2007).

Table 1.8 Summary of human clinical studies with p38 inhibitors

Compound	*Company*	*Clinical indication*	*Study phase*	*Status*	*Reference*
GSK-681323	GlaxoSmithKline	RA, Crohn's psoriasis, COPD	I	Awaited	(Molfino, 2005)
GSK-856553	GlaxoSmithKline	RA, Crohn's psoriasis, COPD	I	Awaited	(Molfino, 2005)
RWJ-67657	Johnson and Johnson	Crohn's, RA, psoriasis, rhinitis	I	Discontinued	(Fijen *et al.,* 2001)
VX-745	Vertex	RA, Crohn's psoriasis, COPD	II	Discontinued-crosses blood brain barrier	(Stelmach *et al.,* 2003)
BIRB-796	Boehringer	Asthma, allergy, RA	II	Awaited	(Kuma *et al.,* 2005)
Scio-469	Scios	RA, Crohn's psoriasis, COPD	II	Awaited	(Nikas and Drosos, 2004)

Abbreviations: RA, rheumatoid arthritis; COPD, chronic obstructive pulmonary disease

1.10 CYTOKINE MIMETICS AS THERAPEUTICS

While the use of TNF against certain tumours seemed logical, two major limitations have prevented its use, that is, TNF resistance and TNF-induced systemic side effects (Schottelius *et al.*, 2004). Similarly, IL-2, a cytokine which stimulates T lymphocyte proliferation, was used in cancer therapy but its therapeutic potential is limited by various side effects such as fever and influenza-like symptoms, a significant vascular leak syndrome, which involves damage to vascular endothelial cells, oedema and organ failure (Rose *et al.*, 2003, Schwinger *et al.*, 2005).

Since most proteins exert their biological activity through the interaction between very small regions of their folded surfaces to their cognate receptors, smaller peptides which mimic the shape of the proteins at the point of contact with the receptors can be used to mimic and/or block the actions of these proteins (Fairlie *et al.,* 1998). Recently, peptide technology has resulted in the discovery of specific inhibitors for cytokine receptors such as a disulphide-cyclised 18-mer peptide AF1721, which inhibits the binding of IL-5 to IL-5Rα and blocks IL-5 dependent eosinophil activation (England *et al.,* 2000). Similarly a 30-mer peptide mimetic of IL-2 that binds to the dimeric IL-2Rβ2 receptor has also recently been discovered. The 30-residue peptide mimetic, like its parent cytokine, activates Shc and $p56^{lck}$ but unlike IL-2, the peptide could not activate the protein kinases, janus kinase-1 (Jak) and Jak-2. Consequently, the signal transduction and activators of transcription (STAT) pathway is not activated by the IL-2 mimetic peptide (Rose *et al.*, 2003). Using phage-display screening with a neutralising anti- IFN-β

monoclonal antibody a 15-mer mimetic peptide of IFNβ was recently isolated (Sato and Sone, 2003). This peptide has been shown to compete with IFN-β for binding to type I IFN receptor in a dose-dependent manner (Sato and Sone, 2003).

With the above precedents, it is envisaged that TNF mimetics can be generated which could produce some of the major actions of TNF. In line with this thinking, (Rathjen *et al.*, 1991) demonstrated, with the use of neutralising anti-human TNF antibodies, that the sequence from amino acids 65 to 85 of the TNF molecule was involved in binding the TNF receptor (Rathjen *et al.*, 1991). This subsequently led to the discovery of a TNF mimetic peptide, TNF_{70-80}, with substitution of leucine-76 for isoleucine to increase stability *in vitro* in the presence of serum (Figure 1.6). This peptide was shown to stimulate and prime neutrophils for increased respiratory burst, which was mirrored *in vivo* as the peptide enhanced neutrophil-mediated killing of intraerythrocytic asexual blood stages of *P. falciparum*, to a degree almost equal to that observed with TNF, and increased the resistance of mice to *P. chabaudi* (Kumaratilake *et al.,* 1995) (Table 1.9). Britton et al (1998) also showed that TNF_{70-80} synergised with interferon γ to stimulate nitric oxide-dependent inhibition of mycobacterial growth. However, several studies have shown that not all the effects of TNF are mimicked by TNF_{70-80}. Kumaratilake et al (1995) demonstrated that TNF_{70-80} failed to increase the expression of E-selectin-1, ICAM-1 and VCAM-1 on endothelial cells and the peptide did not kill tumour cells such as WEHI-164 or trigger death in a sepsis model (Kumaratilake *et al.*, 1995) (Table 1.9).

Another mimetic peptide $TNF_{132-150}$ corresponding to a different region of TNF was found to have properties that differed from those of TNF_{70-80}. $TNF_{132-150}$ lacked the

neutrophil stimulating ability *in vitro*, but interestingly exhibited cytotoxic effects towards tumour cells (Figure 1.6). This effect was mirrored *in vivo*, where the mimetic peptide was shown to be effective in halting tumour growth (Rathjen *et al.*, 1993) (Table 1.9).

Table 1.9 Comparison of the biological effects of TNF, TNF_{70-80} and $TNF_{132-150}$

	TNF	TNF_{70-80}	$TNF_{132-150}$
In vitro			
Induction of CAM expression on EC	+	-	ND
Primes PMN/MØ anti-microbial killing of bacteria, fungi and parasites	+	+	-
Tumour cell cytotoxicity	+	-	+
In vivo			
Promotes clearance of bacteria, fungi and parasites	+	+	ND
Anti-tumour activity	+	-	+
Sepsis	+	-	ND
Cachexia	+	-	ND
Neutrophil and MØ priming	+	+	ND

Abbreviations: CAM, cell adhesion molecules; EC, endothelial cells, PMN, neutrophils; MØ, macrophages; ND, not determined.

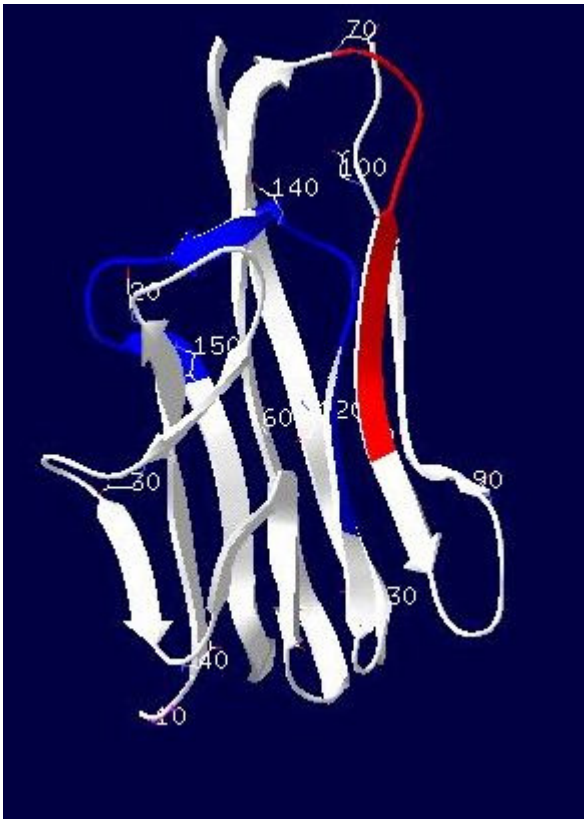

Figure 1.6 The location of TNF_{70-80} and $TNF_{132-150}$ within the TNF structure (monomer). TNF_{70-80} sequence corresponds to the region in red while $TNF_{132-150}$ is depicted in the blue region.

1.11 RATIONALE, AIMS AND HYPOTHESIS

Preliminary data (Haddad, 1998) indicated that while TNF induced the activation of ERK1/ERK2, p38 and JNK, TNF_{70-80} only activated the p38 pathway and in contrast, $TNF_{132-150}$, due to its inability to stimulate neutrophils may not activate the p38 pathway. Thus, it can be rationalised that if TNF_{70-80} functions through the TNFR then it interacts with unique sites on the receptor compared with TNF or $TNF_{132-150}$. Identification of the site could lead to the development of small TNFR mimetic peptides to block TNF-induced p38 activation. Since the TNFR and p38 are key targets in the same inflammatory disorders, it is likely that a more selective inhibition of p38 could be achieved with the TNFR mimetics, sparing the toxicity encountered with inhibiting all response going through p38 or the TNFR.

1.11.1 Hypotheses

The characteristics of the selective biological properties of the TNF mimetics such as TNF_{70-80} and $TNF_{132-150}$ suggest that when they engage the TNFR, the peptides induce a distinct set of intracellular signals. We propose that the difference is related to the ability to recruit adaptor proteins to the cytoplasmic portion of the TNFR. TNF_{70-80} binds specifically to certain amino acid residues on TNFR and that peptides made from such residues on TNFR can be used to inhibit the TNFR-p38 signalling pathway and associated cellular activation and inflammation.

1.11.2 Aims

The general aim of this project is to identify the basis for the difference in the properties between these TNF mimetic peptides, and identify a new approach to developing new therapeutics for chronic inflammatory diseases.

Specifically to:

1. determine whether or not these mimetics bind specifically to TNF receptor.
2. identify whether these peptides stimulate distinct intracellular signalling pathways.
3. attempt to identify the region of the TNFRI which binds these mimetics
4. investigate whether peptides derived from the TNF_{70-80} binding region on TNFRI selectively block TNF responses.
5. examine the anti-inflammatory properties of the TNFR mimetic peptides.

2.0 CHAPTER TWO: MATERIALS AND METHODS

2.1 REAGENTS

2.1.1 Biochemicals and antibodies

Ficoll 400, percoll and lymphoprep were obtained from Pharmacia Biotech (Uppsala, Sweden). RPMI-1640, HBSS, foetal bovine serum (FCS) and DMEM were purchased from JRH Biosciences (Lenexa, KA). N-formyl-methionyl-L-leucyle-L-phenylalanine (fMLP), lucigenin (9, 9'-bis (N-methyl-acridinium nitrate), lipolysaccharide (LPS), 3',3',5',5'-tetramethylbenzidine (TMB) substrate, benzamidine, DL-dithiothreitol (DTT), leupeptin, pepstatin A, *p*-nitro phenylphosphate, phenylmethylsulfonyl fluoride (PMSF) and carrageenan type IV were purchased from Sigma Chemical Company Ltd (St Louis, Mo). Angiografin was obtained from Bayer Schering Pharma (Berlin, Germany), aprotinin (Bovine Lung, 10000 U/5 ml) and agarose were purchased from Calbiochem (San Diego, CA). Anti-p38 antibody, anti-NF-κB (p65) and anti-ERK1/ERK2 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Horseradish peroxidase streptavidin conjugate was obtained from Endogen (Rockford, ILL). PE-labelled anti-CD11b antibody and PE-labelled IgG1 isotype were purchased from Becton Dickinson (San Jose, CA). LipofectAMINE 2000, TRIzol reagent and cytokine primers were purchased from Invitrogen (Mount Waverley, VIC, Australia). Nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH). Isoton II and bovine albumin (BSA) were purchased from Beckman Coulter (Fullerton, CA). iScript cDNA synthesis kit and iQ SYBR Green supermix were obtained from Bio-Rad (Gladesville, NSW, Australia). Sheep blood was obtained from the Institute of Medical and Veterinary Science (IMVS) (Adelaide, SA, Australia).

2.1.2 Plasmids

The pRK5-TRAF2-FLAG and pRK5-TRAF$_{287-501}$-FLAG plasmids were provided by Dr P Xia, Hanson Institute, Adelaide, Australia). The hTNFRI plasmid was kindly provided by Dr. M. Kronke (Institute of Medical Microbiology and Hygiene, Medical Center University or Cologne, Koln, Germany).

2.2 ANIMALS

Female BALB/c mice, 6 - 8 weeks were purchased from the Adelaide University Animal House. The animals were housed in the Children, Youth and Women's Health Services (CYWHS) Animal House. The study was approved by the CYWHS Animal Ethics Committee and the University of Adelaide Animal Ethics Committee.

2.3 CYTOKINES AND PEPTIDES

2.3.1 Cytokines and receptors.

Human recombinant TNF was a gift from Dr. G.R. Adolf (Ernst-Boehringer Ingelheim Institut, Vienna, Austria) and produced by Genetech Inc. (California, USA). The specific activity was 6 x 10^7 U/mg as assessed for cytotoxicity by the supplier on actinomycin-D-treated murine fibrosarcoma cell line, L929. The concentration of TNF stock solution was confirmed by ELISA (Ferrante *et al.*, 1990) to be 1 pg/ml. TNF was also purchased from ProSpec-Tany TechnoGene (Rehovot, Israel). TNF stock solution was stored at -20°C until required. Fresh dilutions of TNF were prepared in HBSS just prior to use. Human recombinant soluble TNFRI (rHusTNFRI, prepared as a chimaeric protein with the 6X histidine tagged Fc portion part of human IgG1) was purchased from Prospec-Tany TechnoGene (Rehovot, Israel).

2.3.2 Peptides

TNF_{70-80} (Kumaratilake *et al.*, 1995) and its control (GGDPGIVTH) were synthesised by Auspep Pty Ltd, (Victoria, Australia). While it is difficult to directly equate the TNF activity to TNF_{70-80} because of the lack of tumour cell cytotoxicity of this peptide, previous comparisons on their ability to stimulate nitric oxide production in macrophage show that 5.0 μg/ml of TNF_{70-80} equates to 1000 U/ml of TNF (Britton *et al.,* 1998). The sequence from the natural TNF-α was modified by substitution of isoleucine for leucine at position 79. The modification extended the serum half-life of the peptide to > 90 min and increased water solubility. Fresh dilutions of the peptide were prepared daily in HBSS or HBSS with 1 % BSA. The His-tagged M4 (HM4) (HHHHHHLKPGTT) and

M4 (LKPGTT) were purchased from Auspep Pty Ltd (Victoria, Australia). $TNF_{132-150}$ (LSAEINRPDYLDFAESGQV), $TNFRI_{209-211}$ peptide (GTT); scrambled control (TGT) and $TNFRI_{206-211}$ (EDSGTT); scrambled control (GEDTST), D-amino form of $TNFRI_{206-211}$ ({d-GLU} {d-ASP} {d-SER} {d-GLY} {d-THR} {d-THR}), $TNFRI_{198-211}$ (QIENVKGTEDSGTT) and tandem repeats of $TNFRI_{209-211}$ (GTTGTTGTTGTT) were purchased from GenScript (New Jersey, USA). Peptides were purified by high-performance liquid chromatography (HPLC) and analysed by mass spectrometry to be > 85 % pure. Peptides were stored at -20ºC and solutions were made in HBSS or RPMI 1640 prior to use.

2.4 PURIFICATION OF HUMAN LEUKOCYTES

2.4.1 Purification of peripheral mononuclear cells and neutrophils from whole blood

Peripheral blood mononuclear leukocytes (MNL) were purified essentially by the methods outlined of Ferrante and Thong (1982). Briefly, whole blood was layered onto Hypaque-Ficoll gradient with a specific gravity of 1.114 and cells separated by centrifugation at 600 g for 35 min. This resolves leukocytes into two bands, the top containing the MNL and the lower band neutrophils. The MNL were harvested, re-suspended in RPMI 1640 and washed in the medium by repeated centrifugation. Neutrophils were similarly harvested and prepared. The MNL and neutrophils were of > 98 % viability as judged by the ability to exclude Trypan blue. In some cases, the

neutrophil preparations were further enriched in purity by centrifugation over lymphoprep which removed any contaminating MNL. In addition in some, cases when erythrocyte contamination was evident these were removed by centrifugation at 80 g for 5 min.

2.4.2 Production of TNF-rich medium (TNF-RM) culture fluids

Cell culture fluids rich in TNF and neutrophil priming properties were prepared essentially as described previously (Bates *et al.,* 1991). Briefly, MNL (4×10^7) in 20 ml were resuspended in 20 ml RPMI 1640 medium with 200 mM L-glutamine solution, 100 U/ml penicillin and 100 µg/ml streptomycin and mixed with heat-killed *Staphylococcus aureus* (2×10^8) in 20 ml RPMI 1640 with 5 % human blood group AB serum. The cell cultures were set up in 250 ml flasks and incubated at 37ºC for 24 h in 5 % CO_2 and high humidity. After incubation the cells were removed by centrifugation and the supernatants collected by using a 0.22 µm filter and stored in 1ml aliquots at -70 ºC. Medium conditioned by *S. aureus*-treated MNL was termed 'TNF-rich medium' (TNF-RM). Medium conditioned by non-stimulated MNL alone was termed control medium (CM).

2.5 CELL LINES AND THEIR MAINTENANCE

2.5.1 WEHI-164

A mouse fibroblastic fibrosarcoma cell line WEHI-164 was obtained from Dr Geeta Chauhdri (John Curtin School of Medical Research, Australian National University, Canberra). The cells were maintained in RPMI 1640 medium (supplemented with 10 % heat-inactivated foetal calf-serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) and passaged every 2-3 days. Cells were detached with 0.25 % trypsin in a 0.02 % EDTA solution for 1 min.

2.5.2 HEK 293T

HEK 293T cells were obtained from the American Type Culture Collection (Virginia, USA) and maintained in DMEM, supplemented with 10 % foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were stably transfected with pM1, pM3, pM4, pWT of the p55 TNFRI or an empty plasmid using LipofectAMINE 2000. HEK293T cells were stably transfected with WT-TRAF2, ΔTRAF2 or empty plasmid (Xia *et al.,* 2002).

2.5.3 Mono Mac 6 cells

The monocytic cell line Mono Mac 6 was obtained from Dr H.W. L. Ziegler-Heitbrock (Institute of Clinical Chemistry and Pathobiochemistry, Klinikum rechts der Isar, Technische Universitat, Munchen, Germany). The cell line was maintained in RPMI 1640 supplemented with 10 % heat-inactivated foetal calf-serum, 1 mM pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Ferrante *et al.*, 1997).

2.5.4 Pre B-cell line 70Z/3

70Z/3 cells, a mouse Pre B cell line that lacks binding sites for human TNF were obtained from Prof. Wallace Langdon (School of Pharmacy and Laboratory Medicine, University of Western Australia) (Kruppa *et al.,* 1992). Cells were maintained in RPMI 1640 with 4.5 g/l glucose, 2 mM L-glutamine, 50 µM 2-mercaptoethanol and 10 % FCS in an atmosphere of 95 % air and 5 % CO_2.

2.5.4.1 Transfection

Cells were plated into 10 cm plate at 4 $x10^7$ cells in 15 ml culture medium. Before transient transfection, plasmid DNA (hTNFR1WT) (20 µg in 1ml of RPMI 1640) were

mixed with LipofectAMINE 2000 (Invitrogen) (60 µl in 1ml of RPMI 1640) and the mixture were left to stand at room temperature for 20 min. This was directly added to cells and incubated for 24 h.

2.5.5 Endothelial cell culture

Endothelial cells were prepared essentially as described previously (Jersmann *et al.*, 2001). Fresh human umbilical cords were obtained from consenting mothers according to the institution's guidelines on human ethics. Human umbilical vein endothelial cells (HUVECs) were obtained from fresh umbilical cords by collagenase digestion and maintained in RPMI 1640, supplemented with 20 % AB serum, L-glutamine, penicillin and streptomycin (Jaffe *et al.,* 1973). Cells were grown on gelatin (0.2 %) coated 75 cm^2 flasks until confluent. They were trypsinised from the first passage of primary cultures. For experiments the cells were transferred to 0.2 % gelatin coated 60 mm culture dishes and grown to confluency (2-3 days after seeding) prior to use.

2.6 CONSTRUCTION OF TNFRI MUTANTS

The signal sequence from the p55 TNF-receptor cDNA was isolated by PCR and cloned, via a NheI site incorporated into the 5' primer and a KpnI site in the 3' primer, into the expression vector pcDNA3.1. A double stranded oligonucleotide encoding His 6-tag,

with KpnI and BamHI sticky ends, in frame with the signal peptide, was cloned downstream of the signal sequence. The transmembrane and cytoplasmic domains of the TNF receptor were also isolated by PCR and cloned, via a 3' BstXI site, and a 3' XhoI site incorporated into the PCR primers, 3' of the His 6 sequence. This construct was designated pcDNA3sigHIScTNF. The mutants designated M1 and M4 which lacked the 1st, and all four cysteine rich domains respectively were isolated using the restriction enzyme sites incorporated into the primers, cloned between the BamHI and 5' BstXI sites of the pcDNA3sigHIScTNF, such that the reading was contiguous with the signal His 6 and transmembrane/cytoplasmic coding sequences.

2.7 SOLID-PHASE LIGAND BINDING ASSAY

Microtitre plates were coated with 100 µl/well of 3 µg/ml rHusTNFRI (prepared as a chimaeric protein with the 6X histidine tagged Fc portion of human IgG1) in 18 mQ-cm H_2O overnight at 4°C and blocked with 1 % BSA and 0.05 % Tween 20 in PBS for 1 hr at 4°C. 10 µM of biotin-labelled TNF_{70-80} plus various concentrations of unlabelled TNF_{70-80} or control peptide were added to each well and incubated for 3 h at 4°C. The wells were washed four times with wash buffer (PBS containing 0.05 % Tween 20) and the plates were then incubated with 100 µl/well of horseradish peroxidase streptavidin conjugate (1: 6000 dilution in 1 % BSA and 0.05 % Tween 20 in PBS) for 45 min at 4°C. Wells were then washed four times with wash buffer and bound enzyme detected by the

addition of 3',3',5',5'-tetramethylbenzidine (TMB) substrate. Absorbance was measured using dual filter at 450/570 nm on a Dynatech MR700 Plate Reader.

2.8 ACTIVATION OF INTRACELLULAR SIGNALLING MOLECULES

2.8.1 Preparation of cell lysates

Cells were treated with TNF or peptide in a serum-free medium for a specific time period. After washing twice with cold HBSS, the cells were harvested by scraping (if applicable) and lysed in 300 µl of buffer A (20 mM HEPES, pH 7.4, 0.5 % (v/v) Nonidet P-40, 100 mM NaCl, 1mM EDTA, 2 mM Na_3VO_4, 2 mM DTT, 1 mM PMSF, and 10 µg/ml *p*-nitrophenylphosphate, leupeptin, aprotonin, pepstatin A, and benzamidine) for 2 h at 4 ºC with constant mixing (Hii *et al.*, 1998). After centrifugation (12,000 g x 5 min), the supernatants were collected and stored at -70 ºC for kinase assays.

2.8.2 Lowry's protein assay

Lysates from nuclear and cytoplasmic fractions were assayed for protein content according to the Lowry method (Harrington, 1990). Protein standards were obtained by diluting BSA (1 mg/ml) with H_2O, resulting in six standards (0 µg, 3.125 µg, 6.25 µg, 12.5 µg. 25 µg, 50 µg). Each unknown sample (5 µl) was diluted in 45 µl of H_2O, then

150 µl $CuSO_4$/Lowry's solution (2 % Na_2CO_3, 1 % SDS, 0.4 % NaOH, 0.16 %, Na/K tartrate (1:100) was added to all tubes and left to stand at room temperature for 15min. Folin and Ciocalteau's Phenol Reagent: H_2O (1:1), (15 µl), was then added and the tubes were left to stand for 20 min. An aliquot (180 µl) of each sample was transferred into a 96-well microtitre plate and the absorbance at 540 nm was measured in a Dynatech MR7000 plate reader (Guernsey, Channel Islands). The absorbance of the proteins standards was plotted on a linear regression nomogram (Instat, GraphPad Software Incorporated, San Diego, CA, USA) and the protein content of the test samples was calculated.

2.8.3 ERK and p38 activity

The activity of ERK1/ERK2 and p38 was determined as previously described (Hii *et al.*, 1998). Cell lysates containing equal amounts of protein (0.5-1 mg) were precleared with 15 µl of protein A sepharose beads (1:1 slurry) (4ºC) before being incubated with anti-p38 antibody (3 µg/sample). After mixing for 2 h (4ºC), the immune complexes were precipitated by the addition of protein A sepharose. The immunoprecipitates were collected by centrifugation (16,000 g x 15 s) and washed once with buffer A (4 °C), once with buffer B (10 mM Tris/HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA and 100 µM Na_3VO_4) and once with assay buffer [(20 mM HEPES, pH 7.2, 20 mM β-glycerophosphate, 10 mM Sigma 104, 10 mM $MgCl_2$, 1 mM DTT, 50 µM orthovanadate, 20 µM cold ATP). The assay was initiated by adding 10 µCi of [γ-^{32}P] ATP to the tubes,

which were then incubated at 30°C for 20 min (Hii *et al.*, 1998). ERK and p38 activities were determined as described using myelin basic protein as a substrate (Hii *et al.*, 1998). The reaction was terminated by the addition of Laemmli buffer (20 mM Tris-HCl, pH 6.8, 40 % sucrose, 6 % SDS and 10 mM β-mercaptoethanol) and the samples heated at 100°C for 5 min. Phosphorylated myelin basic protein was fractionated on a 16 % SDS polyacrylamide gel, and the radioactive bands were detected using an Instant Imager (Packard Instruments, Canberra, Australia).

2.8.4 JNK assay

A solid phase assay was used for the determination of JNK activity as described previously (Hii *et al.*, 1998). Lysates containing equal amounts of protein (0.6 mg-1 mg) were added to 50 µl of GST-c-jun (1-79) beads (1:1 slurry) and $MgCl_2$ and ATP added to a final concentration of 15 mM and 10 µM respectively, and the samples mixed for 2 h at 4°C. The samples were then washed with lysis buffer (2 x 300 µl), with wash buffer (10 mM PIPES, pH 7.0, 100 mM NaCl, 2 mM DTT, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A and 10 µg/ml benzamidine) (2 x 300 µl) and once in assay buffer (300 µl). Kinase assays were initiated and terminated as described above for ERK1/ERK2 and p38. Phosphorylated GST-c-jun (1-79) was separated on a 12 % SDS-PAGE gel run at 175 volts for 90 min and incorporated radioactivity present in the GST-c-jun (1-79) peptide was quantified using a Packard Instant Imager (Packard, Canberra, Australia) and integrated using the imager software (Version 2.0.5).

2.8.5 Measurement of NF-κB activation

The nuclear translocation of NF-κB was used as an indicator of the activation of the NF-κB pathway (Ferrante *et al.*, 2006). Neutrophils (3 x 10^7) were treated with HBSS, TNF_{70-80} (10 μM) or TNF (1000 U/ml) for 30 min or 1 h. The cells were then centrifuged (1400 g for 5 min) and the pellet was resuspended in cold HBSS. Following futher centrifugation the cells were lysed for 30 min on ice by adding 200 μl NF-κB lysis buffer (10 mM HEPES pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 300 mM sucrose, 0.5 % Nonidet P-40, 1 mM dithioreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 10 μg/ml benzamidine). The tubes were centrifuged as before, and the pellets were washed with 200 μl NF-κB lysis buffer, centrifuged and resuspended in 40 μl NF-κB lysis buffer. The nuclei were then disrupted by sonication. After centrifugation (1400 g 2 min), the supernatants containing nuclear fractions were mixed with Laemmli buffer (20 mM Tris-HCl pH 6.8, 40 % sucrose, 6 % SDS and 10 mM β-mercaptoethanol), boiled (100 °C for 5 min) and stored at – 70 °C.

For western blotting, equal amounts of denatured protein from each lysate were separated by 12 % SDS polyacrylamide gel electrophoresis (PAGE) for approximately 60 min. The proteins were transferred to nitrocellulose membranes (3 h at 40 V). After transfer, the blots were stained with ponceau S (0.1 % in 5 % acetic acid) to confirm equal protein loading and complete transfer of all proteins. The membrane was then blocked for 1 h at

room temperature in blocking buffer (25 mM Tris/HCl pH 7.4, 100 mM NaCl, 5 % skim milk). Subsequently blots were incubated (1 h at 37° C) with rabbit primary antibody (NF-κB) at 1:1000 (diluted in blocking buffer + 0.1 % Tween-20). The membrane was then washed (3 x 5 min) and incubated with HRP conjugated secondary antibody (sheep anti-rabbit) at 1:1000, for another hour at 37 °C. After 3 x 5 min washes, the immunocomplexes were visualised with enhanced chemiluminescence. The blots were scanned and quantifed by laser densitometry using Image Quant™ scanner and software (Molecular Dynamics, USA).

2.9 CYTOTOXICITY ASSAY

The cell survival assay was performed using a modified method described by (Espevik and Nissen-Meyer) (1986) and (O'Toole *et al.*) (2001). Briefly, WEHI-164 cells were grown in 96-well culture dishes to a density of 5 x 10^4 cells/well, in growth medium with 1 mg/ml of actinomycin D. After incubation with specific dilutions of TNF and $TNF_{132-150}$ for 20 h at 37 °C, 5 % CO_2, cells were washed once with PBS and incubated with 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS; 20 µl/well) for a further 4 h. The reaction was terminated by the addition of 50 µl of 20 % SDS in 20 mM HCl and absorbance read at 540 nm after overnight incubation to solubilise the reduced MTT.

2.10 CHEMILUMINESCENCE ASSAY

Neutrophil superoxide production was measured by an indirect assay involving the reduction of lucigenin, (9, 9'-bis (N-methyl-acridinium nitrate) and measuring the light output (Gyllenhammar, 1987). Lucigenin-dependent chemiluminescence was measured as described previously (Hardy *et al.,* 1995). Neutrophils ($5x10^5$) were incubated with TNF or other stimuli that had previously been pre-incubated in the absence or presence of receptor peptides for 20 min. After incubation 500 µl of lucigenin (250 µM, final) was added and the final volume was adjusted to 1 ml. The cells were placed in luminometer (Autolumat Plus Model LB 953, Berthold Technologies, Bundoora, Australia) and the resulting chemiluminescence (in relative light units, RLU) measured, the data was automatically stored in Microsoft Excel. The results are expressed as peak chemiluminescence produced unless specified otherwise.

2.11 MEASUREMENT OF CR3 EXPRESSION

The upregulation in the expression of the complement receptor type 3 (CR3; CD18/CD11b) in neutrophils was measured by flow cytometry using anti-CD11b antibodies as described (Moghaddami *et al.,* 2003). Neutrophils ($1x10^6$) were incubated for 30 min with TNF that had previously been pre-incubated in the absence or presence of receptor peptides for 20 min. After treatment, 2 µl of IgG1 isotype control or PE-labelled anti-CD11b antibody was added to neutrophils in 200 µl of Isoton II

supplemented with 0.1 % (w/v) BSA. After 30 min the cells were washed, fixed with formaldehyde (0.1 %) and analysed on a FACScan (Becton Dickinson). Data analysis was performed on WinMDI 2.9 software.

2.12 ANALYSIS OF CYTOKINE AND CHEMOKINE GENE EXPRESSION BY QUANTITATIVE REAL-TIME PCR

2.12.1 Isolation of total RNA

Total RNA was extracted from 1 x 10^6 neutrophils using the TRIzol reagent according to the manufacturer's instructions. Cells ($1x10^6$) were pelleted and lysed in 1 ml Trizol for 5 min. To extract the RNA, 200 µl of chloroform was added, vigorously shaken for 15 s and incubated for 5 min. Samples were then centrifuged at 12,000 g for 10 min at 4°C. The upper aqueous phase was transferred to a new tube, and the RNA was precipitated by adding 500 µl of isopropanol, gently inverted, and then incubated at room temperature for 10 min. After centrifugation (12,000 g for 10 min at 4°C), the supernatant was discarded and the RNA pellet was washed with 1ml of 75 % ethanol. After brief centrifugation (7500 g for 5 min at 4°C) the RNA pellet was allowed to air-dry briefly and then 20 µl of RNAse/DNAse free water was added to each tube and pulse-vortexed briefly (~5 sec). The RNA was dissolved by incubation at 55°C for 10 min and stored at –20°C, until required for cDNA synthesis. Purity of the RNA samples was assessed by determining the optical density at 260 nm and 280 nm expressed as a ratio.

2.12.2 Synthesis of cDNA (Reverse Transcription)

Single-strand (first-strand) cDNA was synthesised from total RNA using iScript cDNA synthesis kit according to the manufacturer's instructions. In this reaction 4 µl of 5 iScript reaction Mix and 1 µl of iScript Reverse Transcriptase was added to 100 fg- 1 µg of RNA and water up to 15 µl. The conditions were as follows: 25°C for 5 min, 42°C for 30 min and 85 °C for 5 min.

2.12.3 Quantitative real-time PCR

Primers for cytokines were designed using Invitrogen Oligo Perfect Designer website (Table 2.1). These primers were synthesized by Invitrogen. The synthesised cDNA was amplified in 20 µl triplicate reactions with iQ SYBR Green supermix, 1µl of cDNA and 500 nM of each specific primer pair and house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using iQ5 system with v.3.1 software. The were as follows: an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s.

Table 2.1 Primer sequences

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
IL-1β	TCA TTG CTC AAG TGT CTG AAG C	TCC TGG AAG GAG CAC TTC AT
IL-8	TCT GTG TGA AGG TGC AGT TTT G	AAT TTC TGT GTT GGC GCA GT

2.13 *IN VIVO* MODELS OF INFLAMMATION

2.13.1 Lipopolysaccharide-induced peritoneal inflammation

Female BALB/c mice (Adelaide University Animal House) were housed under a 12 h light-dark cycle and housed in cages with ad-lib access to food and water in a temperature controlled room until the time of experiments. LPS-induced peritoneal inflammation was induced as previously described (Ferrante *et al.*, 2006). Mice were injected intraperitoneally with 50 μg of LPS or HBSS. At 24 h, the animals were euthanized and the peritoneal cavity was washed with 2 ml HBSS to collect leukocytes. Cytospins of the cellular infiltrates were prepared by centrifugation of 200 μl (vehicle challenge) or 100 μl (LPS challenge) aliquots. Slides were fixed and stained with a Giemsa stain. The total leukocyte number in the peritoneal exudates was enumerated using a haemocytometer chamber.

2.13.2 Delayed-type hypersensitivity

The delayed-type hypersensitivity (DTH) response was induced in 12 week old female BALB/c mice as described previously (Ferrante *et al.*, 2006). Briefly mice were injected with 100 µl of a 10 % haematocrit sheep red blood cell (SRBC) suspension. After 6 days, mice were challenged subcutaneously via the right hind footpad with 25 µl of 40 % suspension of SRBC. The DTH response was determined 24 h post challenge by measuring the footpad thickness of the unchallenged vs. SRBC injected footpads with a calliper and micrometre screw gauge. The inflammatory response was expressed as the difference in thickness (mm) between the unchallenged and challenged footpads.

2.13.3 Carrageenan-induced paw inflammation

Carrageenan-induced paw inflammation was induced as described previously (Costabile *et al.,* 2001). Briefly, BALB/c mice were inoculated with 1 ml/kg of a 1% solution of carrageenan type IV into the right hind footpad. The swelling/inflammation was assessed 24 h later by comparing the thickness between the unchallenged vs. carrageenan challenged footpads as described above.

2.14 STATISTICS AND ANALYSIS

Data are presented as the mean ± standard error of the mean (SEM) values unless stated otherwise. Statistical significance was assessed by analysis of variance (ANOVA). Difference were considered statistically significant when $p < 0.05$. All statistical analyses were performed on GraphPad Prism 5 software.

3.0 CHAPTER THREE: TNF_{70-80} ACTS THROUGH TNF-RECEPTORS

3.1 INTRODUCTION

It has been previously shown that the TNF_{70-80} mimetic peptide demonstrated selective biological properties in comparison to its parent cytokine TNF (Kumaratilake *et al.*, 1995). The results suggested that the TNF mimetic was likely to act via the TNFR. The results showed that an anti-TNF monoclonal antibody (mAb54) and mAb against either the p75 TNFRII or the p55 TNFRII inhibited the ability of TNF_{70-80} to stimulate superoxide production in neutrophils. Other studies provided further evidence for this suggestion (Briscoe *et al.*, 2000). These anti-human TNF mAb54 and a soluble TNFRI human IgG fusion protein blocked the synergistic bacteriostatic action of IFNγ and TNF, and IFNγ and the TNF mimetic peptide on bone marrow derived macrophages infected with mycobacteria (Briscoe *et al.*, 2000). However, direct evidence for a ligand-receptor interaction is lacking. Studies were therefore conducted to address this issue.

3.2 TNF_{70-80} STIMULATES P38 ACTIVATION THROUGH TNFRI

Since TNF and the TNF_{70-80} stimulate p38 and it was previously shown that this is important in the neutrophil antimicrobial activity (Zu *et al.*, 1998, Forsberg *et al.*, 2001), we used p38 activation as a marker for neutrophil activation to study the interaction between TNF_{70-80} and the TNFR. This was addressed by making use of the pre B 70Z/3 cell line which lacks binding sites for and proved to be non-responsive to human TNF (Kruppa *et al.*, 1992). The B cell line and HL-60 cells (positive control), were treated

with TNF for 5 min and p38 activity assayed. The results show that indeed the pre B 70 Z/3 cell line was unable to respond to TNF, while HL-60 responded (data not shown). The pre B cells were then transiently transfect with wild type human TNFRI (hTNFRIWT) using the lipofectamine 2000 reagent. After 24 h the cells (non-transfected and transfected) were treated with diluent, TNF_{70-80} (10 μM), TNF (1000 U/ml) or control peptide (10 μM) at 37°C for 5 min. The cells were then lysed and the protein contents were quantified and p38 activity was assayed. The data show that neither TNF nor TNF_{70-80} activated p38 in the non-transfected 70Z/3 cells (Fig 3.1). In comparison both molecules caused the activation of p38 in cells transfected with hTNFRI. The control peptide failed to activate p38 in cells expressing the TNFRI (data not shown).

3.3 TNF_{70-80}- BINDS TO TNFRI

Previously, attempts to label TNF_{70-80} with an ^{125}I-tyrosine residue to study ligand-receptor binding had resulted in its altered function and failure to associate with neutrophils in binding assays (Kumaratilake *et al.*, 1995). Thus a biotinylated form of TNF_{70-80} was synthesised by Auspep (Victoria, Australia). To determine whether the synthetic TNF_{70-80} peptide was indeed a ligand for the TNFRI, competitive binding of TNF_{70-80} and biotin-labelled TNF_{70-80} to recombinant human soluble TNF receptor inhibitor/Fc chimera (rHusTNFRI) was investigated using a solid-phase binding assay as described in Materials and Methods. The data show that unlabelled TNF_{70-80} was able to inhibit biotin-labelled TNF_{70-80} binding to immobilised rHusTNFRI in a concentration-

dependent manner (Figure 3.2), with the half-maximal inhibition concentration (IC_{50}) of ≈ of 55 μM (GraphPad Prism 5 software). A scrambled control peptide had no effect on biotin-labelled TNF_{70-80} binding at a similar concentration (Figure 3.3).

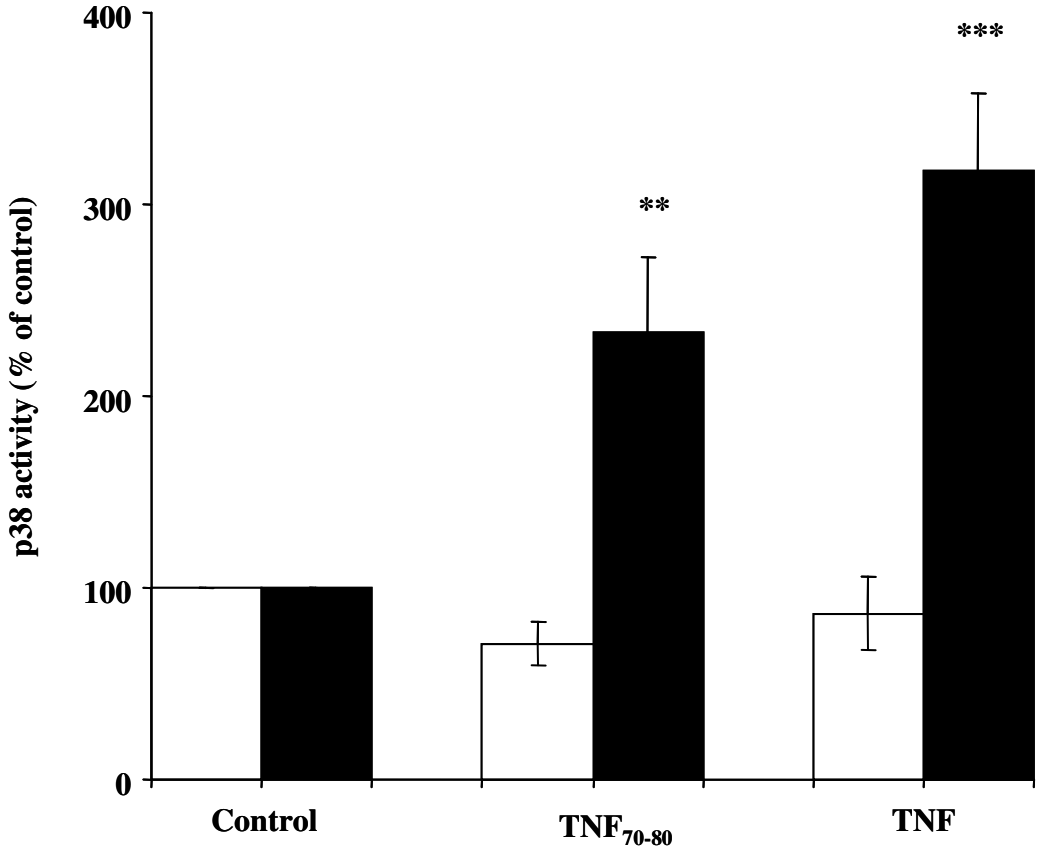

Figure 3.1 Inability of TNF_{70-80} to stimulate p38 activation in cells lacking TNFR. Transfected (hTNFRIWT) (solid bars) or non-transfected (open bars) 70Z/3 cells were treated with TNF_{70-80} (10 μM), TNF (1000 U/ml) or control peptide (10 μM) at 37°C for 5min. The cells were then lysed, protein contents quantified and p38 activity was assayed. The results are presented as the mean ± SEM of 3 experiments. Significance of difference between non-transfected cells and transfected cells: ** $p <$ 0.01 and *** $p <$0.001 (Tukey-Kramer Multiple's Comparison Test).

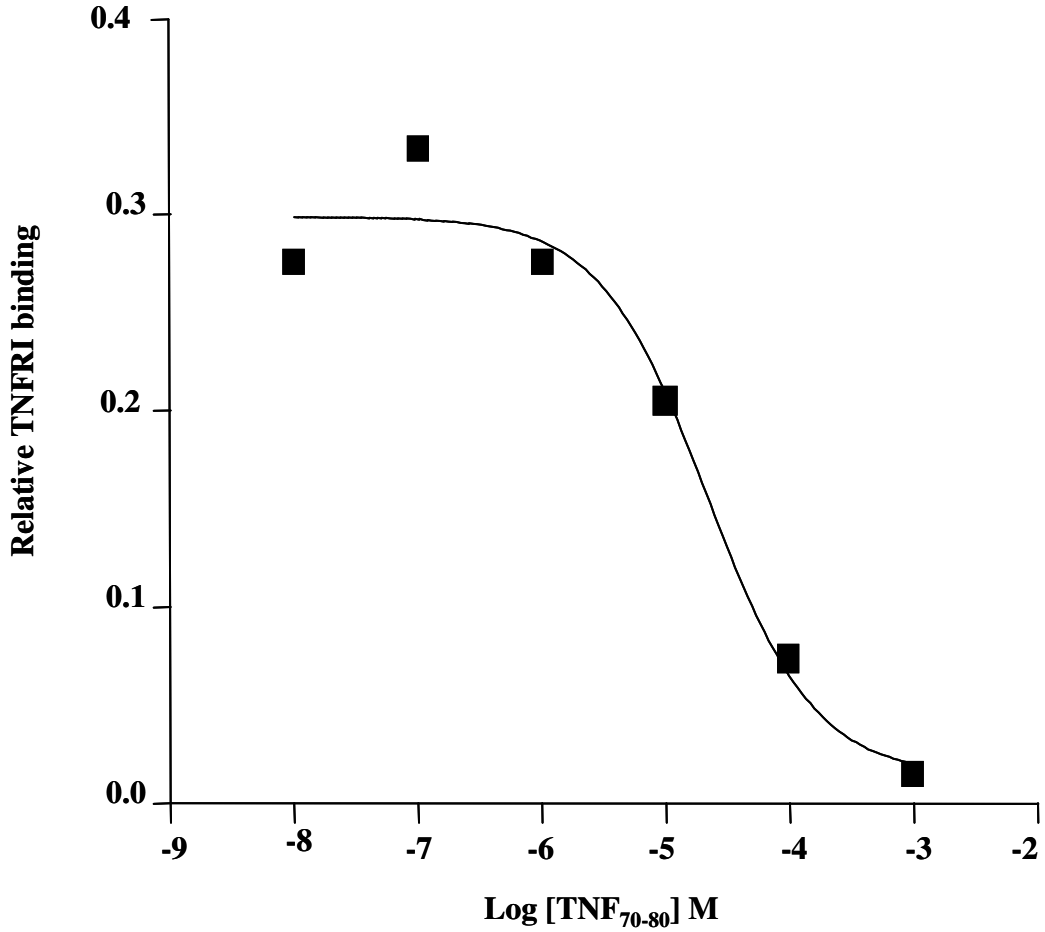

Figure 3.2 Estimation of TNF_{70-80} binding affinity using a simple microtitre-plate based competition binding. Biotinylated TNF_{70-80} (10 μM) as the tracer was incubated with the indicated concentrations of unlabelled TNF_{70-80}. In a microtitre plate with wells coated with rHusTNFRI (3 μg/ml) as described in Chapter 2. Bound biotinylated TNF_{70-80} was detected by the addition of poly-horseradish peroxidase streptavidin and 3',3',5',5'-tetramethylbenzidine (TMB) substrate. The IC_{50} of ≈ 55 μM was obtained (mean of duplicate wells from two experiments) using GraphPad Prism 4.

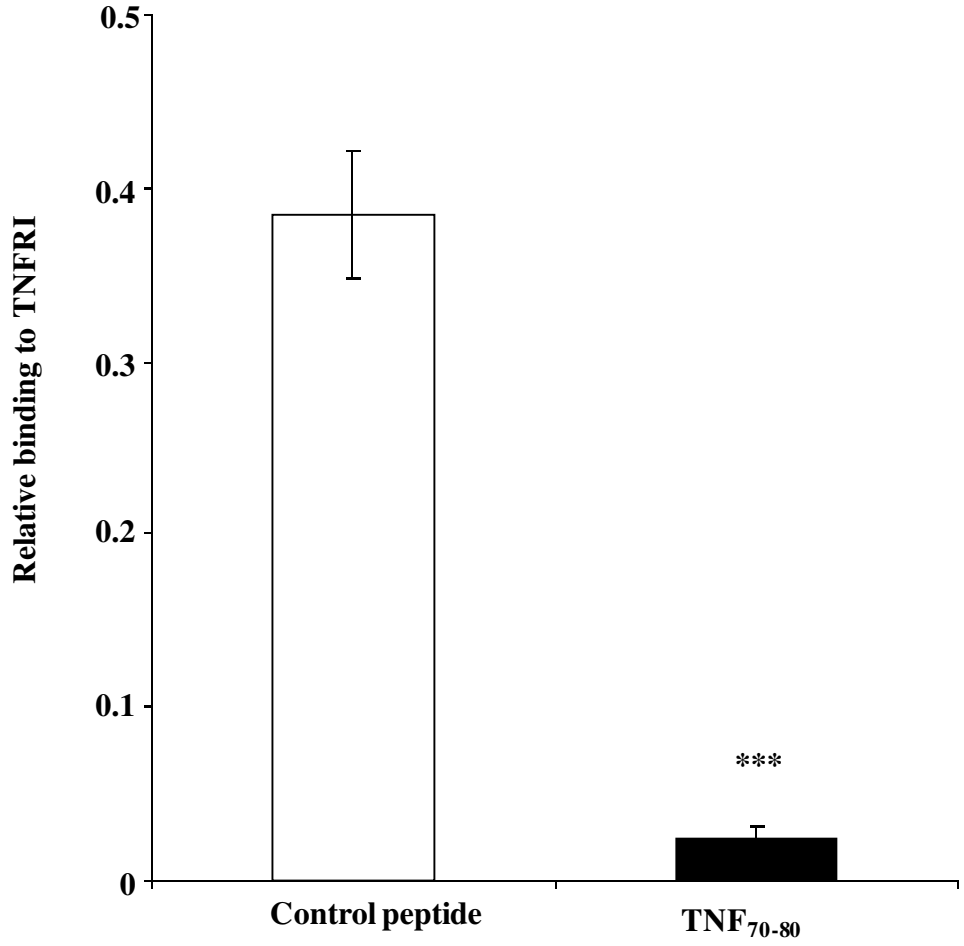

Figure 3.3. Lack of effect of control peptide on the binding of biotin-labelled TNF_{70-80} to immobilised rHusTNFRI. Biotinylated TNF_{70-80} (10 μM) plus 1000 μM of TNF_{70-80} or control peptide were added to microtitre wells previously coated with rHusTNFRI (3 μg/ml). Bound biotinylated TNF_{70-80} was detected as described in legend of Figure 3.2. All data represent means ± SEM from duplicate wells from 3 independent experiments. Significance of difference between control peptide and TNF_{70-80}: *** p <0.001 (Students t test).

3.4 SUMMARY

The data demonstrate that the biological properties of the mimetic peptide TNF_{70-80} were due to its specific interaction with TNFRI. This was shown from a functional aspect in the cell line lacking TNFRs and in solid phase ligand-receptor binding assays.

4.0 CHAPTER FOUR: THE SELECTIVE ACTIVATION OF TNFRI-INDUCED INTRACELLULAR SIGNALLING PATHWAYS BY TNF_{70-80} AND $TNF_{132-150}$

4.1 INTRODUCTION

Although our studies have demonstrated that TNF_{70-80} and TNF exert identical effects in neutrophils both *in vitro* and *in vivo*, not all of the effects of TNF are mimicked by TNF_{70-80}. Previously studies have shown that TNF_{70-80} failed to increase the expression of cell adhesion molecules, E selectin-1, ICAM-1 and VCAM-1, in human endothelial cells and did not kill tumour cells such as WEHI-164 or precipitate death in a sepsis mode (Kumaratilake *et al.*, 1995). These distinct properties suggest a difference in activation of intracellular signalling pathways. The p38 MAPK has been implicated in the activation of neutrophils in response to invading micro-organisms resulting in the production of reactive oxygen intermediates and chemotaxis (Detmers *et al.*, 1998, Zu *et al.*, 1998). In contrast, the JNK MAPK pathway has been implicated in the modulation of apoptosis (Donovan *et al.*, 2002, Lei and Davis, 2003). TNF is known to activate all three MAPK pathways, which mediate most of its pleiotropic effects (Modur *et al.*, 1996, Read *et al.*, 1997, Zu *et al.*, 1998). Therefore we investigated if the selective/restricted biological properties of TNF_{70-80} were due to selective activation of MAPK pathways.

Work form our group has demonstrated in human endothelial cells (HUVEC) and neutrophils that TNF (100-1000 U/ml) and TNF_{70-80} (1-50 μM) caused similar activation of the p38 MAPK module (Haddad, 1998). But in contrast, to TNF, TNF_{70-80} was unable to stimulate JNK activity and ERK1/ERK2 in HUVEC. Hence the ability of TNF_{70-80} to stimulate neutrophil anti-microbial activity and its lack of cytotoxic effects are mirrored in the selective activation of the p38 and not the JNK and ERK/ERK2 pathways.

4.2 ACTIVATION OF NFκB PATHWAY BY TNF_{70-80}

TNF receptors are coupled to the NFκB pathway via TRAF2 and it has been proposed that this action protects cells against TNF-induced apoptosis (Baud and Karin, 2001). We investigated whether TNF_{70-80} was able to couple TNF receptors to the NFκB pathway. This pathway, similar to the p38 pathway is dependent on TRAF-2 and RIP (Kyriakis and Avruch, 2001). We measured the nuclear translocation of NFκB as an indicator of the activation of this pathway. Neutrophils were treated with either TNF or TNF_{70-80}. After incubation, cells were lysed and nuclear fractions were prepared and subjected to western blotting to determine the amount of NF-κB translocated to the nucleus. The results showed that TNF_{70-80} caused the translocation of NFκB (p65) to the nucleus in a similar manner to TNF (Figure 4.1).

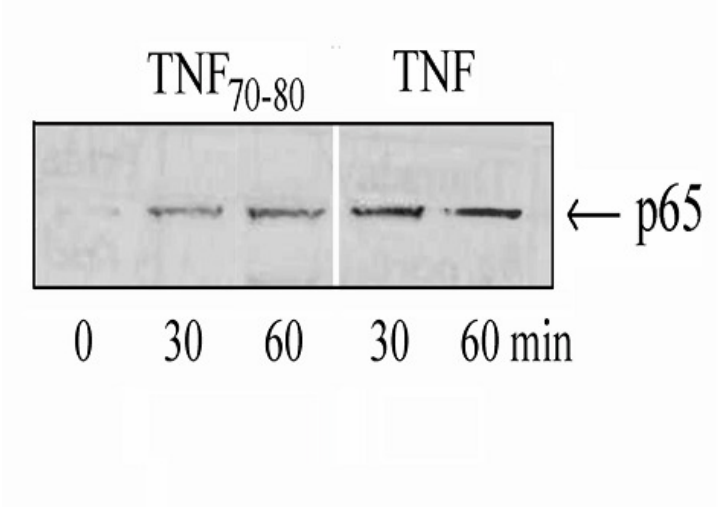

Figure 4.1 Activation of NFκB by TNF and TNF_{70-80} in human neutrophils. Cells were stimulated with either TNF (100 U/ml) or TNF_{70-80} (10 μM) at 37°C for the times indicated. After cell lysis, nuclear fractions were prepared and NF-κB (p65) detected by Western blotting. Results shown are from one experimental run, representative of three experiments.

4.3 ABILITY OF TNF_{70-80} TO RECRUIT ADAPTOR PROTEINS

4.3.1 TNF_{70-80} stimulates p38 activity via TRAF2

The selective biological activity of TNF_{70-80} suggests an inability by the peptide-ligated receptors to recruit key adaptor proteins to the ligand-receptor complex and thus activate downstream signalling pathway. The actions of TNF are mediated via the TNF receptor associated factor 2 (TRAF2) and activation of p38 by TNF has been demonstrated to involve TRAF2, receptor interacting protein (RIP) and apoptosis signal regulating kinase-1 (ASK-1) (Kyriakis and Avruch, 2001). On the other hand, JNK can be activated by TNF via at least two parallel pathways, one involving TRAF2, germinal centre kinase (a member of the STE20 family of kinases) and MAP kinase/ERK kinase kinase 1 (MEKK1), and the other involving TRAF2 and ASK-1 (Kyriakis and Avruch, 2001). To attempt to understand how TNF_{70-80} selectively activated the p38 module, it is important to determine whether TRAF2 was involved in the action of the peptide. After establishing that p38 can be activated in HEK293T cells, we stably transfected these cells with wild-type TRAF2, a dominant-negative TRAF2 ($TRAF_{287-501}$, Δ TRAF2) or an empty vector. The cells were treated with diluent or TNF_{70-80} (10 μM) for 5 min at 37 °C, lysed and p38 kinase activity was assayed after immunoprecipitation. The data (Figure 4.2) demonstrate that while over expression of TRAF2 enhanced the activation of p38 by TNF_{70-80} compared to cells transfected with an empty plasmid, expression of Δ TRAF2 inhibited this response. These data imply that TRAF2 was recruited to TNFR when the receptors were engaged by TNF_{70-80}.

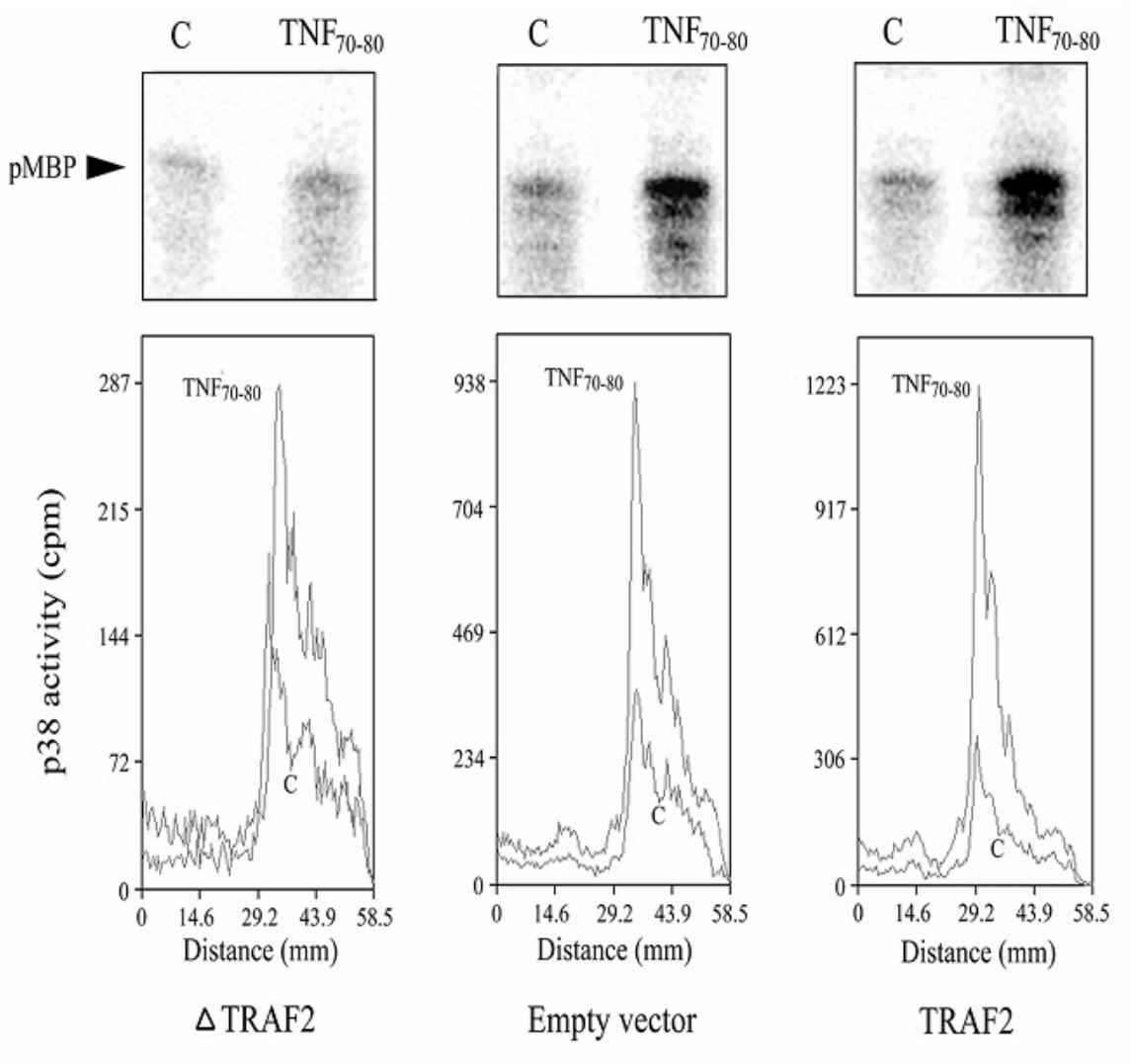

Figure 4.2 **TNF_{70-80} stimulates p38 activity via TRAF2.** HEK 293T cells were stably transfected with wild-type TRAF2, a dominant-negative TRAF2 (ΔTRAF2) or an empty vector. TNF_{70-80} (10 μM) was added and the cells were incubated for 5 min. at 37 °C, lysed and p38 immunoprecipitated. Kinase activity was assayed using myelin basic protein as a substrate. The data (digital radiogram and radioactivity profile of phosphorylated MBP) shown, are representative of 2 experiments.

4.3.2 Lack of activation of germinal centre kinase (GCK), an upstream kinase in the JNK pathway

The STE20 family of kinases constitutes a group of upstream regulators of the JNK module, acting directly on MEKK1 (Kyriakis and Avruch, 2001). Although TNF has been demonstrated to stimulate the activities of some of these kinases, which include germinal centre kinase (GCK), GCK-related kinase (GCKR) and GCK-like kinase (GLK) in some cell-type (Kyriakis and Avruch, 2001), such an effect has not been reported in neutrophils. We therefore investigated whether TNF and TNF_{70-80} were able to stimulate the activity of GLK and GCKR. Neutrophils were pre-adhered to plasma-coated dishes, stimulated with TNF, lysed and the lysates were incubated with an anti-GCKR antibody which detects both GCKR and GLK. The data in (Figure 4.3) show that TNF_{70-80} in comparison to TNF did not cause the activation of GCKR/GLK in adherent neutrophils.

4.3.3 Lack of activation of ASK-1 by TNF_{70-80}

The p38 and JNK modules can also be activated by TNF via the upstream apoptosis signal-regulating kinase (ASK) , and this has been demonstrated in cell types such as HEK293T cells (Tobiume *et al.,* 2001). ASK-1 has also been reported to bind to and is activated via TRAF2 (Kyriakis and Avruch, 2001). We therefore compared the ability of TNF and TNF_{70-80} to activate ASK-1 in HEK293T cells, by determining the level of

autophosphorylation of the kinase, a hallmark of ASK-1 activation (Kyriakis and Avruch, 2001). While TNF caused a transient increase in ASK-1 autophosphorylation, peaking at 3 min, TNF_{70-80} did not affect the level of ASK-1 phosphorylation (Figure 4.4). In contrast, treatment of HEK293T cells with TNF_{70-80} for 5 min increased p38 activity by 243 ± 27 % over control ($p < 0.05$).

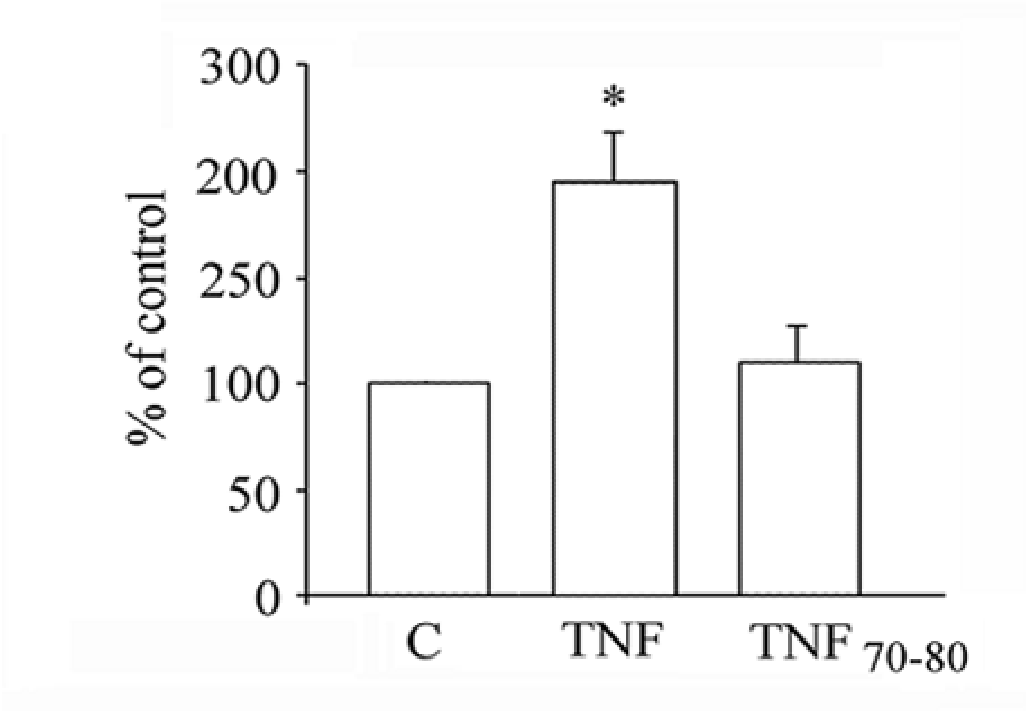

Figure 4.3 Lack of activation of GCKR by TNF_{70-80}. TNF but not TNF_{70-80} activated GCKR in neutrophils. Neutrophils were adhered to autologous plasma coated culture dishes and treated for 15 min with either HBSS, TNF (1000 U/ml) or TNF_{70-80} (10 μM). GCKR was immunoprecipitated from cell lysates and activity assayed using myelin basic protein (MBP) as the substrate. The data for the phosphorylated MBP is presented as mean kinase activity ± SEM of three experiments. Significance of difference between control and TNF or TNF_{70-80} treated cells: * $p < 0.05$.

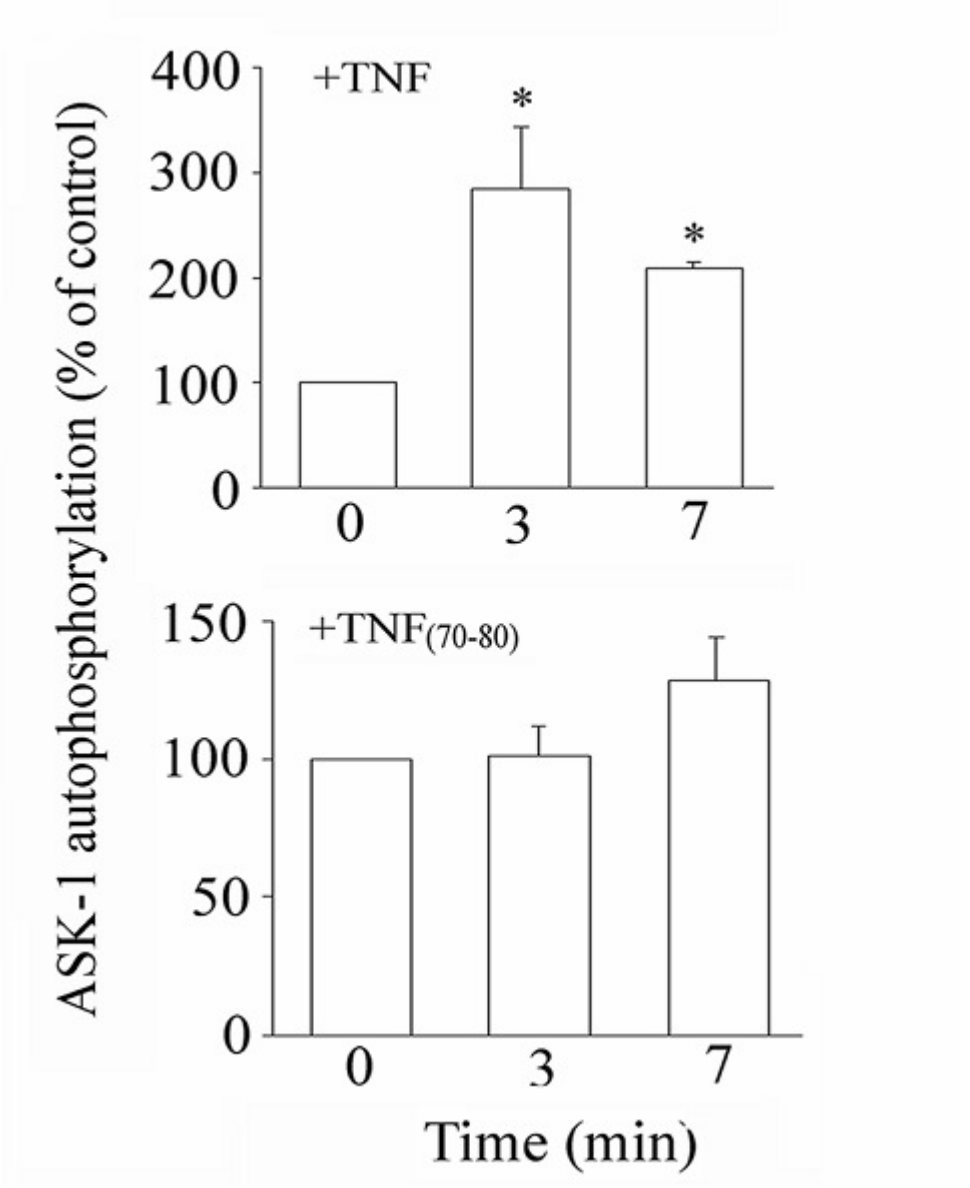

Figure 4.4 Lack of activation of ASK-1 by TNF_{70-80}. TNF but not TNF_{70-80} caused the autophosphorylation of ASK-1 in HEK293T cells. HEK293T cells were incubated with TNF or TNF_{70-80} for 3 or 7 min, lysed and autophosphorylation of immunoprecipitated ASK-1 was determined. Results shown are the means ± SEM of 3 experiments. Significance of difference between control and TNF or TNF_{70-80} treated cells: * $p < 0.05$.

4.4 EFFECT OF $TNF_{132-150}$ ON MAPK ACTIVATION IN WEHI-164 CELLS

Previously, (Rathjen *et al.*, 1993) demonstrated that peptide $TNF_{132-150}$ lacked the neutrophil stimulating properties of TNF and mimetic TNF_{70-80}, to confirm this 10 μM of $TNF_{132-150}$, TNF_{70-80} or diluent was added to neutrophils (5×10^5) and 250 μl of lucigenin in HBSS (250 μM final), was added and the final volume adjusted to 500 μl and the resultant chemiluminescence production measured in a luminometer. The kinetics of chemiluminescence production over the incubation period is shown in (Figure 4.5). It is evident that TNF_{70-80} caused a dramatic increase in chemiluminescence response which reached a peak within approximately 5 min, while $TNF_{132-150}$ failed to stimulate chemiluminescence production at similar concentrations. In comparison, while $TNF_{132-150}$ was cytotoxic for target cells (Figure 4.6 and 4.7), TNF_{70-80} did not exhibit similar properties (Rathjen *et al.*, 1993).

To investigate the effect of $TNF_{132-150}$ on MAPK activation, WEHI-164 cells were treated under the same conditions as those in the cytotoxic experiments. The results show that both TNF and $TN_{132-150}$ were able to activate JNK activity to a similar degree (Figure 4.8). Under similar conditions ERK1/ERK2 activity was increased after treatment with TNF or $TNF_{132-150}$ (Figure 4.9). The activity of p38 was increased by TNF at a concentration of 1000 U/ml. Interestingly, 100 μg/ml of $TNF_{132-150}$ failed to stimulate the activity of p38 in WEHI-164 cells (Figure 4.10).

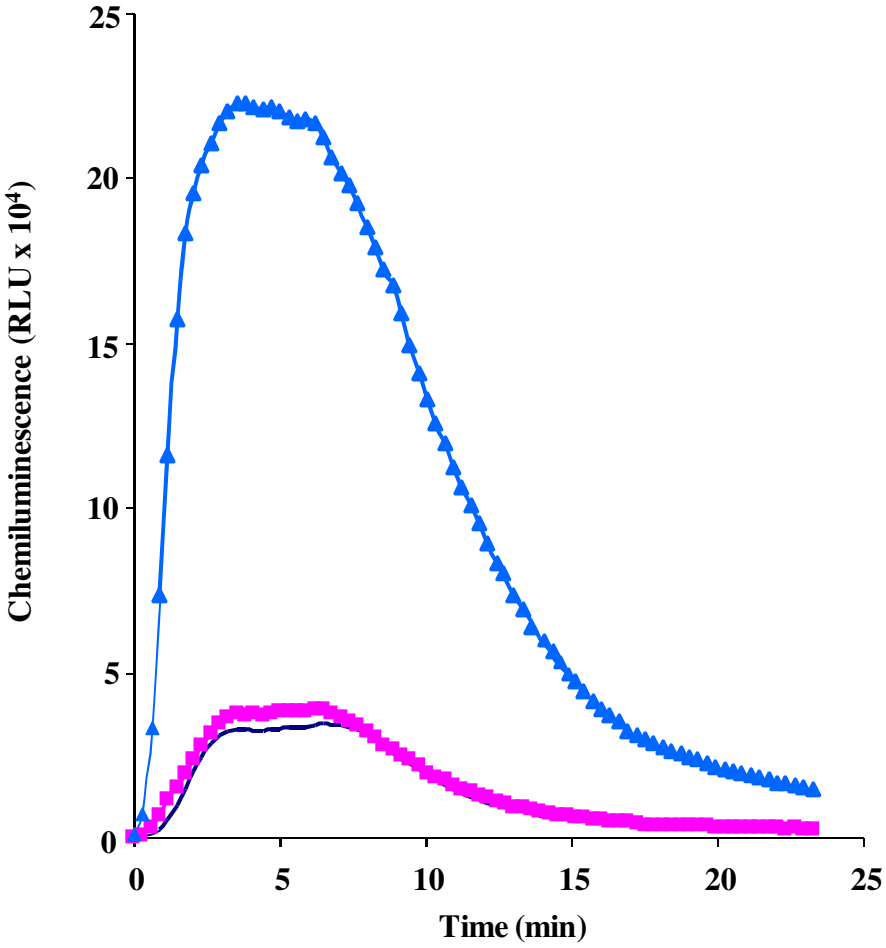

Figure 4.5 The effect of peptides TNF_{70-80} and $TNF_{132-150}$ on superoxide (chemiluminescence) production in neutrophils. To 50 µl of neutrophils ($5x10^5$ in HBSS), 50 µl of peptide (10 µM final) or diluent and 250 µl of lucigenin (250 µM final) were added and the resultant chemiluminescence produced measured. Data from a representative experiment of three are presented. Diluent control (—), $TNF_{132-150}$ (■), TNF_{70-80} (▲).

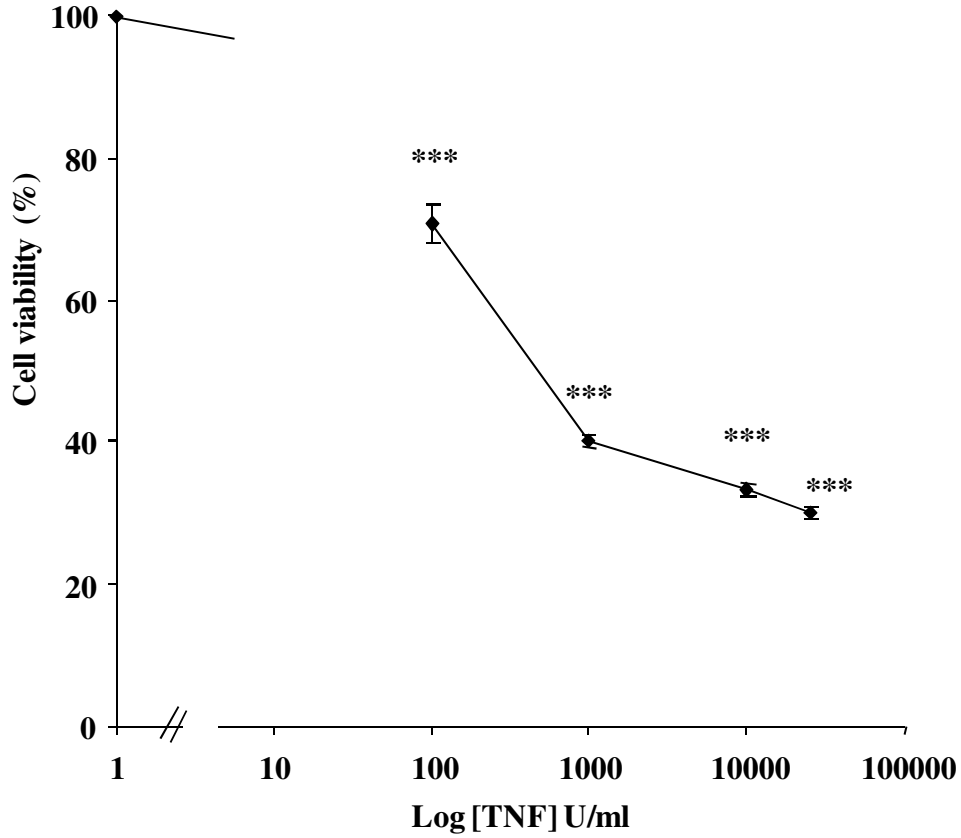

Figure 4.6 The cytotoxic effect of TNF on WEHI- 164 fibrosarcoma cells. WEHI-164 cells ($5x10^4$ cells/well) were cultured in 96-well plates and pre-treated with actinomycin D (1 µg/ml) for 15 min before the addition of the different dilutions of TNF and incubated for 20 h. After, 20 µl of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide dye (MTT) (5mg/ml) was added and incubated for a further 4 h. The reaction was terminated by the addition of 50 µl of 20 % SDS in 20 mM HCl and absorbance read at 540 nm after overnight incubation to solubilise the reduced MTT. Percentage viability was determined by comparison with untreated control cells. Results shown are means ± SEM for three experiments. Significance of difference between control and TNF: *** $p < 0.001$ (Dunnett's Multiple Comparisons Test).

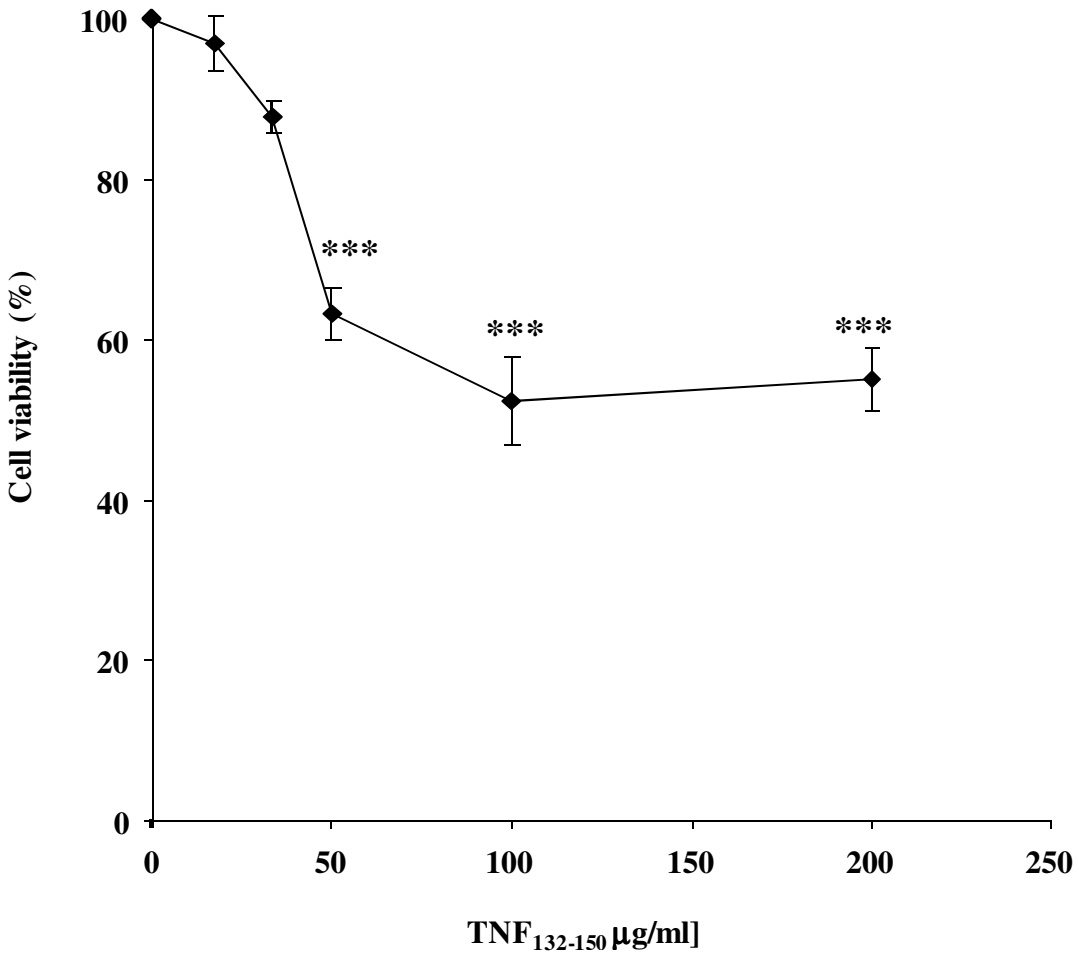

Figure 4.7 The cytotoxic effect of $TNF_{132-150}$ on WEHI- 164 fibrosarcoma cells. WEHI-164 cells ($5x10^4$ cells/well) were cultured in 96-well plates and pre-treated with actinomycin D (1 µg/ml) for 15 min before the addition of the different dilutions of $TNF_{132-150}$ and incubated for 20 h. After, 20 µl of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide dye (MTT) (5 mg/ml) was added and incubated for a further 4 h. The reaction was terminated by the addition of 50 µl of 20 % SDS in 20 mM HCl and absorbance read at 540 nm after overnight incubation to solubilise the reduced MTT. Percentage viability was determined by comparison with untreated control cells. Results shown are means ± SEM for three experiments. Significance of difference between control and $TNF_{132-150}$: *** $p < 0.001$ (Dunnett's Multiple Comparisons Test).

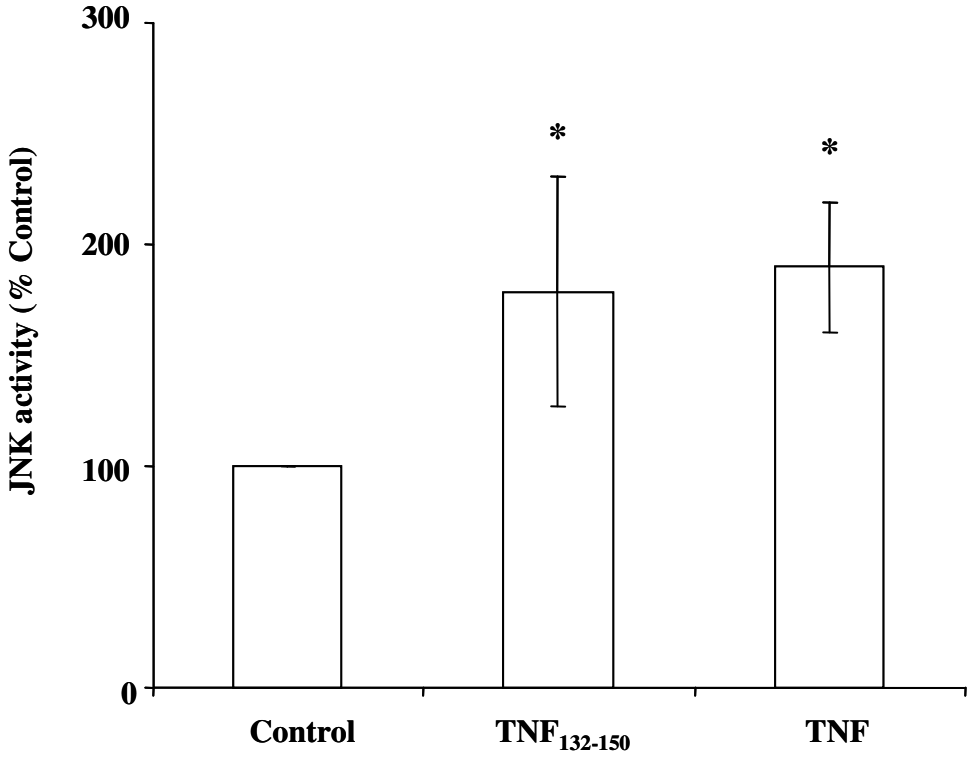

Figure 4.8 Activation of JNK by $TNF_{132-150}$ in WEHI-164 cells. WEHI-164 cells ($2x\ 10^6$) were cultured in 28 cm^2 dishes to approximately 80 % confluence. Serum starved cells were incubated with 1000 U/ml TNF or $TNF_{132-150}$ (100 μg/ml) for 15 min at 37°C, the cells were lysed and the degree of JNK activity was assayed using GST-jun (1-79) as a substrate. Data are presented as mean ± SEM of 3 experiments. Significance of difference between control and TNF or $TNF_{132-150}$ treated cells: * $p < 0.05$ (Dunnett's Multiple Comparisons Test).

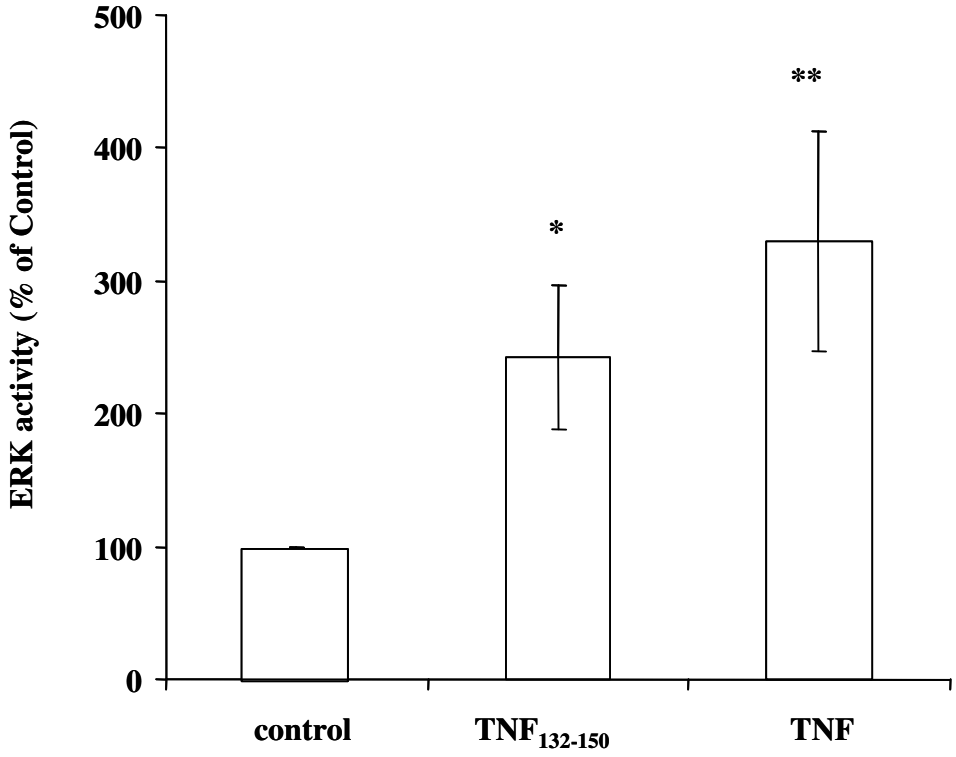

Figure 4.9 ERK1/ERK2 activation by $TNF_{132-150}$ in WEHI-164 cells. WEHI-164 cells (2x 10^6) were cultured in 28 cm^2 dishes to approximately 80 % confluence. Serum-starved cells were incubated in the presence or absence of TNF (1000 U/ml) or $TNF_{132-150}$ (100 µg/ml) for 15 min at 37°C, the cells were lysed and ERK1/ERK2 activation was determined after immmunopreciptation, by kinase activity using myelin basic protein as a substrate. Data are presented as means ± SEM of 3 experiments. Significance of difference between control and TNF or $TNF_{132-150}$: * p <0.05; ** p <0.01 (Dunnett's Multiple Comparison Test).

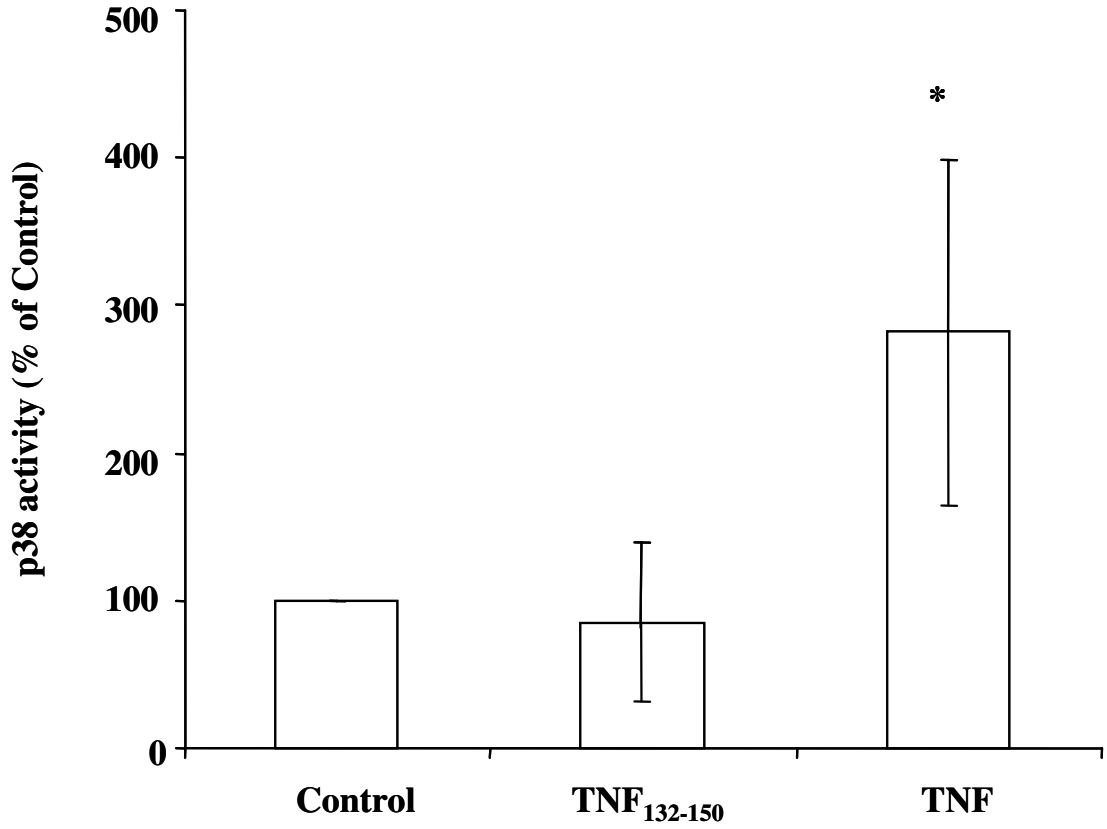

Figure 4.10 Lack of effect of $TNF_{132-150}$ on p38 kinase activity in WEHI-164 cells. WEHI-164 cells (2x 10^6) were cultured in 28 cm^2 dishes to approximately 80 % confluence. Serum starved cells were incubated with 1000 U/ml TNF or $TNF_{132-150}$ (100 µg/ml) for 15. The cells were lysed, p38 immunoprecipitated and kinase activity was assayed using myelin basic protein as a substrate. Data presented as means ± SEM of 3 experiments). Significance of difference between control and TNF or $TNF_{132-150}$: * $p <$ 0.05 (Dunnett's Multiple Comparisons Test).

4.5 SUMMARY

The data presented here show that indeed there is a selective activation of MAPK occurring in cells treated with TNF_{70-80} compared to TNF. TNF_{70-80} was able to selectively activate the p38 and NF-κB pathway in neutrophils and endothelial cells. These data imply that the TNF_{70-80}-ligated TNF receptors were able to functionally couple TRAF2 to the p38 and NFκB pathway. The inability of TNF_{70-80} to cause coupling of GCK and ASK 1 to TRAF2 is likely to explain the lack of activation of the JNK and ERK1/ERK2 pathways. These results are summarised in Figure 4.11.

While TNF was able to activate all three MAP kinases in adherent WEHI-64 cells, $TNF_{132-150}$ only enhanced the activity of JNK and ERK1/2 and not p38 at concentrations which have previously been shown to cause cytotoxicity in WEHI-164 cells following pre-treatment with actinomycin D. This data is consistent with the inability of $TNF_{132-150}$ to stimulate neutrophil chemiluminescence production, a response which is dependent on p38.

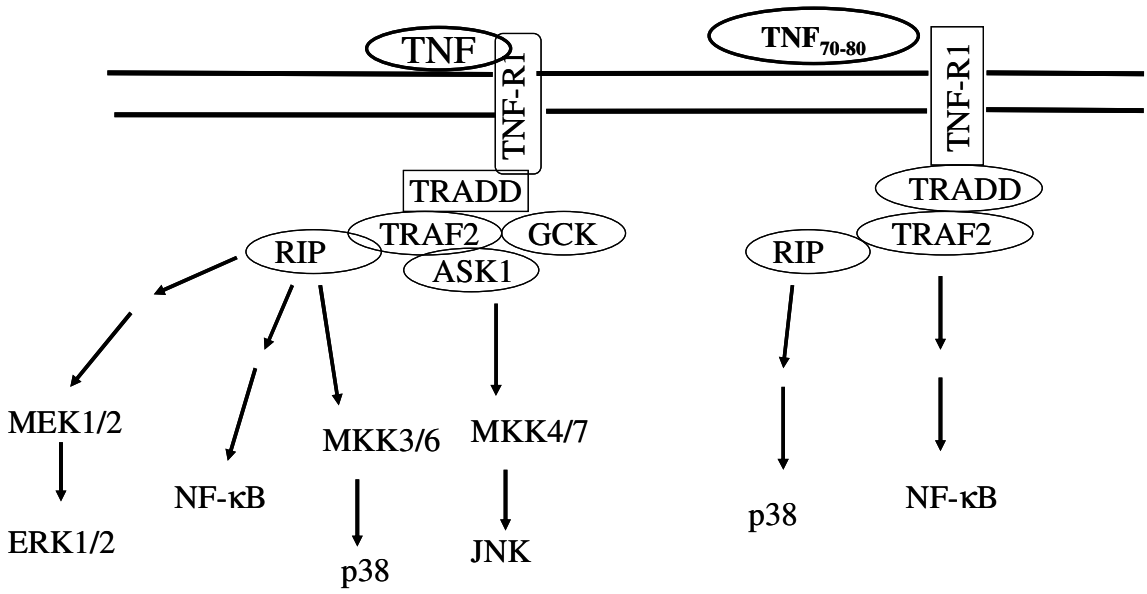

Figure 4.11 Differential coupling of TNFRI to downstream signalling pathways by TNF and TNF_{70-80}. Binding of TNF to TNFRI results in the activation of the p38, ERK1/ERK2, JNK and IκB kinase-NFκB pathways. TRAF2 recruits GCKR, RIP, ASK1, MEKK1 and a putative upstream molecule that links TRAF2 to the ERK1/ERK2 module. Binding of TNF_{70-80} to TNFRI results in the activation of the p38 and IκB kinase-NFκB pathways, implying efficient recruitment of RIP by TRAF2. However, TNF_{70-80} fails to stimulate GCKR or ASK-1 and hence the JNK module, implying that GCKR/GLK or ASK-1 activation is essential for activation of the JNK module. ASK-1 is dispensable for p38 activation by TNF_{70-80}.

5.0 CHAPTER FIVE: IDENTIFICATION OF THE TNF_{70-80} BINDING REGION AND GENERATION OF PEPTIDES TO THESE REGIONS

5.1 INTRODUCTION

The neutralisation of TNF has become a major strategy in the treatment of many inflammatory diseases (Chapter 1, section 1.7) (LaDuca and Gaspari, 2001, Aggarwal *et al.*, 2006). Use of antagonists based on monoclonal anti-TNF antibodies and recombinant soluble TNF receptors is well documented. Pharmacologically, administration of proteins [cytokines] as drugs has many drawbacks such as conformational instability, susceptibility to proteolytic degradation, antigenicity, high cost and poor membrane penetration (Sedmak *et al.*, 1981, Fairlie *et al.*, 1998). Clinically, protein-based drugs have to be administered parentally e.g. by subcutaneous, intramuscular or intravenous injection (Sato and Sone, 2003). Thus small molecules that can be prepared synthetically and administered at high concentrations might offer a more appropriate alternative for anti-TNF therapy.

Studies on the characteristics of binding between TNF and TNFR have led to the discovery of regions and consequently peptides to those regions that mimic the function of TNFRs and could consequently lead to the development of new therapeutic agents. For example, a 20 amino acid synthetic peptide corresponding to residues 175-194 of TNFRI was capable of inhibiting TNF-induced cytolysis, as well as displacing the binding of ^{125}I-labelled TNF to mouse L929 cells (Hwang *et al.,* 1991). Another peptide, corresponding to residues 159-178 of TNFRI was also reported to similarly displace the binding of ^{125}I-TNF and inhibit TNF cytotoxicity to L-M cells (Lie *et al.,* 1992). These receptor-derived mimetics highlight the potential usefulness of small molecule inhibitors

of TNF functions, giving an alternative approach to treating inflammation associated with autoimmune and malignant diseases.

5.2 CONSTRUCTION OF TNFRI MUTANTS

Initially, truncated mutants of TNFRI were constructed by sequentially deleting the cysteine rich domains on the extracellular portion of TNFRI (Figure 5.1). This would allow us to test whether TNF_{70-80} was able to activate cells via the TNFR mutants, and allow the identification of the minimal portion of TNFRI that is required for TNF_{70-80} action. That is, the truncation of mutants could permit the identification of the region on TNFRI that TNF_{70-80} binds.

70Z/3 pre B cells were then transiently transfected with wild type (WT) or a mutant of TNFRI using lipofectamine 2000. After 24 h the cells were treated with diluent, TNF_{70-80} (10 μM) or TNF (1000 U/ml) at 37°C for 5 min. The cells were then lysed and the protein contents were quantified and p38 activity was assayed after immunoprecipitation.

The data showed that TNF-induced activation of p38 was most in WT TNFRI transfected cells (Figure 5.2), with reduced effect using the M1 mutant and essentially no activity with further truncation (M4). TNF_{70-80}-induced activation of p38 was also clearly evident in the WT TNFRI transfected cells and while there was still substantial activity with M1 transfected cells, it was evident that M4 transfected cells still showed a significant p38

response (Figure 5.2). Since all three mutants contained the amino acid residues present in the M4 mutant, the minimum region required by TNF_{70-80} to activate p38 was likely to be within the M4 region. These results imply that TNF_{70-80} binds to the remnant amino acid residues in the M4 mutant. This therefore raised the possibility that peptides of this region may be also to block the function of TNF_{70-80}. To test this peptides corresponding to the extracellular portion of the M4 mutant were made and their ability to inhibit TNF_{70-80} induced superoxide production in neutrophils was investigated. Figure 5.3 shows the various peptides that were made.

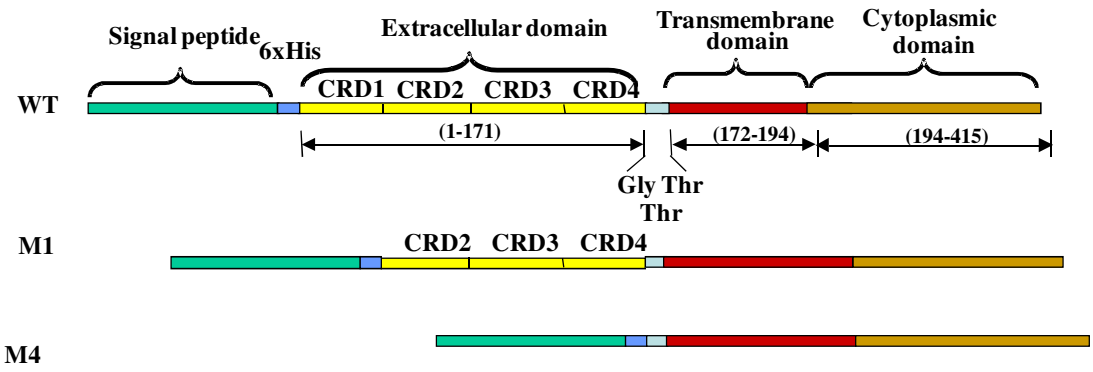

Figure 5.1 Diagrammatic generation of TNFRI mutants. The signal peptide is cleaved from the mature receptor protein. Abbreviations: WT, wild type; CRD, cysteine rich domains; His, histidine; M, mutant.

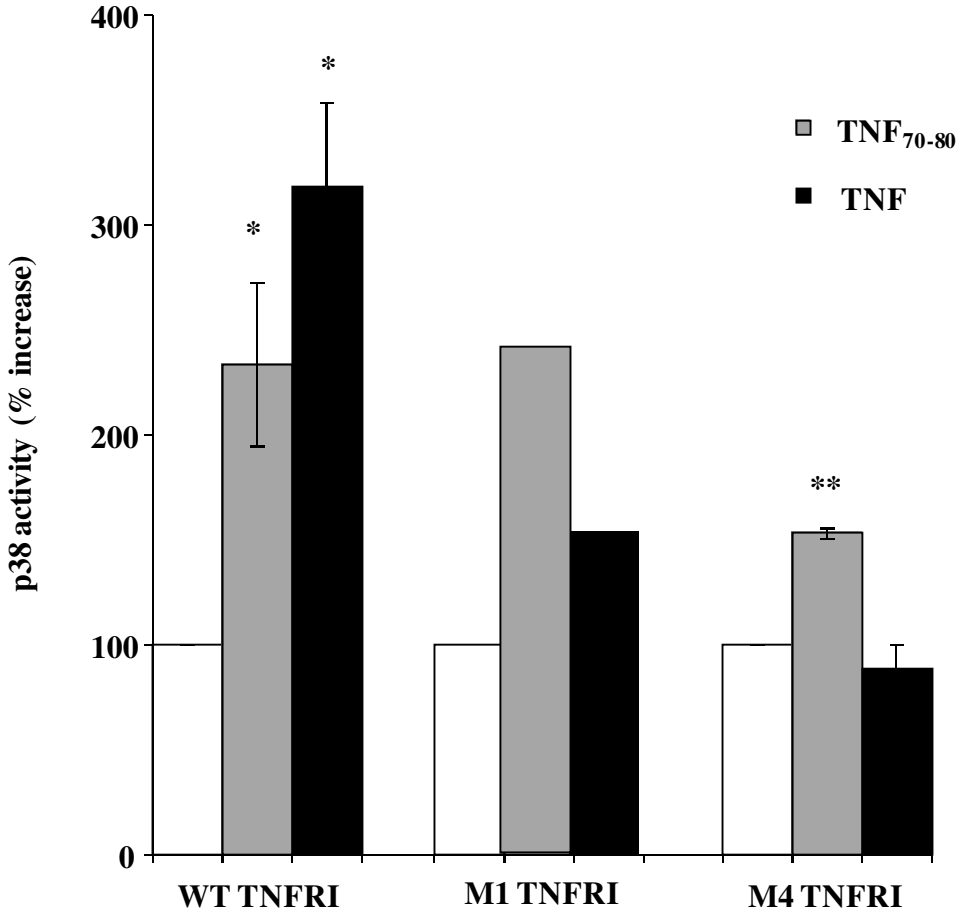

Figure 5.2 p38 activation in cells transfected with WT or mutant TNFRI. 70Z/3 cells transfected with WT TNFRI or different mutant forms of TNFRI, M1, and M4 were treated with HBSS, $TNF_{70\text{-}80}$ (10 μM) or TNF (1000 U/ml) at 37°C for 5 min. The cells were then lysed, protein contents quantified. After immunoprecipitation p38 activity was assayed. The results are presented as the mean ± SEM of 3(WT), 1(M1), 3 (M4) experiments. Significance of difference between control and $TNF_{70\text{-}80}$ or TNF: * $p < 0.05$, ** $p < 0.01$ (Tukey's Multiple Comparison Test).

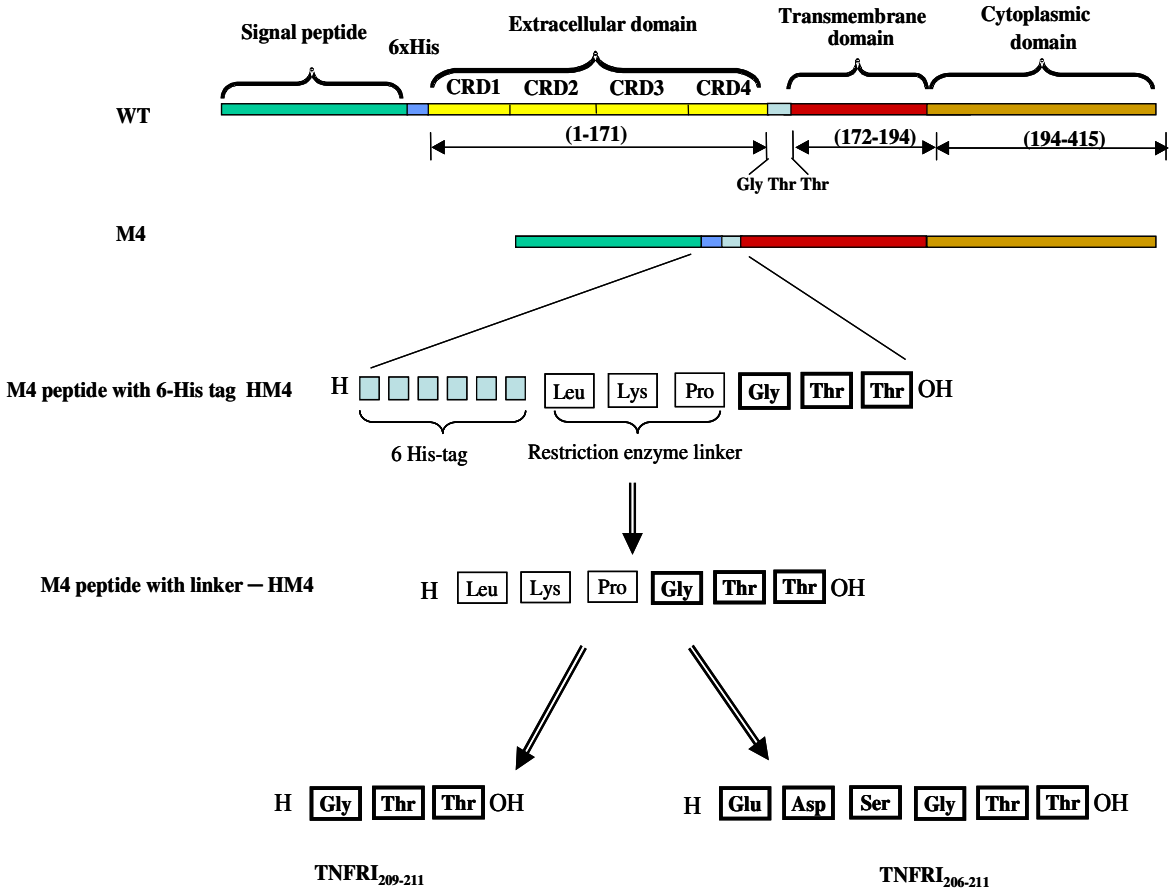

Figure 5.3 Diagrammatic representation of generation of TNFRI-derived peptides. The signal peptide was cleaved from the mature receptor protein. The boldfaced text represents residues in the natural TNFRI sequence. Leu-Lys-Pro were introduced as a consequence of generating a restriction site for coupling to the His-tag. Abbreviations: WT, wild type; CRD, cysteine rich domains; His, histidine; M, mutant.

5.3 THE EFFECT OF HM4 ON TNF_{70-80}-INDUCED SUPEROXIDE PRODUCTION IN NEUTROPHILS

The first peptide included a 6 histidine tag which was used for purification and extraction purposes and a restriction enzyme linker consisting of 3 amino acids linked to the remnant 3 amino acids from the TNFRI sequence (HHHHHHLKPGTT) (Figure 5.3). Neutrophils were incubated with varying concentrations of HM4 in HBSS at 37 °C for 30 min and then tested for their ability to produce superoxide when challenged with TNF_{70-80}, determined by the lucigenin-based chemiluminescence response. The kinetics of chemiluminescence production over the incubation period is shown in (Figure 5.4). It is evident that TNF_{70-80} caused a dramatic increase in chemiluminescence response which reached a peak after approximately 8 min. The results showed that incubation with HM4 caused a reduction in the TNF_{70-80}-induced chemiluminescence production (Figure 5.5). Maximum inhibition was observed with 3.5 μM HM4. The degree of inhibition diminished as the concentration of HM4 increased. The reason for this inverse relationship is not clear but could be due to the His tag overcoming the inhibitory action of GTT. This is consistent with the results from subsequent experiments using peptides devoid of the His tag (Figure 5.6-7).

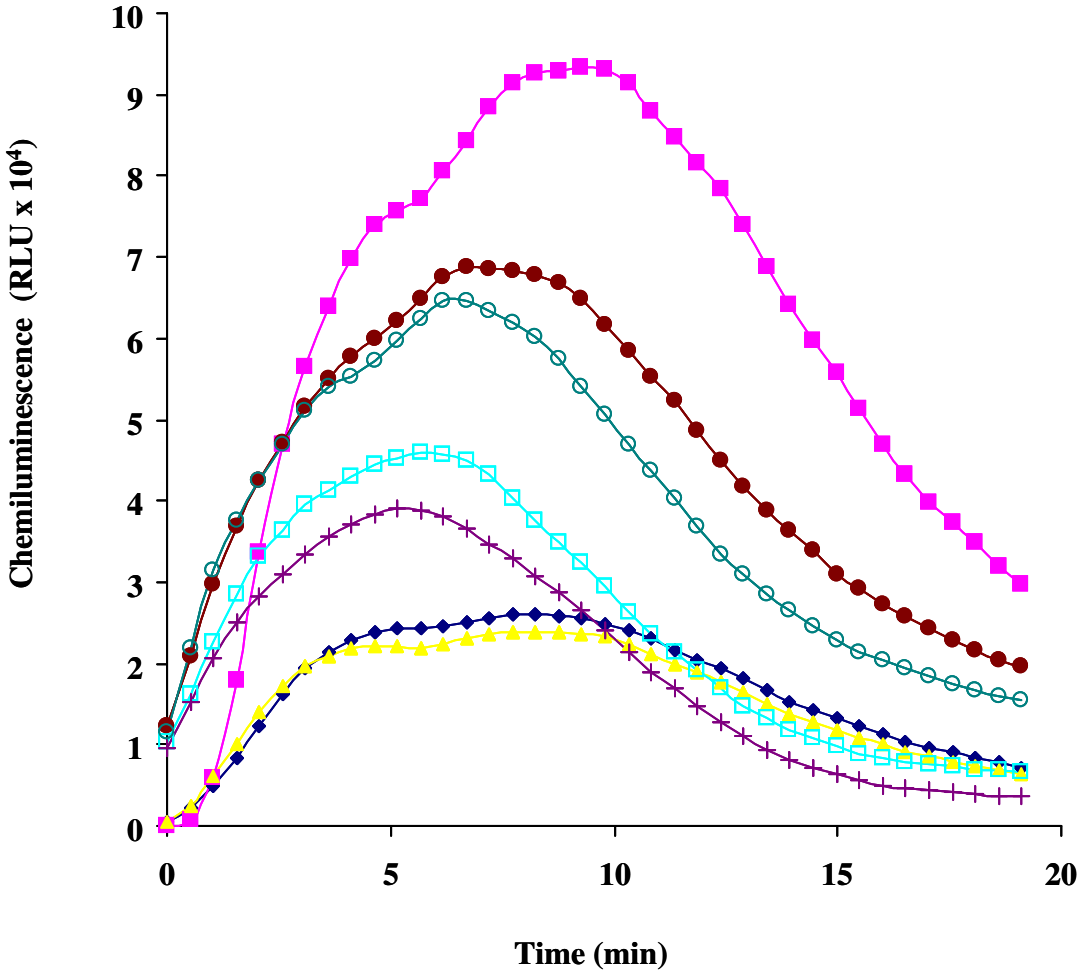

Figure 5.4 Kinetics of TNF_{70-80}-induced superoxide (chemiluminescence) production from neutrophils in the presence of varying concentrations of TNFRI fragment HM4. Neutrophils ($1x10^6$ in 100 µl HBSS) were treated with 100 µl of HM4 at 37 °C for 30 min. Lucigenin and 100 µl of TNF_{70-80} (10 µM) were then added and the resultant chemiluminescence production measured in a luminometer. Data from a representative experiment of three are presented. Diluent (♦); 70 µM HM4 (▲); TNF_{70-80} (■); TNF_{70-80} + 3.5 µM HM4 (+); TNF_{70-80} + 35 µM HM4 ( ); TNF_{70-80} + 70 µM HM4 (●); TNF_{70-80} + 105 µM HM4 (o).

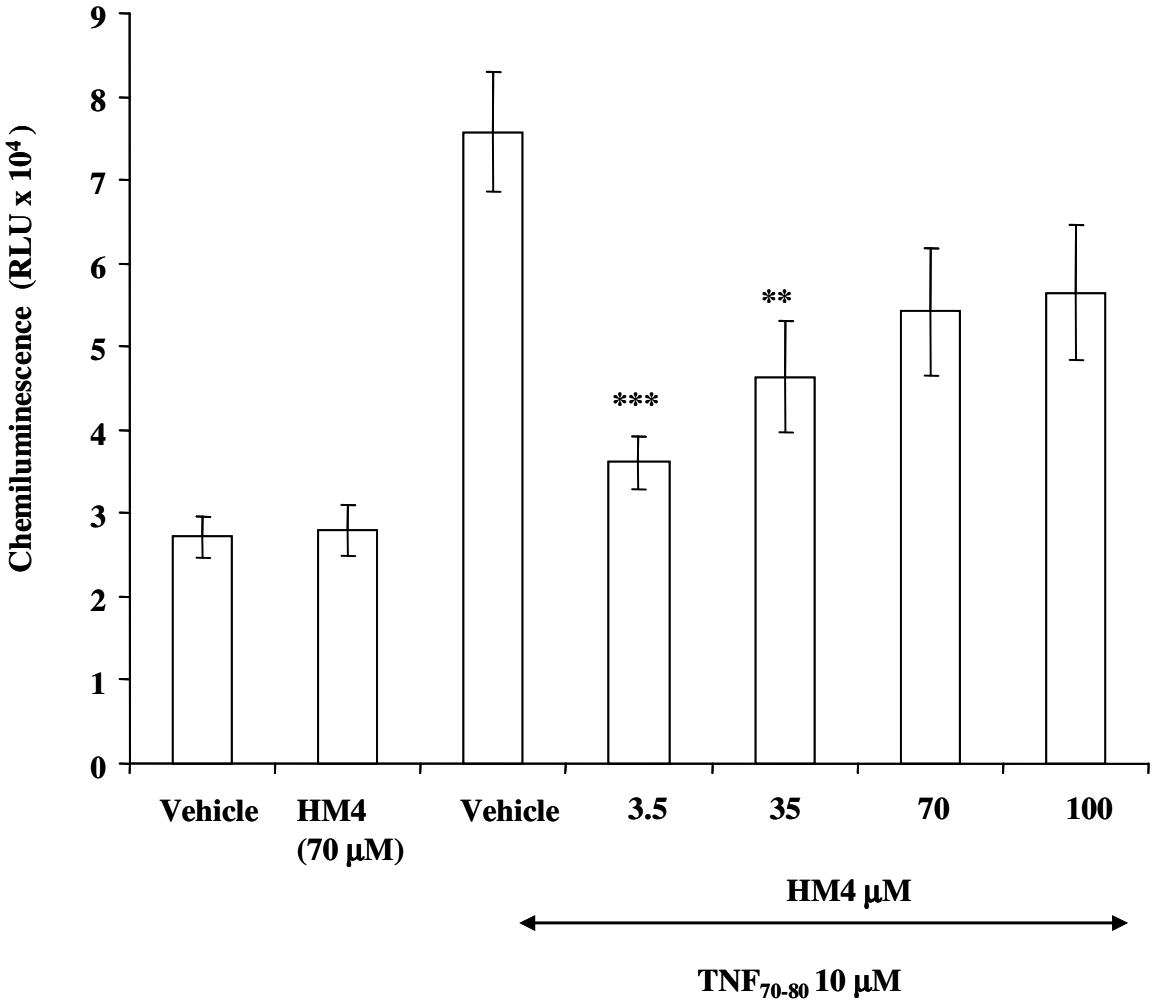

Figure 5.5 The effect of his-tagged TNFRI fragment- HM4 on TNF_{70-80}-induced superoxide (chemiluminescence) production in neutrophils. Neutrophils ($1x10^6$ in 100 µl HBSS) were treated with 100 µl of HM4 at 37 °C for 30 min. After incubation, lucigenin and 100 µl of TNF_{70-80} (10 µM) were added and the resultant chemiluminescence production measured in a luminometer. The results are expressed as maximal rate of chemiluminescence production. Significance of difference between TNF_{70-80} and TNF_{70-80} + HM4: ** $p < 0.01$, *** $p < 0.001$ (Dunnett's Multiple Comparison Test).

5.4 THE EFFECT OF –HM4 ON TNF_{70-80}-INDUCED SUPEROXIDE PRODUCTION IN NEUTROPHILS

The above results prompted us to test the effects of –HM4 (LKPGTT) that lacked the His tag (Figure 5.3). Neutrophils were incubated with 8-50 μM of –HM4 in HBSS or vehicle at 37°C for 30 min. After incubation, 500 μl of lucigenin (250 μM, final), 100 μl of TNF_{70-80} (10 μM) in HBSS were added, the final volume was adjusted to 1 ml and the resultant chemiluminescence was measured in a luminometer. TNF_{70-80} caused a marked increase in chemiluminescence response which reached a peak after approximately 6 min (Figure 5.6). In contrast to the results in Figure 5.5, -HM4 caused a dose dependent suppression of the ability of TNF_{70-80} to stimulate superoxide production. The results showed that incubation of cells in the presence of 25 μM and 50 μM of M4 caused a reduction in the TNF_{70-80} induced response by 17 % and 66 %, respectively (Figure 5.7).

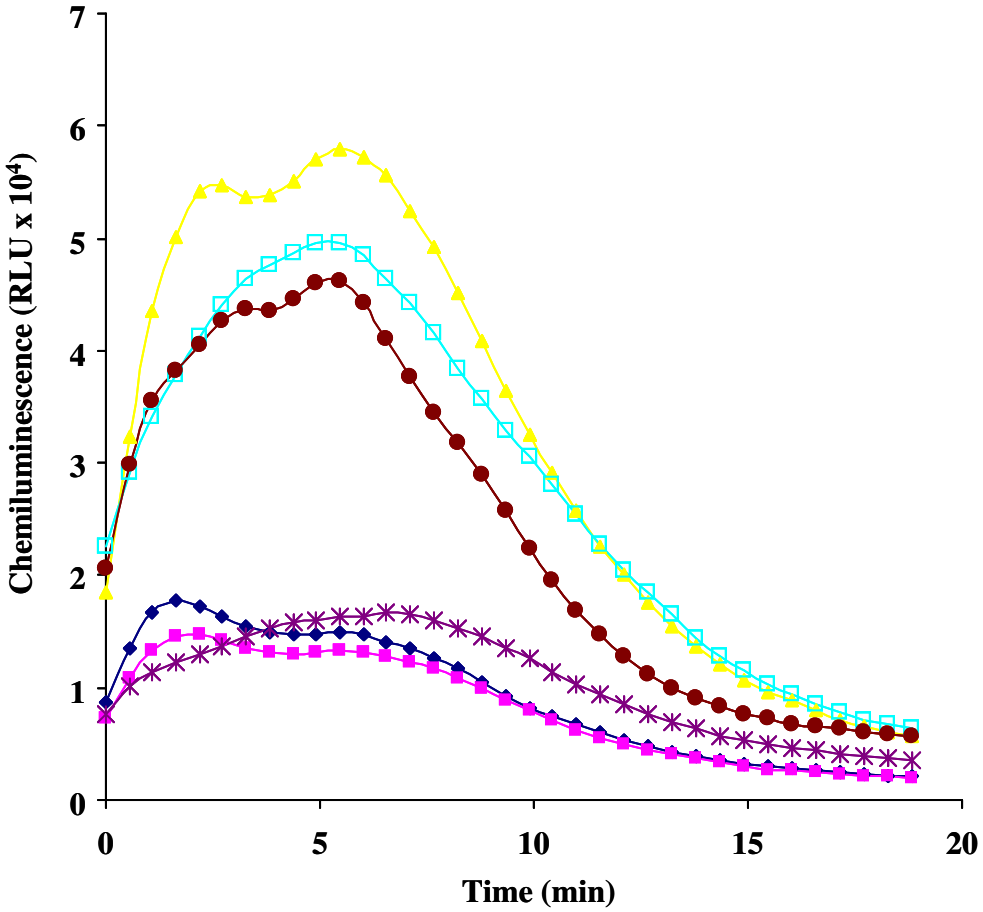

Figure 5.6 Kinetics of TNF_{70-80}-induced superoxide (chemiluminescence) production from neutrophils in the presence of varying concentrations of TNFRI fragment -HM4. Neutrophils ($1x10^6$ in 100 µl HBSS) were treated with 100 µl of -HM4 at 37 °C for 30 min. After incubation, lucigenin and 100 µl of TNF_{70-80} (10 µM) were added and the resultant chemiluminescence was measured in a luminometer. Data from a representative experiment of three are presented. Diluent control (■); 50 µM -HM4 (■); TNF_{70-80} (▲); TNF_{70-80} + 8 µM –HM4 (□); TNF_{70-80} + 25 µM –HM4 (●); TNF_{70-80} + 50 µM –HM4 (*).

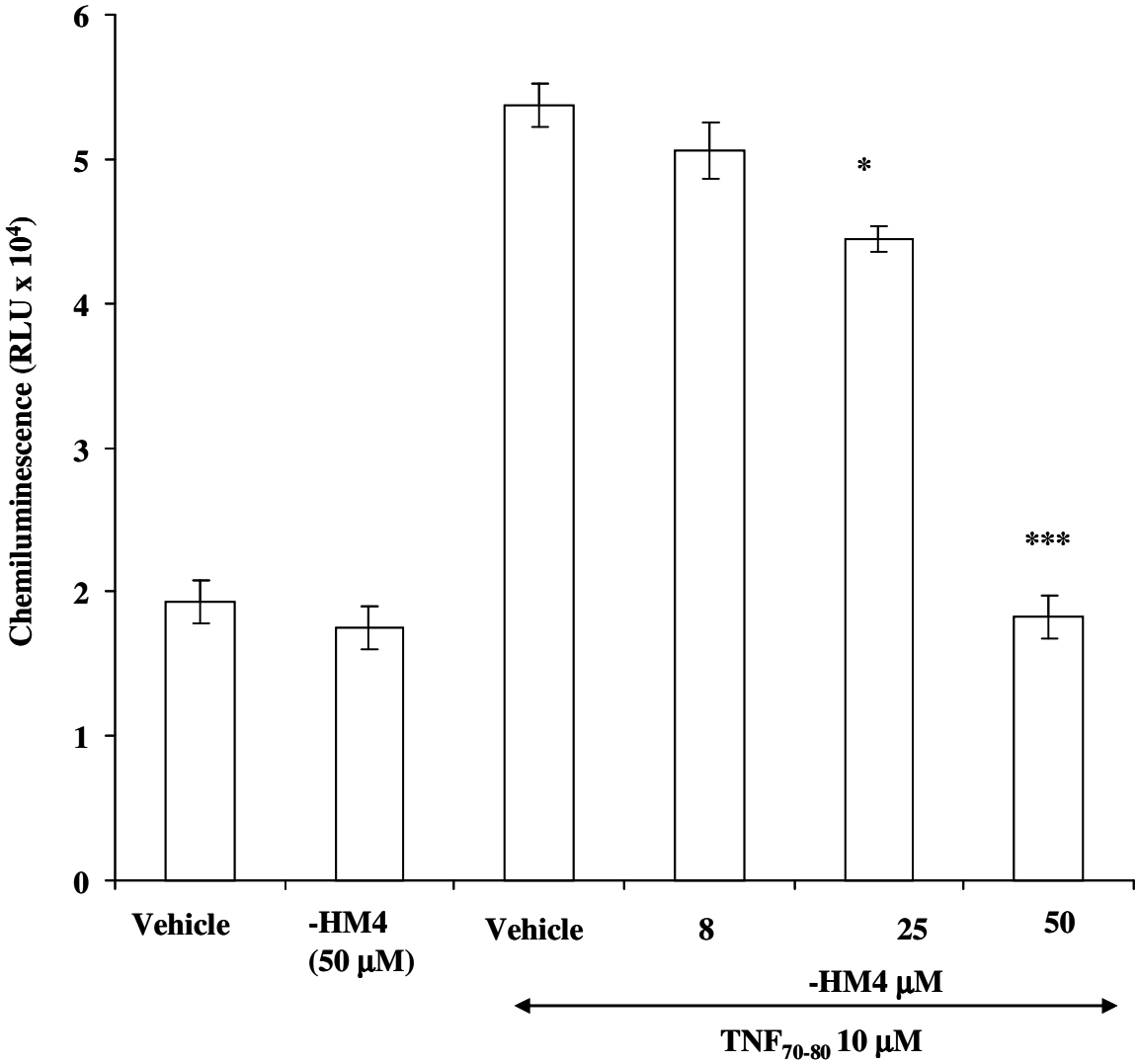

Figure 5.7 The effects of TNFRI fragment -HM4 on TNF_{70-80}-induced superoxide (chemiluminescence) production in neutrophils. Neutrophils ($1x10^6$ in 100 μl HBSS) were treated with 100 μl of -HM4 at 37 °C for 30 min. After incubation, 500 μl of lucigenin (250 μM, final) and 100 μl of TNF_{70-80} (10 μM) were added and the resultant chemiluminescence measured in a luminometer. The results are expressed as maximal rate of chemiluminescence production. Significance of difference between TNF_{70-80} and TNF_{70-80} + -HM4: * $p < 0.05$, *** $p < 0.001$ (Dunnett's Multiple Comparisons Test).

5.5 THE EFFECT OF TNFRI$_{209-211}$ ON TNF-INDUCED SUPEROXIDE PRODUCTION IN NEUTROPHILS

Having obtained the above results with –HM4, it was of interest to investigate whether the effect was due to the three linker amino acids (LKP) or to GTT (TFNRI$_{209-211}$) (Figure 5.3). The above results also raised the more important question of whether a peptide based entirely on TNFRI was able to block the actions of TNF given that the ultimate aim was to design therapeutic agents to block the actions of TNF. We therefore synthesised and tested the effect of TNFRI$_{209-211}$ on TNF-mediated effects on neutrophil superoxide production.

TNFRI$_{209-211}$ and a control peptide (TGT) were reconstituted just prior to conducting the experiment by dissolving in HBSS with 1 % bovine serum albumin (BSA). In these experiments, 50 µl of TNFRI$_{209-211}$ (50-400 µM) or diluent were incubated with 50 µl of TNF (1 ng/ml) at 37 °C for 20 min to optimise the interaction and then 50 µl of neutrophils (5x 10^5) in HBSS were added. Following a further incubation for 30 min at 37 °C, 250 µl of lucigenin in HBSS (250 µM final) was added and the final volume adjusted to 500 µl. The resultant chemiluminescence measured in a luminometer.

The results showed that the treatment of TNF with TNFRI$_{209-211}$ caused a dose-dependent inhibition of TNF-induced increase in chemiluminescence (Figure 5.8). Maximum inhibition occurred at TNFRI$_{209-211}$ of 200 µM and above and the half maximal inhibition (IC_{50}) occurred at 122.5 µM. A scrambled control peptide of the TNFRI$_{209-211}$ was tested

to determine the specificity of the natural sequence of the receptor on TNF-induced superoxide production. This peptide contained the sequence TGT. This control peptide was shown to have no inhibitory effect on chemiluminescence induced by TNF at the same concentration as that which $TNFRI_{209\text{-}211}$ inhibited the response (Figure 5.9).

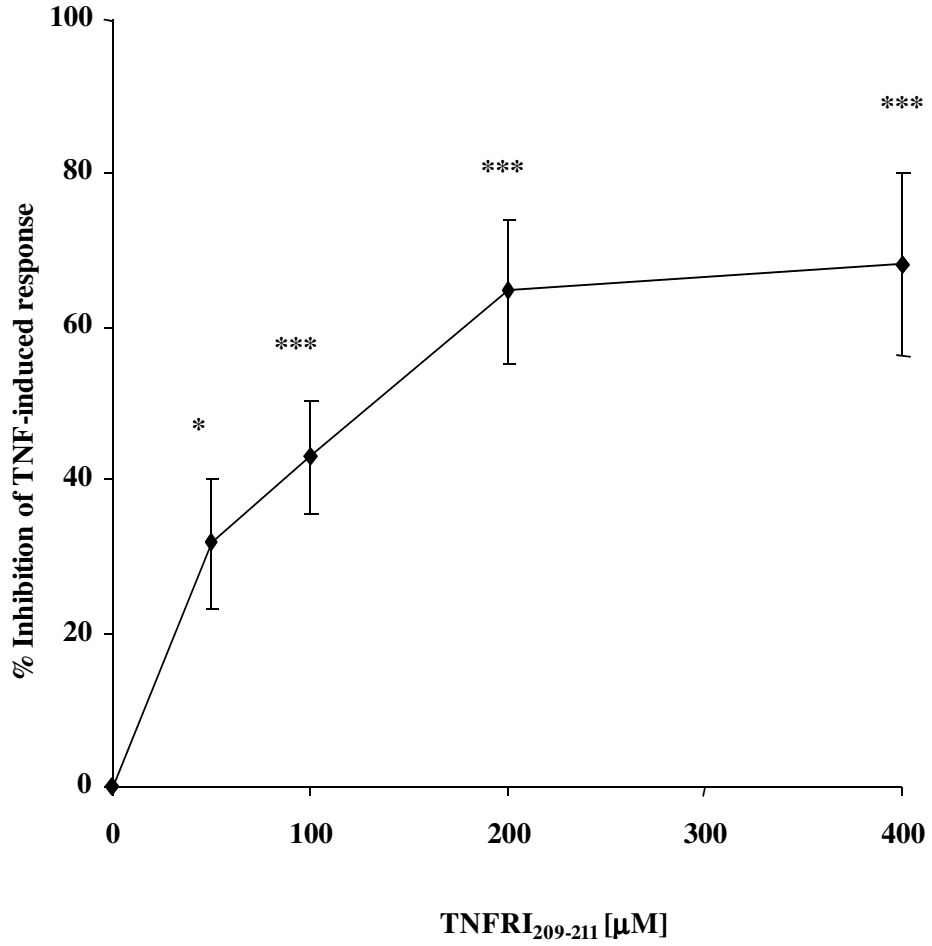

Figure 5.8 The effects of $TNFRI_{209-211}$ on TNF-induced superoxide (chemiluminescence) production in neutrophils. TNF (1 ng/ml) was treated with varying concentrations (50, 100, 200 and 400 μM) of $TNFRI_{209-211}$ at 37°C for 20 min. Neutrophils were added and further incubated for 30 min at 37°C. Then lucigenin was added and the chemiluminescence generated measured in a luminometer. Data represent the % inhibition of the TNF response and are presented as mean ± SEM of three experiments. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 654 ± 120 RLU and in TNF-stimulated cells was 3767 ± 673 RLU. Significance of difference between TNF and TNF + $TNFRI_{209-211}$: * $p < 0.05$, *** $p < 0.001$ (Dunnett's Multiple Comparisons test).

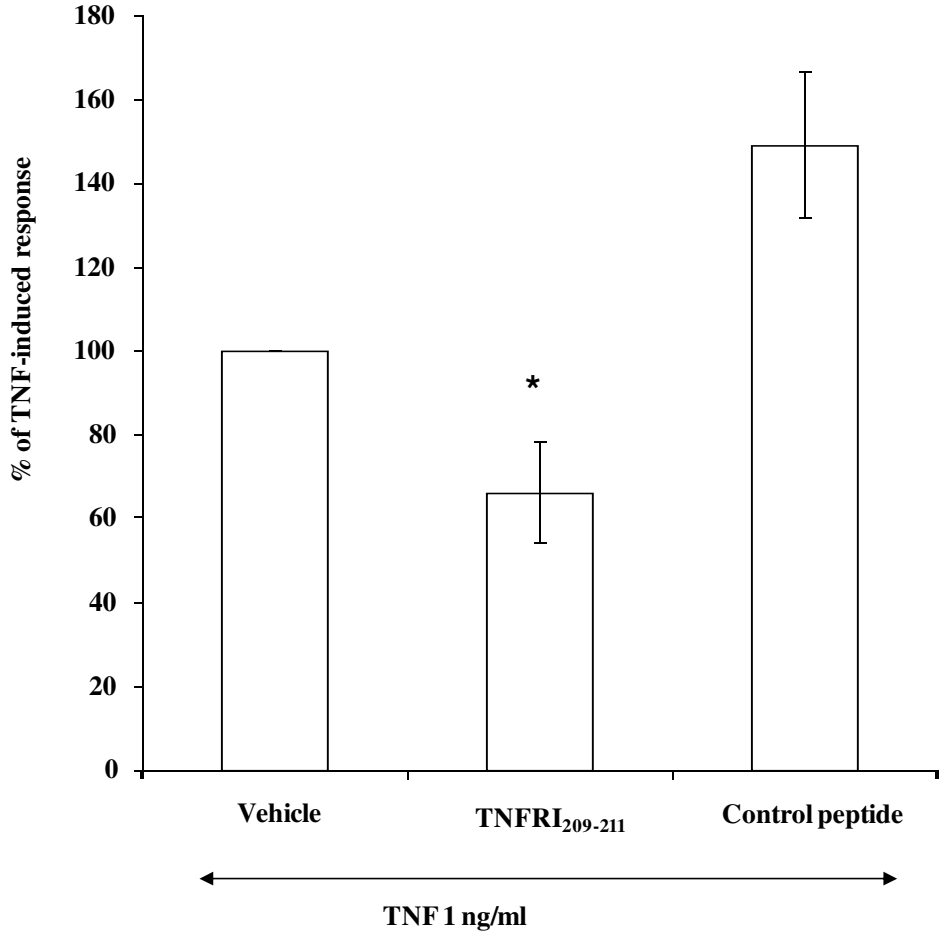

Figure 5.9 Comparison of the effects of $TNFRI_{209-211}$ and a control peptide on TNF-induced superoxide (chemiluminescence) production in neutrophils. TNF (1 ng/ml) was treated with diluent, 200 μM of either $TNFRI_{209-211}$ or control peptide for 20 min at 37°C. Neutrophils were added and incubated for 30 min at 37°C. Following this, lucigenin was added and the resultant chemiluminescence production measured. Data are presented as mean ± SEM of three experiments and expressed as a % of the control response. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 144 ± 46 RLU and in TNF-stimulated cells was 1270 ± 237 RLU. Significance of difference between TNF and TNF+ $TNFRI_{209-211}$: * $p < 0.05$ (Dunnett's Multiple Comparisons test).

5.6 THE EFFECT OF $TNFRI_{206-211}$ ON TNF-INDUCED SUPEROXIDE PRODUCTION IN NEUTROPHILS

The above results prompted us to synthesise and test a longer TNFRI peptide that contained 3 more amino acids N-terminal to GTT. This peptide was $TNFRI_{206-211}$ (Figure 5.3). TNF (1 ng/ml) was incubated with varying amounts of $TNFRI_{206-211}$ for 20 min at 37°C prior to the addition of neutrophils. The cells were examined for production chemiluminescence. Maximum inhibition occurred at $TNFRI_{206-211}$ of 400 μM and the half maximal inhibition (IC_{50}) occurred at 171.4 μM (Figure 5.10). $TNFRI_{209-211}$ caused a greater inhibition compared to $TNFRI_{206-211}$ at 50 μM ($p < 0.05$). A scrambled control peptide for $TNFRI_{206-211}$ (GEDTST) was shown to have no effect on TNF-induced chemiluminescence in neutrophils at similar concentrations, suggesting that the action of $TNFRI_{206-211}$ on TNF is not a non-specific effect (Figure 5.11).

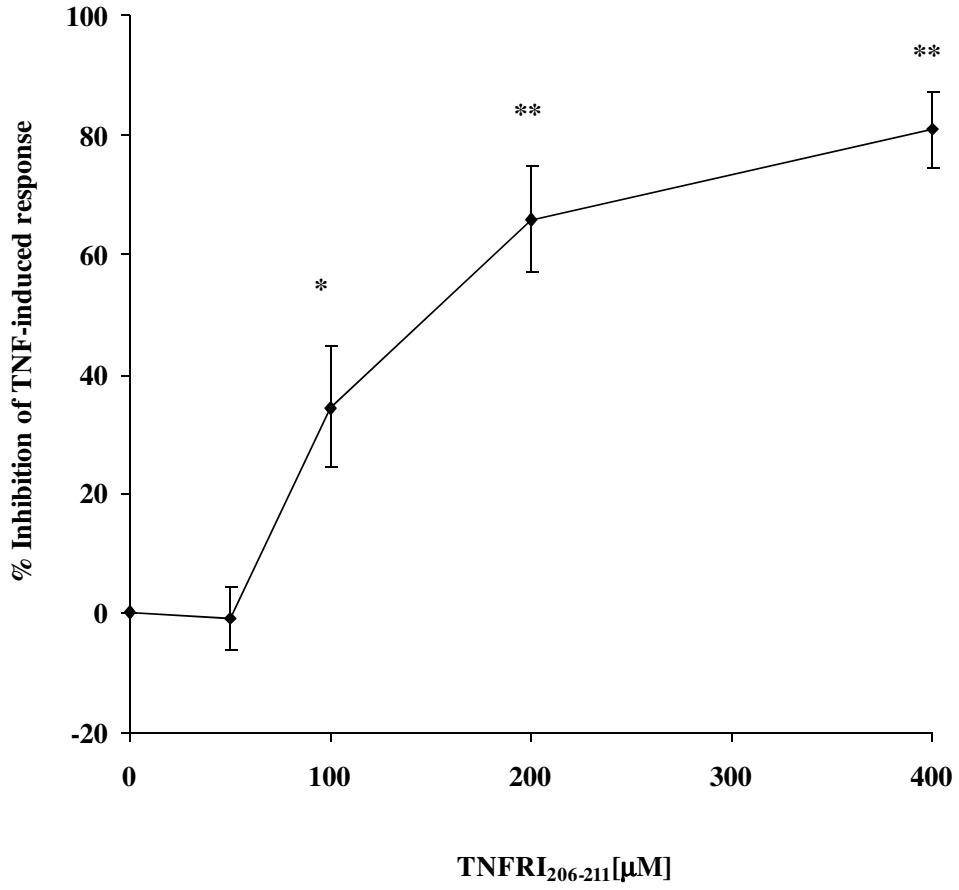

Figure 5.10 The effects of $TNFRI_{206-211}$ on TNF-induced superoxide (chemiluminescence) production in neutrophils. TNF (1 ng/ml) was treated with varying concentrations (50, 100, 200 and 400 μM) of $TNFRI_{206-211}$ at 37°C for 20 min. Neutrophils were added and further incubated for 30 min at 37°C. After incubation, lucigenin was added and the resultant chemiluminescence production measured. Data represent the % inhibition of the TNF response and are presented as mean ± SEM of three experiments. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 361 ± 63 RLU and in TNF-stimulated cells was 3536 ± 706 RLU. Significance of difference between TNF and TNF + $TNFRI_{206-211}$: * $p < 0.05$, ** $p < 0.01$ (Dunnett's Multiple Comparisons test).

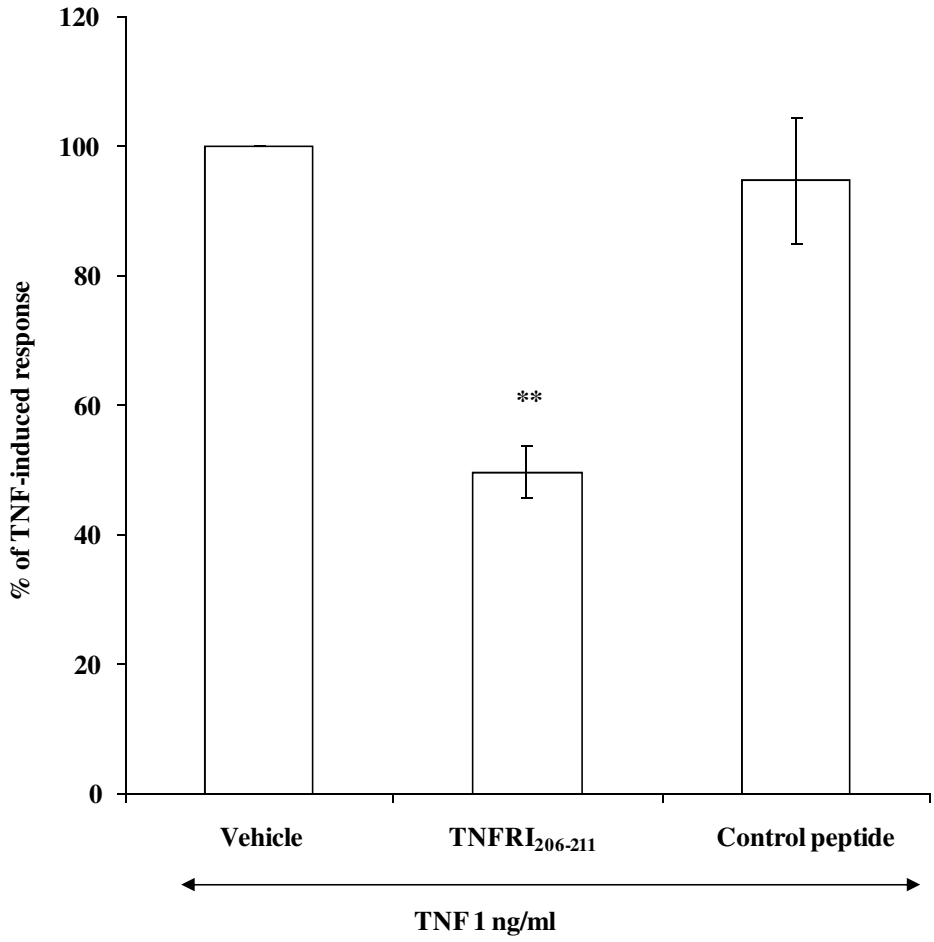

Figure 5.11 The effects of $TNFRI_{206-211}$ and control peptide on TNF-induced superoxide (chemiluminescence) production in neutrophils. TNF (1 ng/ml) was treated with 200 μM of either $TNFRI_{206-211}$ or control peptide for 20 min. Neutrophils were added and incubated for 30 min at 37°C. After incubation lucigenin was added and the resultant chemiluminescence production measured in a luminometer. Data represent the % inhibition of the TNF response and are presented as mean ± SEM of three experiments. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 219 ± 23 RLU and in TNF-stimulated cells was 1516 ± 613 RLU. Significance of difference between TNF and TNF + $TNFRI_{206-211}$: ** $p < 0.01$ (Dunnett's Multiple Comparisons test).

5.7 THE EFFECT OF $TNFRI_{209-211}$ AND $TNFRI_{206-211}$ ON THE ABILITY OF CYTOKINE CONTAINING MNL CONDITIONED MEDIUM TO STIMULATE NEUTROPHIL SUPEROXIDE PRODUCTION

It has been shown that stimulation of MNL with Staphylococcus aureus results in a conditioned medium containing TNF as the major cytokine responsible for stimulating neutrophils (Ferrante *et al.*, 1987, Ferrante *et al.*, 1990). The amount of TNF was evident in culture within 2 h and reached a maximum of 10-20 ng/ml after 1-2 days which remained stable over a 7 day culture period. No lymphotxin was detected but IFNγ and IL-1β were present (Ferrante *et al.*, 1990, Bates *et al.*, 1991). It was of interest to see if the TNFR peptides could also inhibit TNF action when the cytokine was present within an environment of activated leukocytes.

The TNF-rich medium (TNF-RM) caused a direct stimulation of neutrophil chemiluminescence (Figure 5.12). When neutrophils were pre-treated with TNF- RM for 30 min the chemiluminescence response was 333.3 ± 18 RLU in control medium (CM) and 19726 ± 2221 RLU in the TNF-RM. Pre-treatment of TNF-RM (1:10 dilution in HBSS) with different concentrations of $TNFRI_{206-211}$ before incubation with neutrophils resulted in dose-dependent inhibition of the ability of TNF-rich medium to stimulate neutrophil superoxide production, reaching a maximum effect at 200 μM (Figure 5.13). Pre-treatment of TNF-RM with receptor peptide $TNFRI_{209-211}$ resulted in a similar dose-dependent reduction of the neutrophil stimulating activity (Figure 5.14).

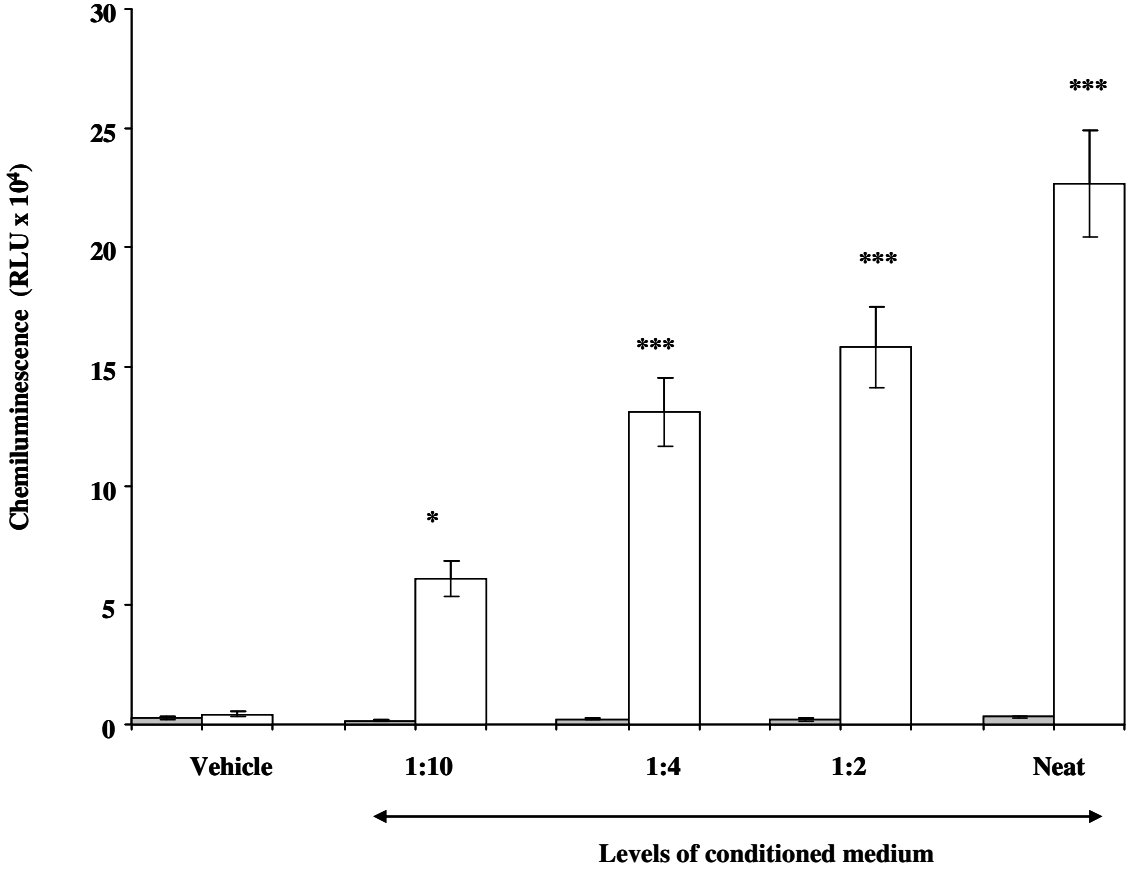

Figure 5.12 Neutrophil stimulating activities of TNF-rich medium and control medium on neutrophil superoxide (chemiluminescence) production. Neutrophils from an individual donor were treated with CM (shaded bars) or TNF-RM (open bars) for 30 min and chemiluminescence assayed. This data represents maximal rates peak of chemiluminescence production and expressed as mean ± SEM of triplicates. Significance of difference between each dilution of TNF-RM and its corresponding CM dilution: * $p < 0.05$, *** $p < 0.001$ (Tukey's Multiple Comparison Test).

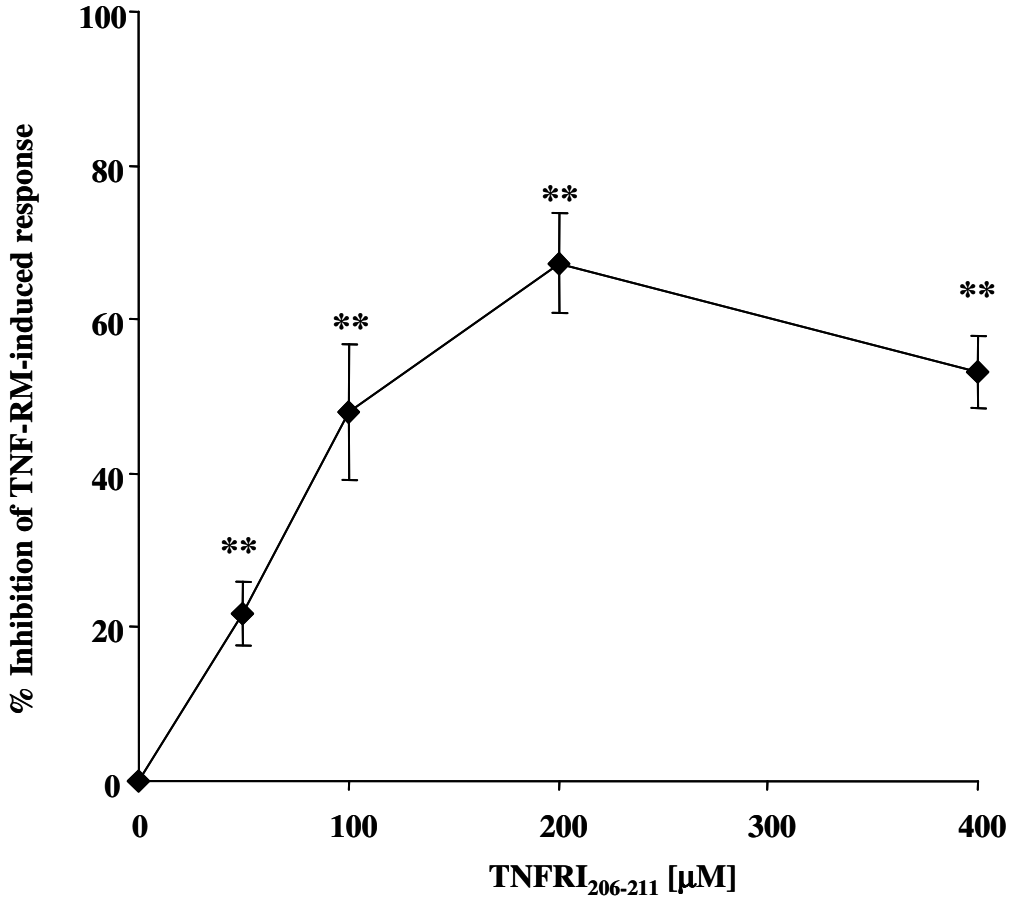

Figure 5.13 Effect of $TNFRI_{206-211}$ on TNF-RM-induced superoxide (chemiluminescence) production in neutrophils. TNF-RM (1:10 dilution in HBSS) was treated with various concentrations of $TNFRI_{206-211}$ and then tested for ability to stimulate neutrophil superoxide (chemiluminescence) production. Data represent the % inhibition of the TNF-RM response and expressed as mean ± SEM of three separate experiments. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 444 ± 69 RLU and in TNF-rich medium-stimulated cells was 8593 ± 1854 RLU. Significance of difference between TNF-RM and TNF-RM + $TNFRI_{206-211}$: ** $p < 0.01$ (Dunnett's Multiple Comparisons test).

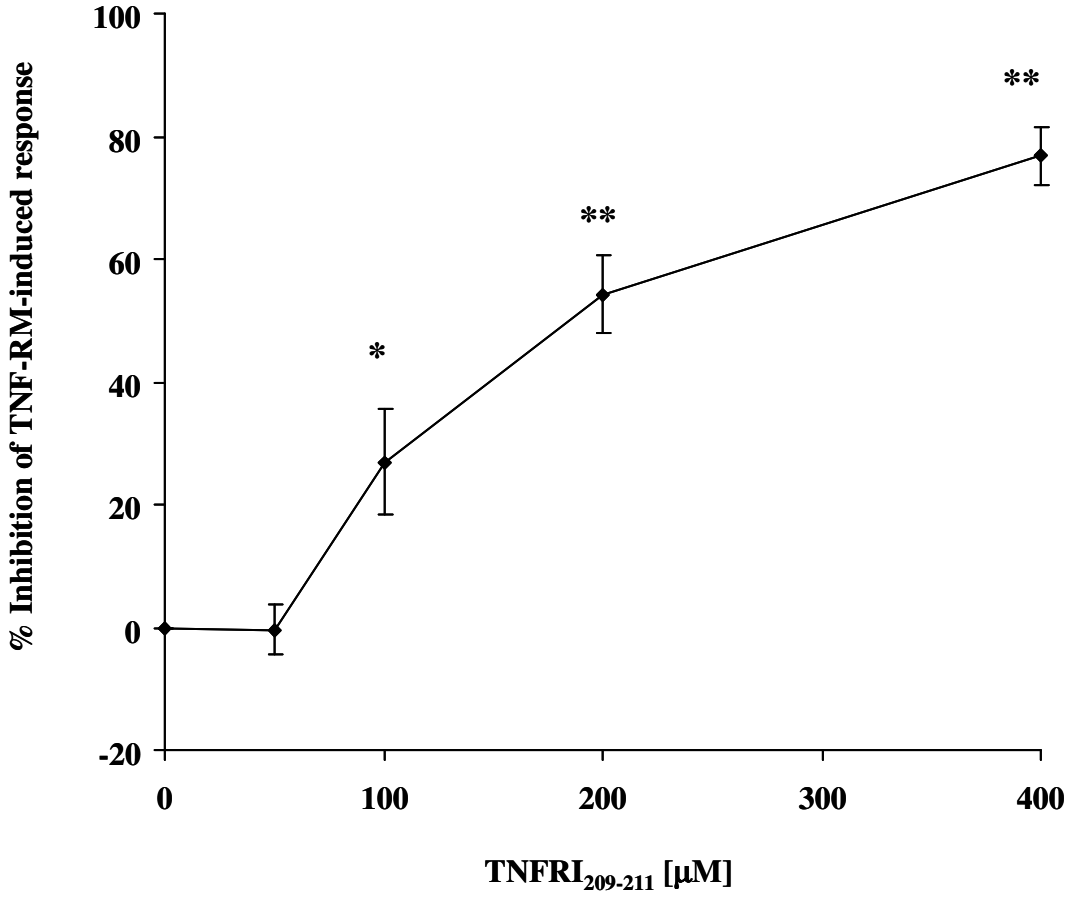

Figure 5.14 Effect of receptor peptide $TNFRI_{209-211}$ on TNF-RM-induced superoxide (chemiluminescence) production in neutrophils. TNF-RM (1:10 dilution in HBSS) was treated with various concentrations of $TNFRI_{209-211}$ and tested for ability to stimulate neutrophil chemiluminescence. Data represent the % inhibition of the TNF-RM-induced response and expressed as mean ± SEM for three separate experiments. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 261 ± 24 RLU and in TNF-RM-stimulated cells was 6328 ± 1495 RLU. Significance of difference between TNF-RM and TNF-RM + $TNFRI_{209-211}$: * $p < 0.05$, ** $p < 0.01$ (Dunnett's Multiple Comparisons test).

5.8 EFFECT OF $TNFRI_{209-211}$ AND $TNFRI_{206-211}$ ON FMLP-INDUCED SUPEROXIDE PRODUCTION IN NEUTROPHILS

The specificity of these receptor peptides was further assessed by examining their effect on the fMLP-stimulated chemiluminescence production. fMLP activates neutrophils by binding to its surface receptor- formyl peptide receptor (FPR). Pre-treatment of fMLP with $TNFRI_{209-211}$ did not affect the ability of neutrophils to respond to fMLP, even at $TNFRI_{209-211}$ concentrations up to 400 μM (Figure 5.15). Similarly $TNFRI_{206-211}$ did not affect the fMLP-induced response at these concentrations (Figure 5.16). Incubation with the peptides alone neither increased nor decreased the baseline chemiluminescence in all experiments (data not shown).

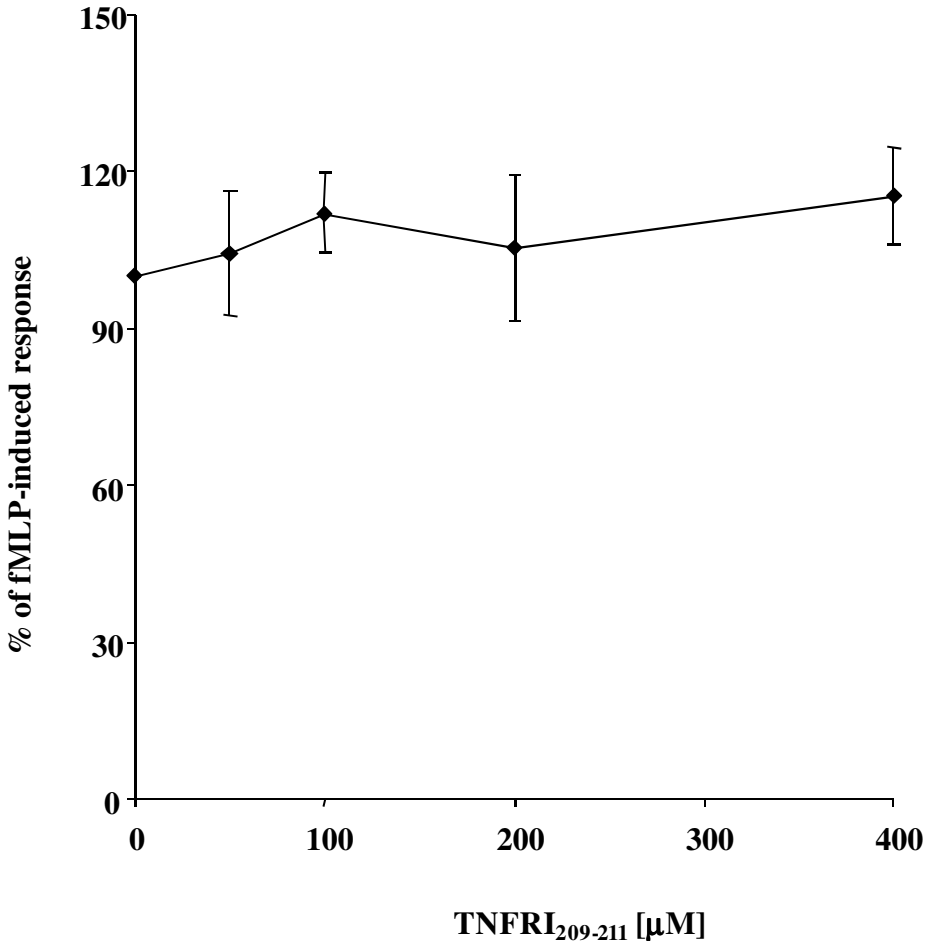

Figure 5.15 Effect of $TNFRI_{209-211}$ on fMLP-induced superoxide (chemiluminescence) generation in neutrophils. fMLP (5 x 10^{-6}M final) was incubated with different concentrations of $TNFRI_{209-211}$ at 37°C for 5 min. Neutrophils and lucigenin were added and the resultant chemiluminescence production measured. The results are expressed as a % of fMLP-induced chemiluminescence production. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 1106± 272 RLU and in fMLP-stimulated cells was 5617± 495 RLU. Data are presented as mean ± SEM of three experiments.

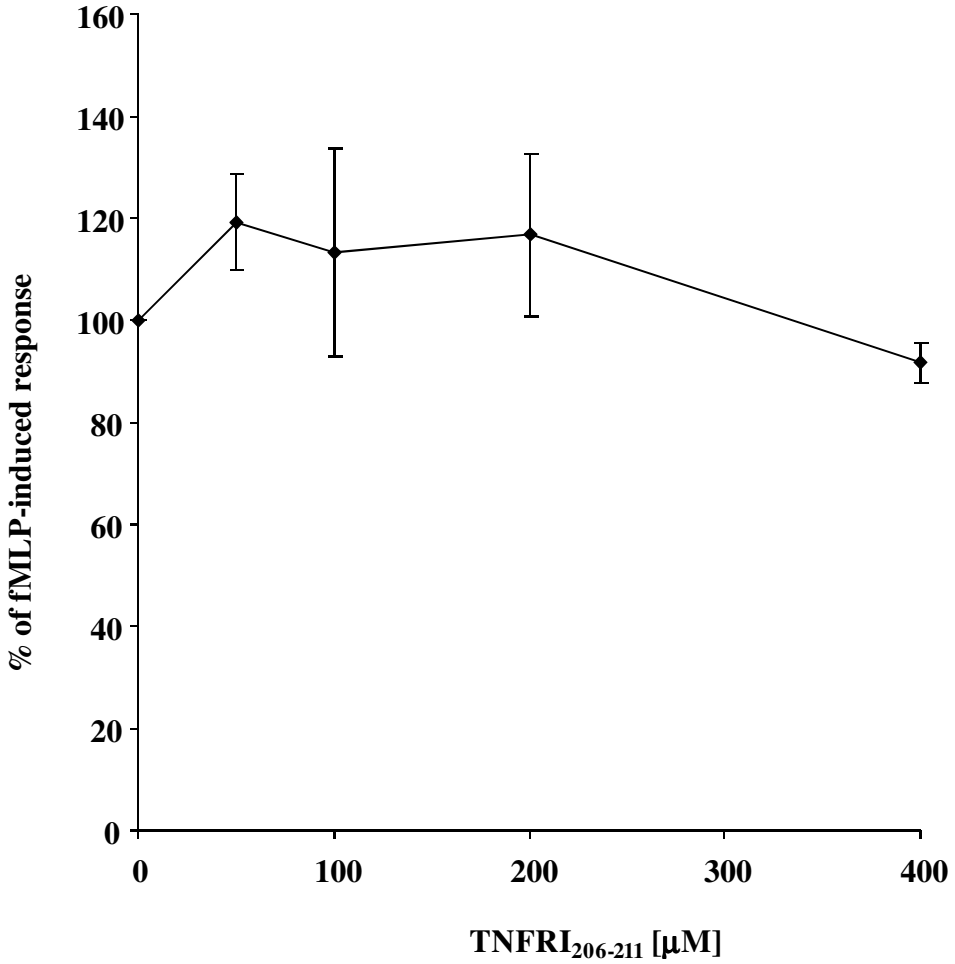

Figure 5.16 Effect of $TNFRI_{206-211}$ on the fMLP-induced superoxide (chemiluminescence) generation in neutrophils. To test specificity of $TNFRI_{206-211}$ fMLP (5 x 10^{-6}M final) was incubated with different concentrations of $TNFRI_{206-211}$ at 37°C for 5 min. Neutrophils and lucigenin were added and the resultant chemiluminescence production measured in a luminometer. The results are expressed as a % of fMLP-induced chemiluminescence production. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 1106± 272 RLU and in fMLP-stimulated cells was 5617± 495 RLU. Data are presented as mean ± SEM of three experiments.

5.9 EFFECT OF $TNFRI_{206-211}$ AND $TNFRI_{209-211}$ ON TNF-INDUCED P38 ACTIVATION

To gain a better understanding of the activities of the TNFR mimetics we examined their ability to prevent TNF from stimulating p38 MAPK. Initial experiments were conducted to determine the kinetics of p38 activation in neutrophils treated with TNF. Neutrophils were treated with TNF (1 ng/ml) for 0, 5, 10 and 15 min, and then assayed for p38 activity (Figure 5.17). p38 activity was found to be rapidly increase following treatment with TNF, reached a maximum after 5 min and decreased from 10 min onwards. The basal level of p38 activity did not vary much over this time period. In subsequent experiments the cells were incubated with TNF for 5 min.

To investigate the effect of TNFRI peptides, TNF was pre-incubated with $TNFRI_{209-211}$ or $TNFRI_{206-211}$ for 20 min before being added to neutrophils. The cells were then incubated for 5 min at 37°C, lysed and p38 precipitated for kinase activity assay. Incubation with $TNFRI_{206-211}$ and $TNFRI_{209-211}$ inhibited p38 activity by 53 % and 40 % respectively (Figure 5.18).

To determine if this effects could be extended to mononuclear phagocytes, we investigated the effects of $TNFRI_{206-211}$ on TNF-induced p38 activation in the monocytic cell line, Mono Mac 6 cells. In accordance with recent studies on monocytes or monocytic cell lines the amount of TNF required to activate p38 was 5 ng/ml (Nguyen *et al.*, 2006, Tahan *et al.*, 2008). The cells were treated with 5 ng/ml of TNF which had

been pre-treated with 200 μM of $TNFRI_{206-211}$ for 20 min. $TNFRI_{206-211}$ reduced TNF induced p38 activity by 70 % ($p < 0.05$) (Figure 5.19)

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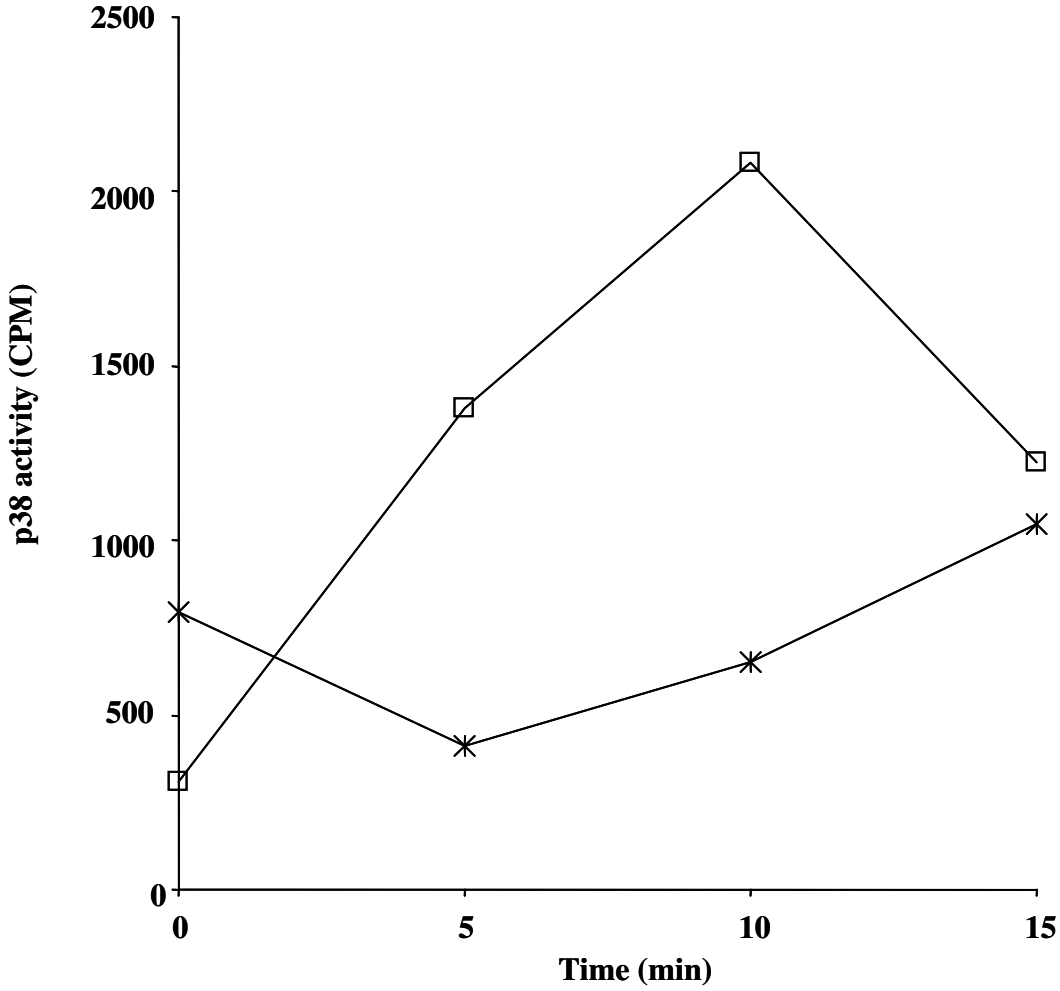

Figure 5.17 Kinetics of p38 activation in neutrophils following stimulation with TNF. Neutrophils were treated with 1 ng/ml of TNF for 0, 5, 10 and 15 min at 37°C. The cells were lysed, p38 immunoprecipitated and kinase activity was assayed using myelin basic protein as a substrate. Control (x), TNF (□). Data from a representative experiment of 3.

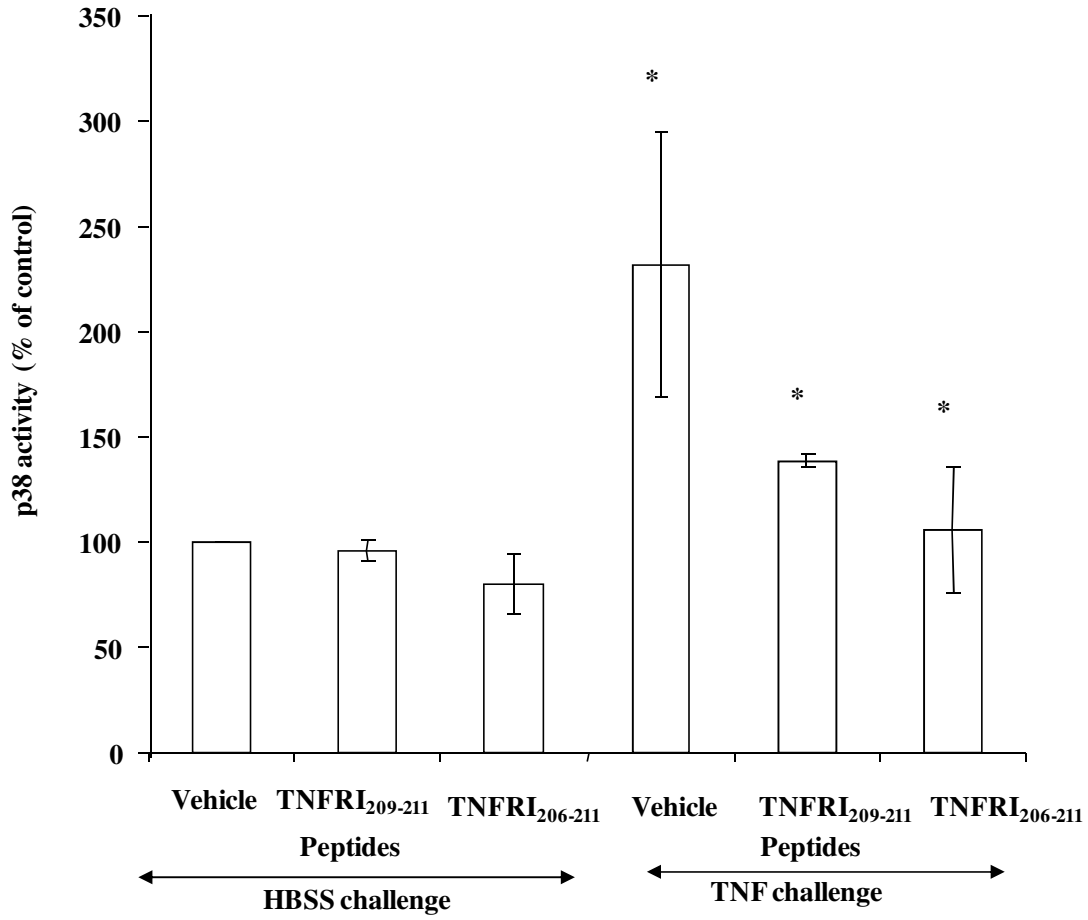

Figure 5.18 Inhibition of TNF-induced p38 activation in neutrophils by TNFRI peptides. Cells were incubated for 5 min in the presence of TNF (1 ng/ml) which had been pre-treated with 200 μM of $TNFRI_{209\text{-}211}$ or $TNFRI_{206\text{-}211}$ for 20 min at 37°C. The cells were lysed, p38 immunoprecipitated and kinase activity was assayed using myelin basic protein as a substrate. Data are expressed as % of vehicle control in the absence of TNF, presented as the mean ± SEM of 3 experiments. Significance of difference between control and TNF or TNF and TNF + $TNFRI_{209\text{-}211}$ or TNF + $TNFRI_{206\text{-}211}$: * $p < 0.05$ (Dunnett's Multiple Comparison test).

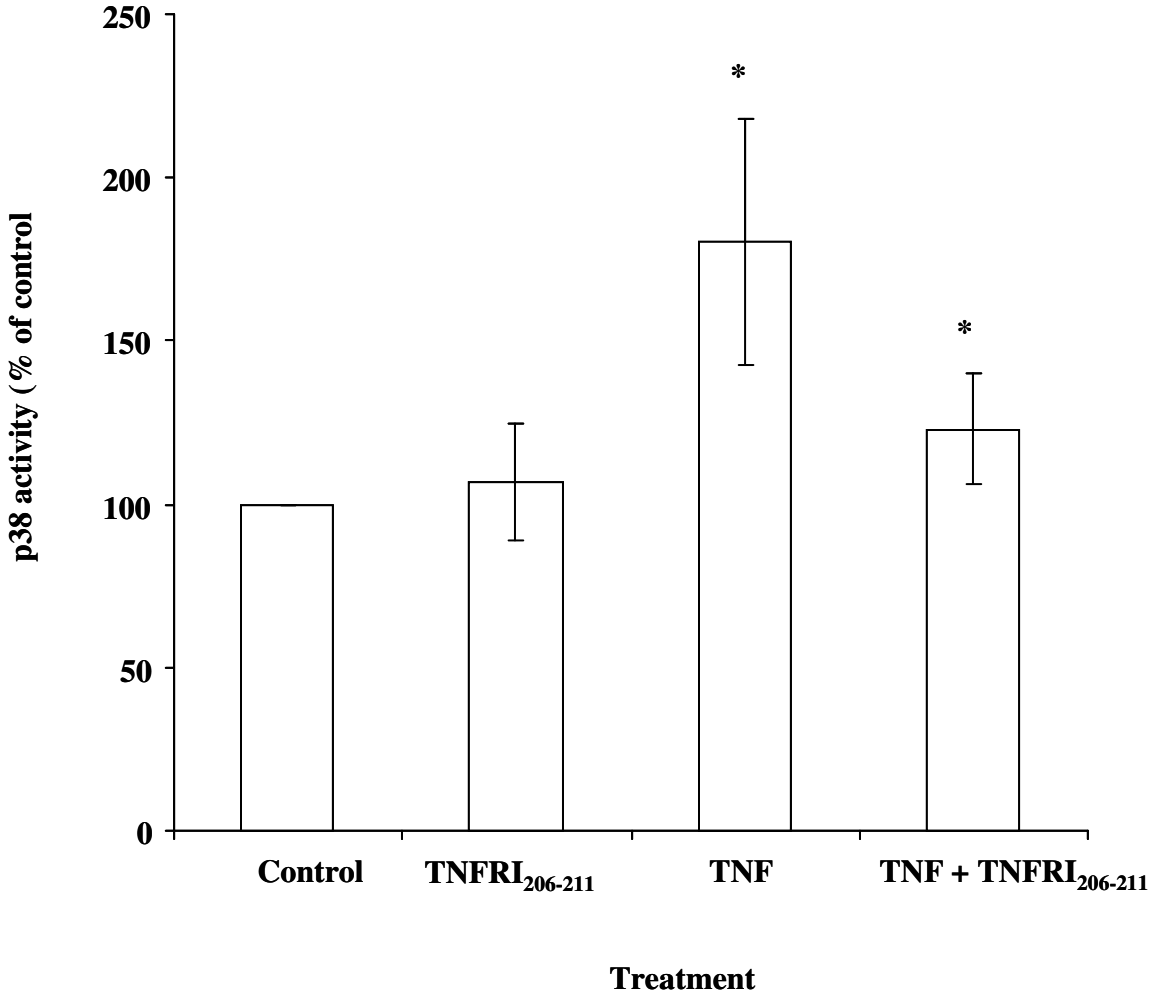

Figure 5.19 The effect of $TNFRI_{206-211}$ on TNF-induced p38 activation in Mono Mac 6 cells. Cells were incubated for 15 min in the presence of TNF (5 ng/ml) which had been pre-treated with 200 μM of $TNFRI_{206-211}$ for 20 min at 37°C. The cells were lysed, p38 immunoprecipitated and kinase activity was assayed using myelin basic protein as a substrate. Data are expressed as % of vehicle control in the absence of TNF, presented as the mean ± SEM of 3 experiments. Significance of difference between control and TNF or TNF and TNF + $TNFRI_{206-211}$: * $p < 0.05$ (Dunnett's Multiple Comparison test).

5.10 EFFECT OF $TNFRI_{206-211}$ ON CR3 (CD11B/CD18) EXPRESSION

Stimulation of neutrophils by TNF primes the cells and induces β2 integrin expression/activation and adhesion-dependent functions such as spreading degranulation and oxidative burst. The depressed oxidative burst observed in neutrophils treated with TNF in the presence of $TNFRI_{206-211}$ may have been due to the down regulation of CD11b expression or the inability to up-regulate its expression. TNF-induced CR3 upregulation has been shown to be mediated by the p38 pathway as pre-treating neutrophils with p38 inhibitor SB203580 blocked the observed TNF-induced CR3 upregulation (Forsberg *et al.*, 2001) .Thus the effect of $TNFRI_{206-211}$ or its scrambled control (50-200 μM) on TNF (5 ng/ml)-induced CR3 upregulation was examined by measuring the expression of CD11b, which together with the β2 integrin, CD18 constitute CR3.

The data show that while $TNFRI_{206-211}$ did not affect basal levels of CD11b expression, it inhibited TNF-induced expression by 56 % at 200 μM (Figure 5.20). A scrambled control of this sequence neither affected the basal levels nor the TNF-induced upregulation (Figure 5.21). The effect of $TNFFRI_{206-211}$ was concentration-dependent (Figure 5.22).

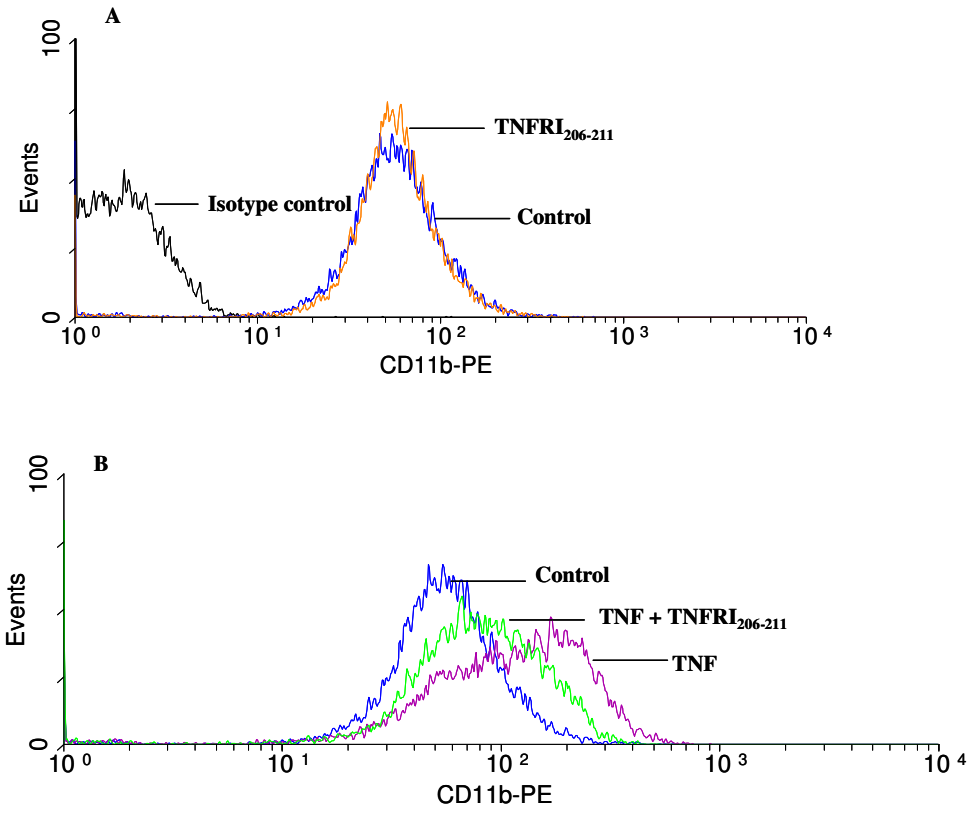

Figure 5.20 The effect of $TNFRI_{206-211}$ on (A) Basal levels of CD11b expression and (B) TNF stimulated up-regulation of neutrophil CD11b expression. TNF (5 ng/ml) was pre-incubated with 200 µM $TNFRI_{206-211}$ for 20 min at 37°C before neutrophils were added. The cells were then stained with a PE-labelled mouse anti-human CD11b antibody and CD11b expression analysed by flow cytometry. A representative histogram from 3 experiments. (Basal mean fluorescence intensity (MFI) = 52, + TNF = 100).

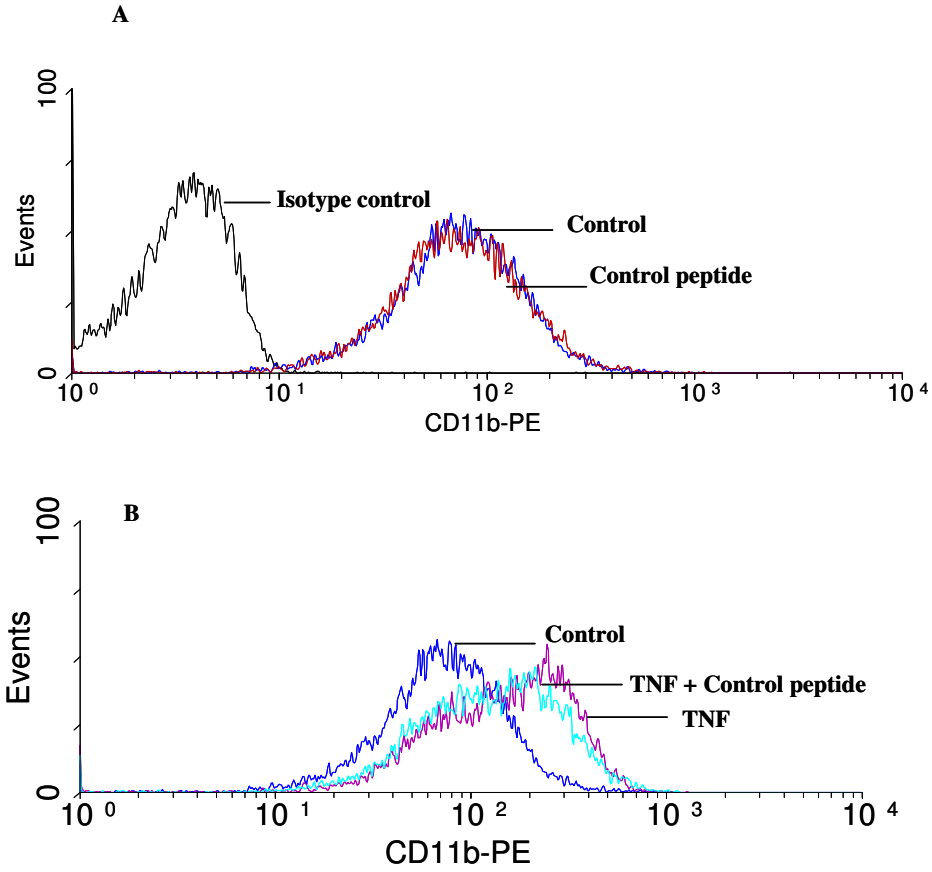

Figure 5.21 The effect of the scrambled control peptide on (A) Basal levels of CD11b expression and (B) TNF stimulated up-regulation of neutrophil CD11b expression. TNF (5 ng/ml) was pre-incubated with 200 µM control peptide for 20 min at 37°C before neutrophils were added. The cells were then stained with a PE-labelled mouse anti-human CD11b antibody and CD11b expression analysed by flow cytometry. A representative histogram from 3 experiments. (Basal MFI = 70.15, + TNF = 136.21).

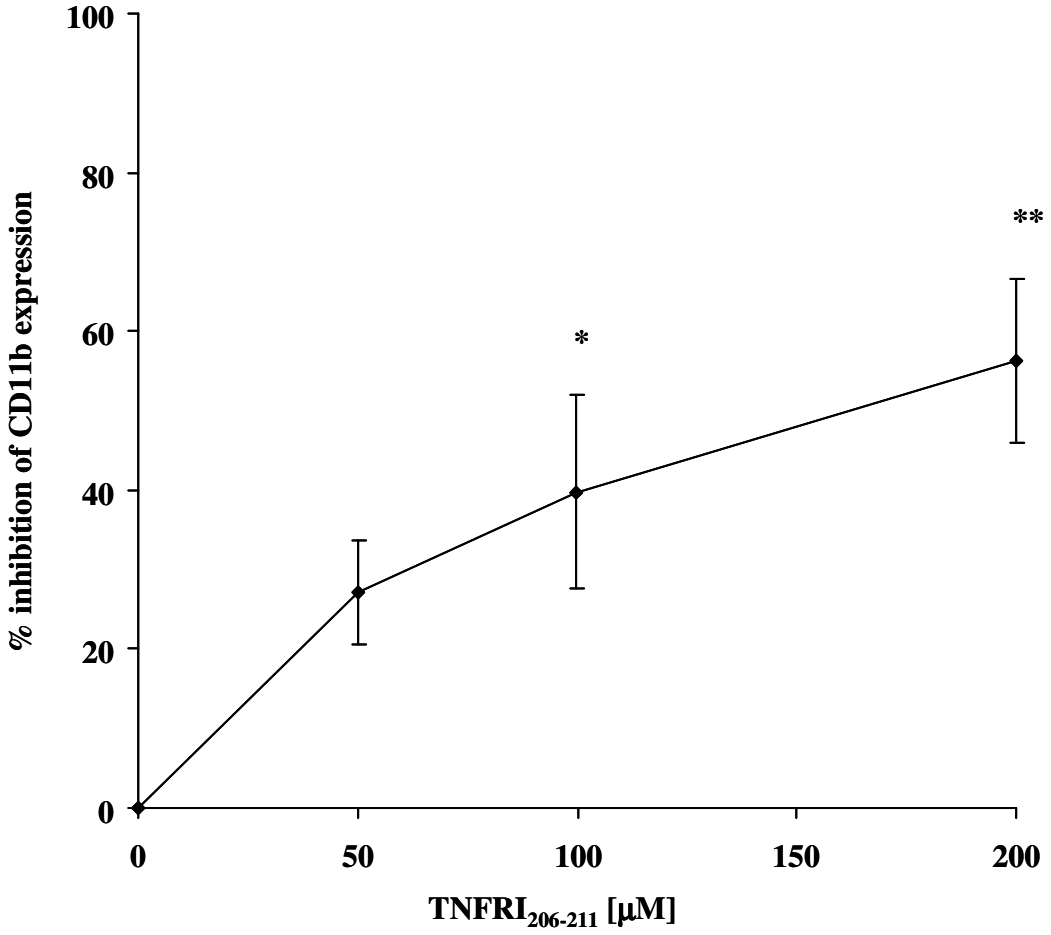

Figure 5.22 The effects of $TNFRI_{206-211}$ on TNF induced up-regulation of neutrophil CD11b expression in neutrophils. Different concentrations of $TNFRI_{206-211}$ were incubated with TNF (5 ng/ml) at 37°C for 20 min. Neutrophils were added and further incubated for 30 min at 37°C. The cells were then stained with a PE-labelled mouse anti-human CD11b antibody and CD11b expression analysed by flow cytometry. Data are expressed as % inhibition of the response seen with TNF alone, presented as mean ± SEM of three experiments. Significance of difference between TNF and TNF + $TNFRI_{206-211}$: * $p < 0.05$, ** $p < 0.01$ (Dunnett's Multiple Comparisons test).

5.11 EFFECT OF TNFRI$_{206-211}$ ON TNF-INDUCED CYTOKINE PRODUCTION IN NEUTROPHILS

A large range of stimuli has been reported to induce cytokine synthesis in neutrophils. These include classic agonist such as lipopolysaccharide, cytokines themselves, phagocytic particles, micro-organisms, fMLP, leukotriene B4 and complement component C5a. The kinetics and magnitude of cytokine release vary depending on the stimulus used (Cassatella, 1995). Recently NF-κB, p38 and ERK1/EK2 pathways have been demonstrated to be involved in the generation of inflammatory cytokines by neutrophils (Cloutier *et al.,* 2007). It was our objective to determine if the TNFRI derived peptide, TNFRI$_{206-211}$, was able to inhibit TNF-induced cytokine production by neutrophils. *In vitro* assessment of *de novo* synthesis of cytokines and chemokines by neutrophils is a major contentious issue which might partly explain recent contradictory results (Altstaedt *et al.*, 1996, Xing and Remick, 2003, Ethuin, 2005). As neutrophils themselves do not produce copious amounts of cytokines like other cell types such as peripheral mononuclear cells, it is highly imperative to work with highly purified neutrophil preparations (> 99.5 %) and avoid pre-stimulation of cells during experiments.

5.11.1 Effect of TNFRI$_{206-211}$ on TNF-induced IL-Iβ production

Highly purified preparations of neutrophils were treated with 0, 0.1, 1, 10 ng/ml of TNF for 1 h. The RNA was extracted and levels of IL-1β mRNA expression were determined

by quantitative real-time PCR. The results show that the level of Il-1β expression was dose-dependently increased by TNF (Figure 5.23). The kinetics of IL-1β expression was also investigated at a TNF concentration of 1 ng/ml. The results show that mRNA expression of IL-1β could be detected as early as 30 min and peaked within 1 h after stimulation and began to decrease after 2 h (Figure 5.24). When TNF was pre-treated with 200 μM of $TNFRI_{206-211}$, there was a significant, 30 %, reduction in IL-1β mRNA production (Figure 5.25).

5.11.2 Effect of $TNFRI_{206-211}$ on TNF-induced IL-8 production

Neutrophils were treated with 0, 0.1, 1, 10 ng/ml of TNF for 1 h. The RNA was extracted and levels of IL-8 mRNA expression was determined by quanitative real-time PCR. The results show that the level of IL-8 expression was increased by a 4 fold at the highest TNF concentration tested (figure 5.26). The kinetics of IL-8 production showed a similar pattern to that of IL-1β production. Thus, IL-8 could be detected as early as 30 min and peaked within 1 h after stimulation and began to decrease after 2 h (Figure 5.27). When TNF was pre-treated with 200 μM of $TNFRI_{206-211}$, there was a significant, 26 %, reduction in IL-8 mRNA production (Figure 5.28)

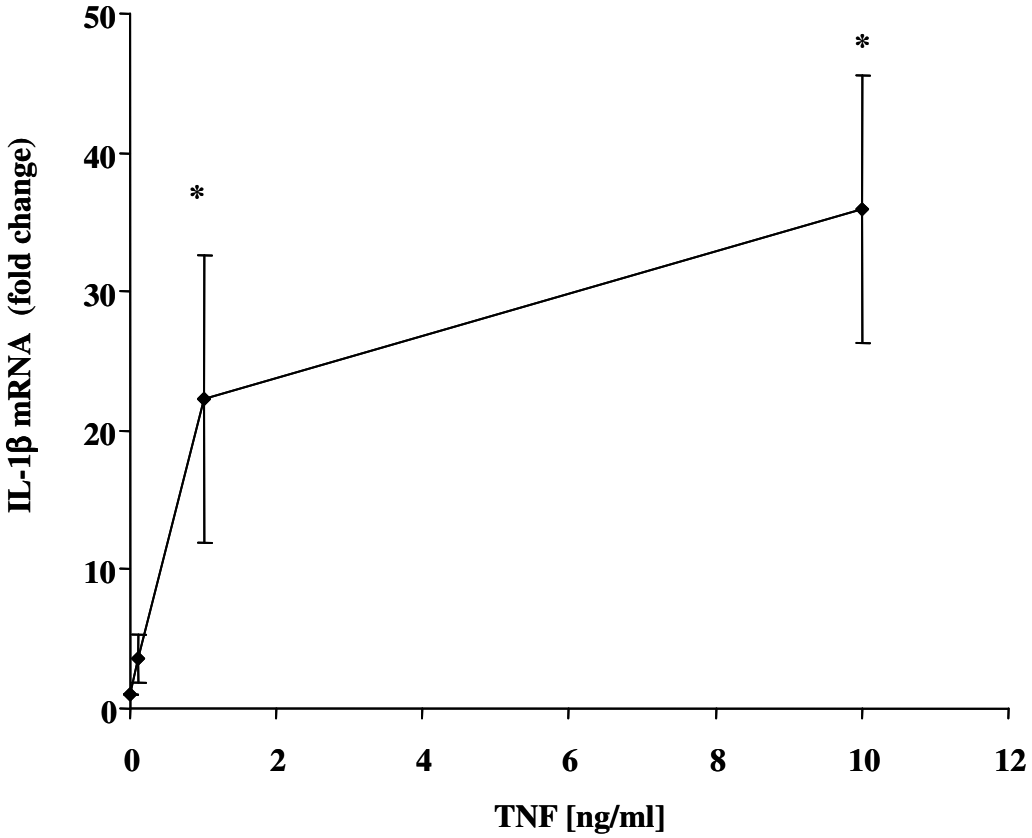

Figure 5.23 The effect of TNF on IL-1β expression in neutrophils. Cells (1 x 10^6 in RPMI 1640 supplemented with medium with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 % AB serum) were stimulated for 1 h with 0, 0.1, 1 or 10 ng/ml of TNF. Levels of IL-1β mRNA were determined by real-time quantitative PCR. The housekeeping gene, GAPDH, was used to normalize the samples. The data are expressed as % fold change ($2^{-\Delta\Delta Ct}$), in which ΔCt = the Ct for cytokine gene minus the Ct of the housekeeping gene, GAPDH: ΔΔCt = the ΔCt of stimulated cells at different concentrations minus the ΔCt of unstimulated cells. Data are expressed as % of vehicle control in the absence of TNF, presented as mean ± SEM of three experiments. Significance of difference between control and TNF: * $p < 0.05$ (Dunnett's Multiple Comparison test).

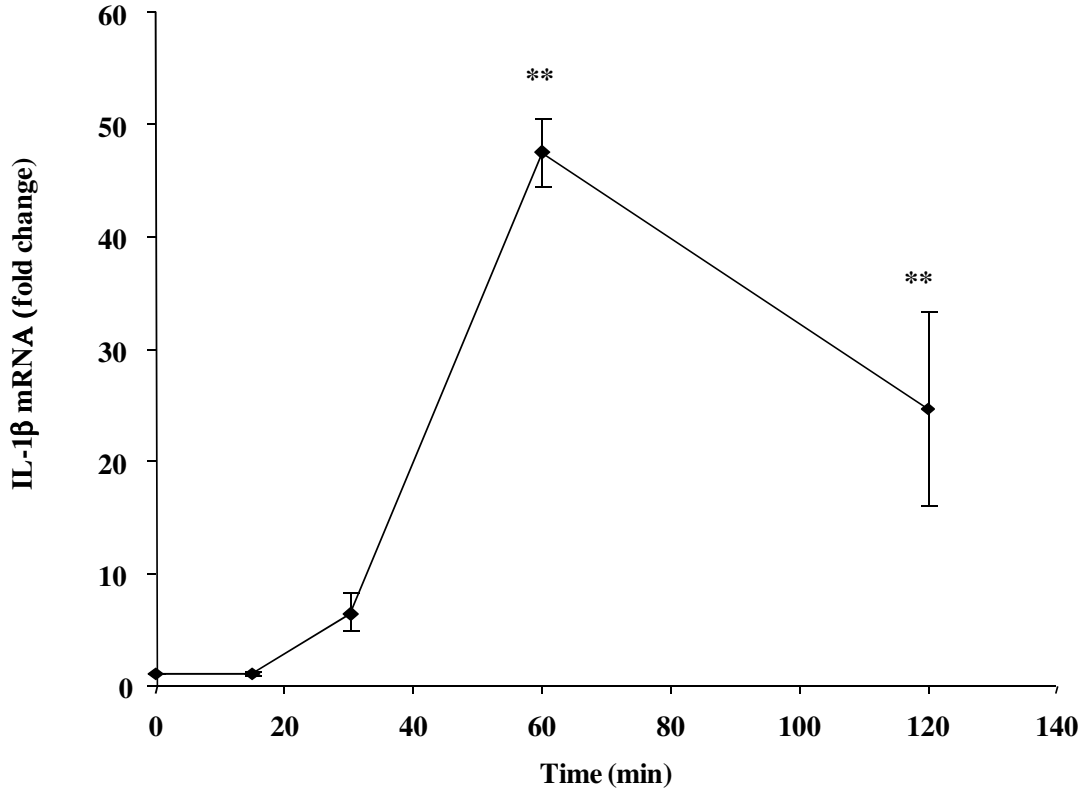

Figure 5.24 Kinetics of IL-1β expression in TNF-stimulated neutrophils. Cells (1 x 10^6 in RPMI 1640 supplemented with medium with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 % AB serum) were stimulated for 0, 15 min, 30 min, 1 or 2 h with 1ng/ml TNF. Levels of IL-1β mRNA were determined by real-time quantitative PCR. The housekeeping gene, GAPDH, was used to normalize the samples. The data are expressed as % fold change ($2^{-\Delta\Delta Ct}$), in which ΔCt = the Ct for cytokine gene minus the Ct of the housekeeping gene, GAPDH: ΔΔCt = the ΔCt of stimulated cells at different time points minus the ΔCt of cells at 0 h. Data are expressed as % of time zero control, presented as mean ± SEM of three experiments. Significance of difference at time zero and the different time points: ** $p < 0.01$ (Dunnett's Multiple Comparison test).

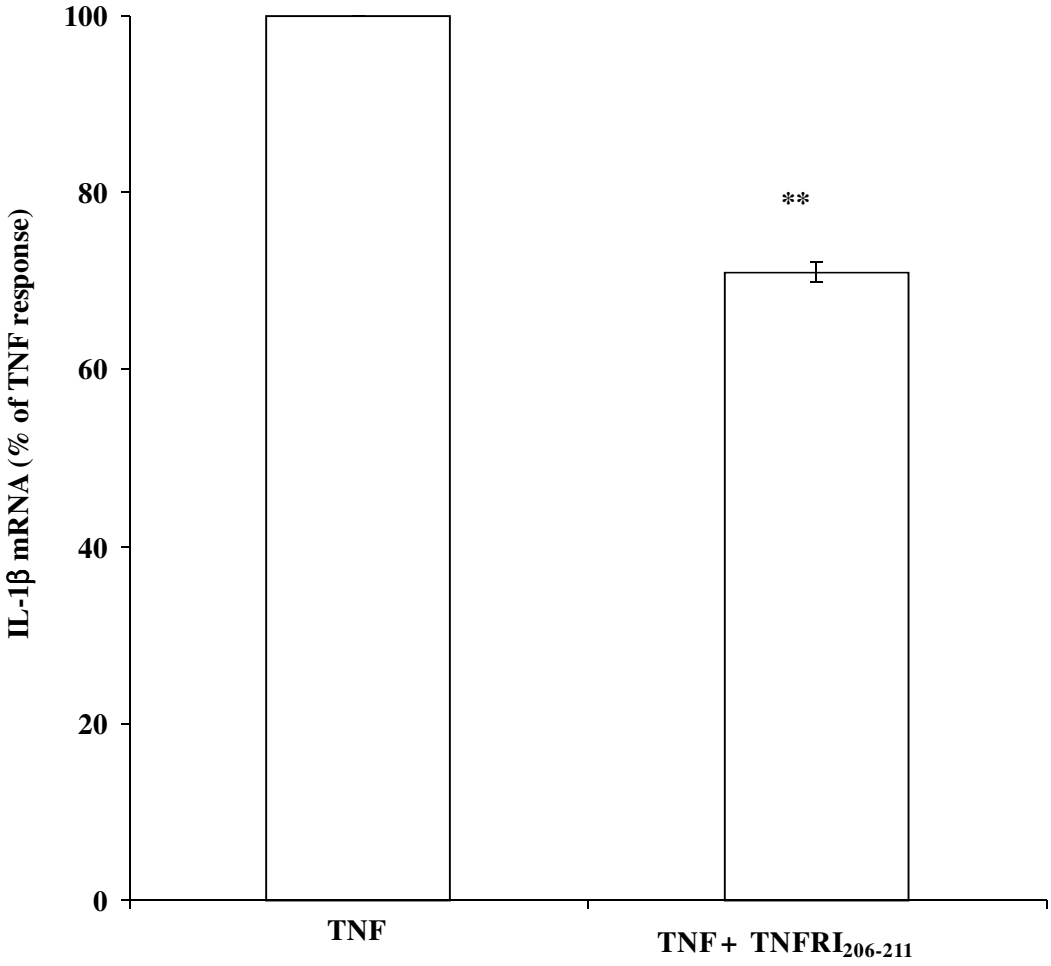

Figure 5.25 The effect of $TNFRI_{206-211}$ on TNF-induced IL-1β mRNA in neutrophils. TNF (1 ng/ml) was pre-incubated with 200μM $TNFRI_{206-211}$ for 20 min at 37°C, added to neutrophils in RPMI 1640 supplemented with medium with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 % AB serum and incubated for 1 h. Levels of IL-1β mRNA were determined by quantitative real-time PCR. The housekeeping gene, GAPDH, was used to normalize the samples. The data (mean ± SEM) are expressed as the % of the TNF response of three experiments. Significance of difference between TNF and TNF + $TNFRI_{206-211}$: ** $p < 0.01$ (Student t test).

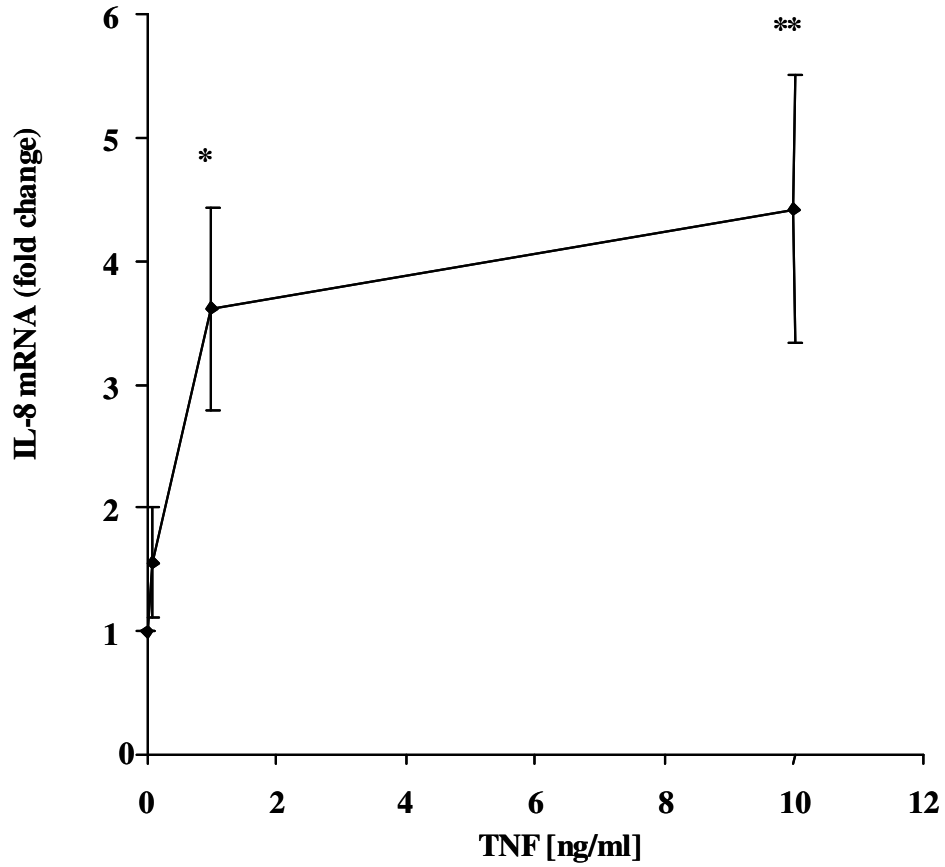

Figure 5.26 The effect of TNF on IL-8 expression in neutrophils. Cells (1 x 10^6 in RPMI 1640 supplemented with medium with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 % AB serum) were stimulated for 1 hour with 0, 0.1, 1 or 10ng/ml of TNF. Levels of IL-8 mRNA were determined by real-time quantitative PCR. The housekeeping gene, GAPDH, was used to normalize the samples. The data are expressed as % fold change ($2^{-\Delta\Delta Ct}$), in which ΔCt = the Ct for cytokine gene minus the Ct of the housekeeping gene, GAPDH: ΔΔCt = the ΔCt of stimulated cells at different concentrations minus the ΔCt of unstimulated cells. Data are expressed as % of vehicle control in the absence of TNF, presented as mean ± SEM of three experiments. Significance of difference between control and TNF: * $p < 0.05$; ** $p < 0.01$ (Dunnett's Multiple Comparison test).

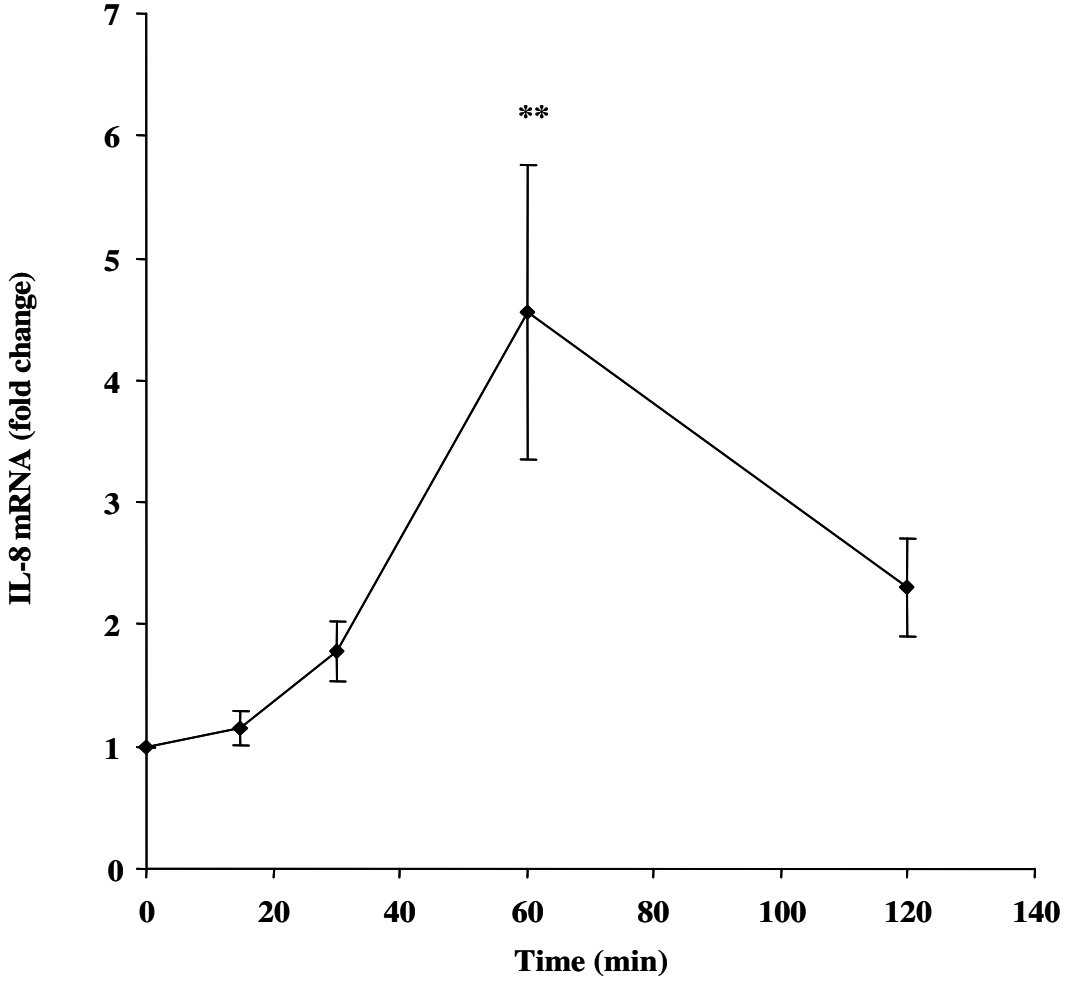

Figure 5.27 Kinetics of IL-8 expression in TNF-stimulated neutrophils. Cells (1 x 10^6 in RPMI 1640 supplemented with medium with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 % AB serum) were stimulated for 0, 15 min, 30 min, 1 or 2 h with 1ng/ml TNF. Levels of IL-8 mRNA were determined by real-time quantitative PCR. The housekeeping gene, GAPDH, was used to normalize the samples. The data are expressed as % fold change ($2^{-\Delta\Delta Ct}$), in which ΔCt = the Ct for cytokine gene minus the Ct of the housekeeping gene, GAPDH: ΔΔCt = the ΔCt of stimulated cells at different time points minus the ΔCt of cells at 0 h. Data are expressed as % of time zero control, presented as mean ± SEM of three experiments. Significance between time zero and the different time points: ** $p < 0.01$ (Dunnett's Multiple Comparison test).

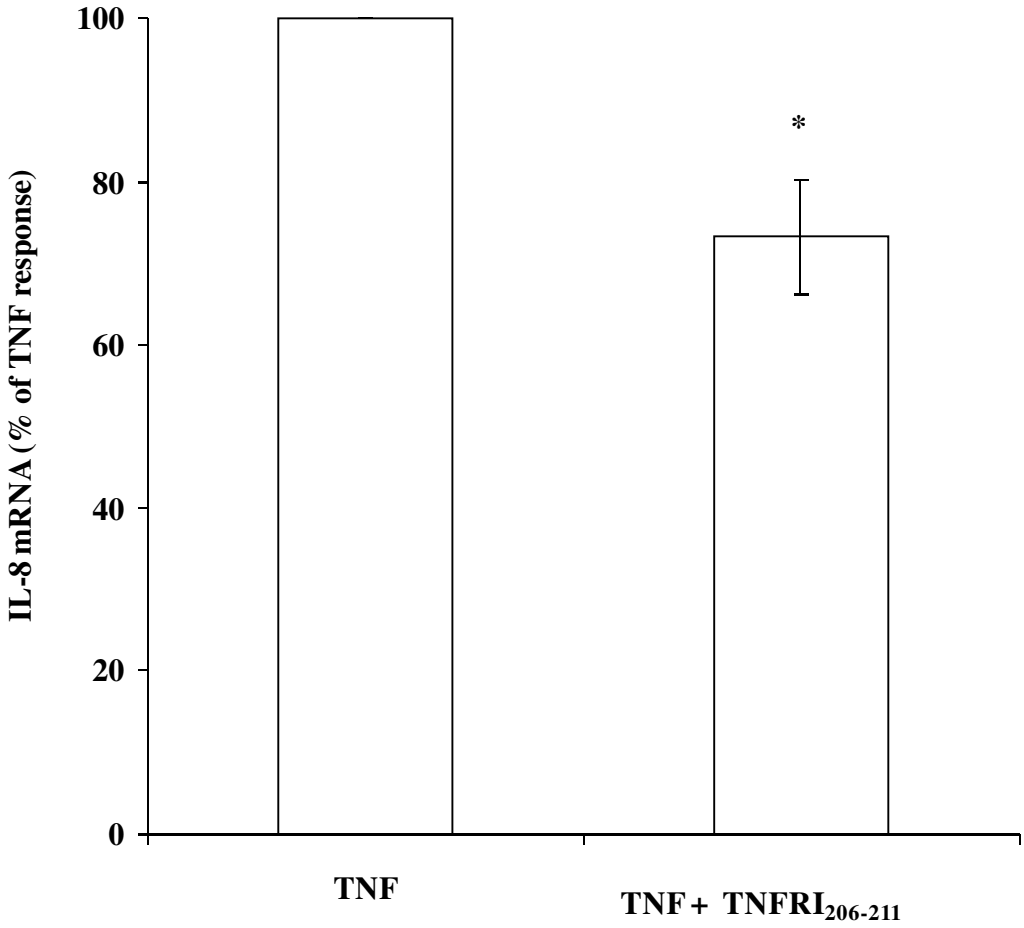

Figure 5.28 The effect of $TNFRI_{206-211}$ on the production of TNF-induced IL-8 mRNA in neutrophils. TNF (1 ng/ml) was pre-incubated with 200μM $TNFRI_{206-211}$ for 20 min at 37°C before neutrophils were added. Neutrophils (1 x 10^6 in RPMI 1640 supplemented with medium with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 % AB serum) were stimulated for 1 h. Levels of IL-8 mRNA were determined by quantitative real-time PCR. The housekeeping gene, GAPDH, was used to normalize the samples. The data (mean ± SEM) are expressed as the % of the TNF response of three experiments. Significance of difference between TNF and TNF + $TNFRI_{206-211}$: * $p < 0.05$ (Student t test).

5.12 EFFECT OF THE D-AMINO FORM OF TNFRI$_{206-211}$ AND OTHER VARIANTS OF TNFRI ON TNF-INDUCED SUPEROXIDE PRODUCTION

Studies using polypeptides or peptides to manipulate the interaction between proteins have demonstrated that small peptides in their natural (L-form) are susceptible to proteolytic degradation. When administered *in vivo*, the L-forms easily succumb to rapid hepatic and renal clearance. Substitution of susceptible residues in the N or C-terminal residues with the D-amino forms results in peptides of increased stability (Hong *et al.*, 1999, Hamamoto *et al.*, 2002, Fischer, 2003, Lien and Lowman, 2003). Thus, since TNFRI$_{206-211}$ exhibited the most potent and consistent inhibitory properties, we synthesised the D-form of this peptide (D-TNFRI$_{206-211}$), with all residues (EDSGTT) substituted.

A longer peptide (TNFRI$_{198-211}$), containing the sequence QIENVKGTEDSGTT was also synthesised. As the common sequence in these peptides was GTT, a peptide consisting of tandem repeats of this sequence repeated was also synthesised (GTTGTTGTTGTT) and tested for the ability to inhibit TNF-induced chemiluminescence generation.

TNF (1 ng/ml) was incubated with D-amino TNFRI$_{206-211}$ (5-50 μM) for 20 min and then tested for ability to induce superoxide (chemiluminescence) production in neutrophils. The data show that at lower concentrations the D-TNFRI$_{206-211}$ did not affect the ability of TNF to induce chemiluminescence production. However, pre-treatment of TNF with 50 μM of D-TNFRI$_{206-211}$ led to a 50 % reduction in chemiluminescence. At this

concentration its natural counterpart did not exhibit an inhibitory effect (Figure 5.29 vs. Figure 5.10).

The effect of the longer $TNFRI_{198-211}$ was tested on the ability of TNF to stimulate superoxide production. While $TNFRI_{198-211}$ did not affect this response when tested at 5 and 10 μM, at concentrations of 20 and 50 μM it caused a 50 % and 75 % reduction respectively (Figure 5.30). Similarly, the tandem repeats of $TNFRI_{209-211}$ had no effects on the neutrophil response when tested at concentrations up to 20 μM. However, this peptide caused a 45 % reduction in the ability of TNF to stimulate superoxide production when tested at 50 μM (Figure 5.31).

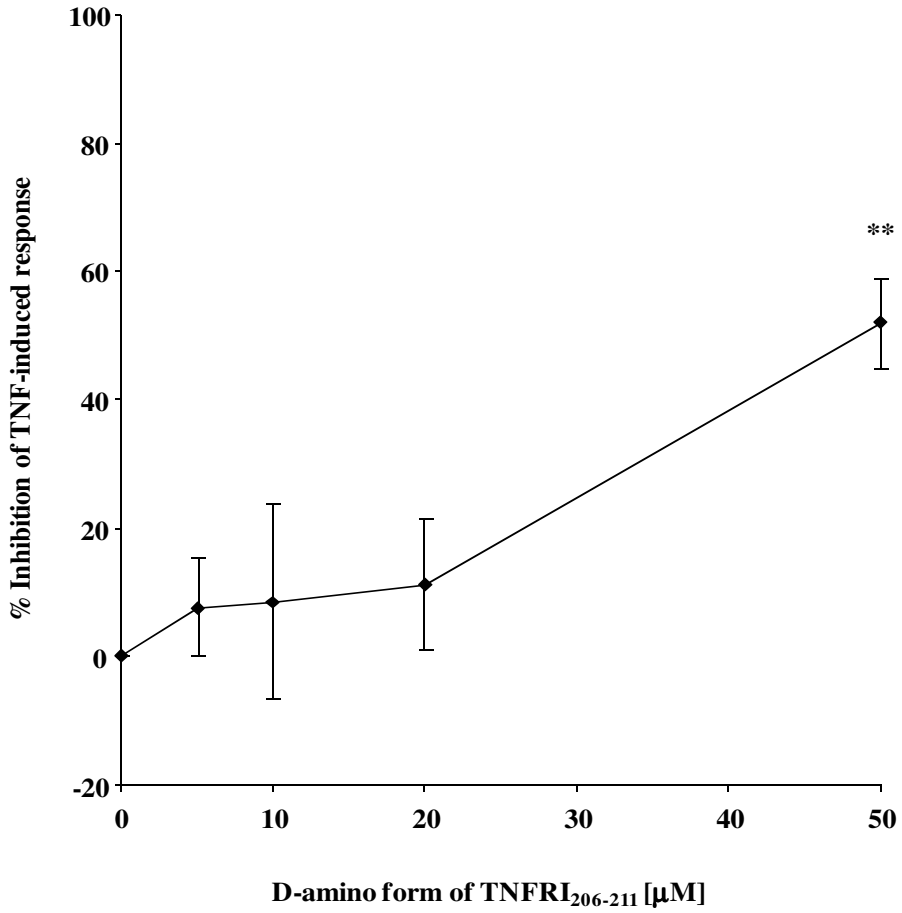

Figure 5.29 Effect of D-amino form of $TNFRI_{206-211}$ on TNF-induced superoxide (chemiluminescence) production. TNF (1 ng/ml) was treated with various concentrations (5, 10, 20 and 50 μM) of D-amino $TNFRI_{206-211}$ at 37°C for 20 min before being added to neutrophils. The cells were incubated for 30 min at 37°C. Lucigenin was then added and the resultant chemiluminescence was measured in a luminometer. Data represent the % inhibition of the TNF response and are presented as mean ± SEM of three experiments. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 101 ± 16 RLU and in TNF-stimulated cells was 3051 ± 606 RLU. Significance of difference between TNF and TNF + D-$TNFRI_{206-211}$: ** $p < 0.01$ (Dunnett's Multiple Comparisons test).

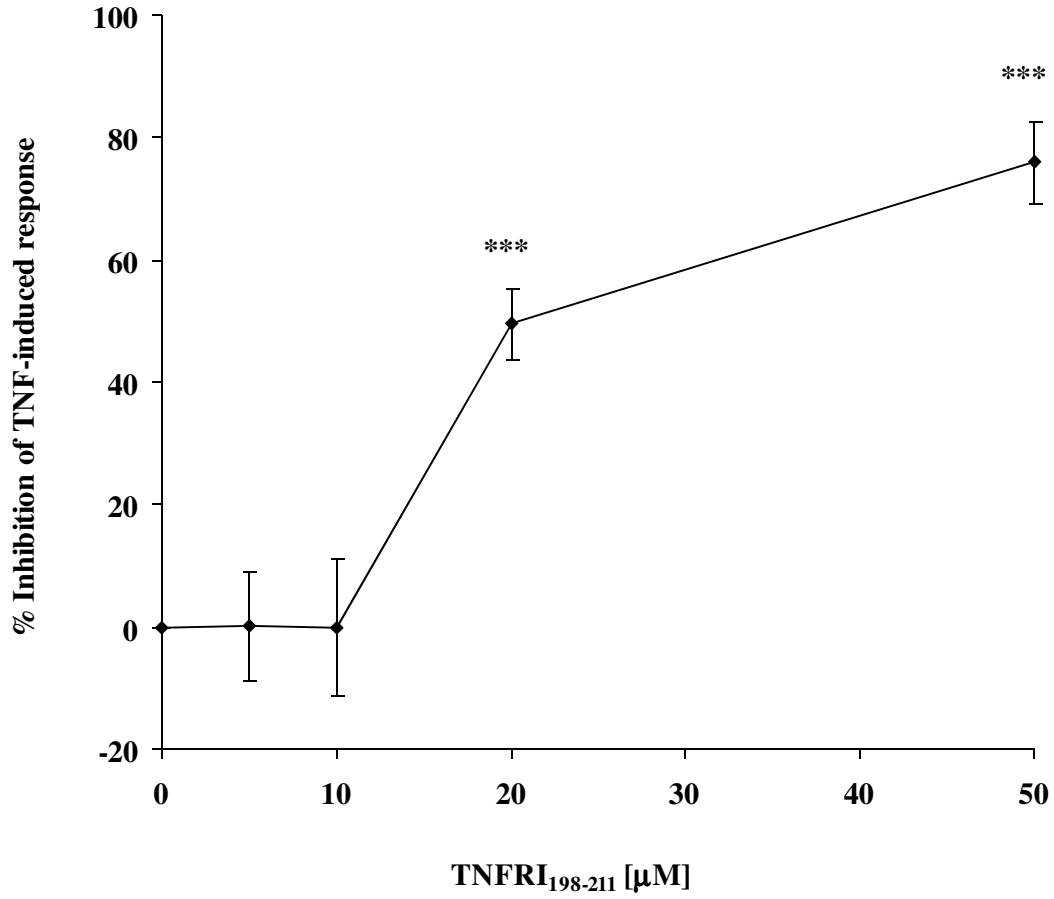

Figure 5.30 Effect of $TNFRI_{198-211}$ on TNF-induced superoxide (chemiluminescence) production. TNF (1 ng/ml) was treated with various concentrations (5, 10, 20 and 50 μM) of $TNFRI_{198-211}$ at 37°C for 20 min, before being added to neutrophils. The cells were incubated for 30 min at 37°C. Lucigenin was then added and the resultant chemiluminescence was measured in a luminometer. Data represent the % inhibition of the TNF response and are presented as mean ± SEM of three experiments. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 72 ± 6 RLU and in TNF-stimulated cells was 1954 ± 547 RLU. Significance of difference between TNF and TNF + $TNFRI_{198-211}$: *** $p < 0.001$ (Dunnett's Multiple Comparisons test).

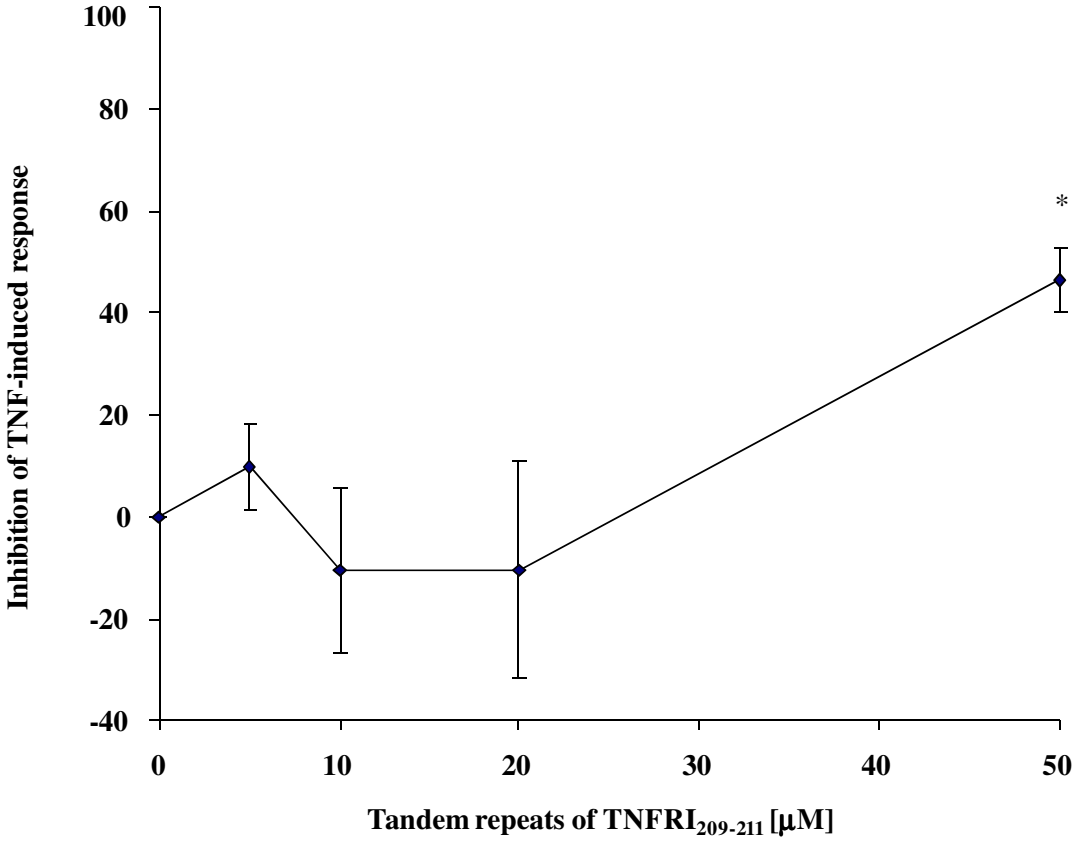

Figure 5.31 Effect of a tandem repeat form of $TNFRI_{209-211}$ on TNF-induced superoxide (chemiluminescence) production. TNF (1 ng/ml) was treated with various concentrations (5, 10, 20 and 50 μM) of tandem repeat form of $TNFRI_{209-211}$ at 37°C for 20 min, before being added to neutrophils. The cells were incubated for 30 min at 37°C. Lucigenin was then added and the resultant chemiluminescence was measured. Data represent the % inhibition of the TNF response and are presented as mean ± SEM of three experiments. The maximal rate of chemiluminescence production in non-stimulated neutrophils was 112 ± 80 RLU and in TNF-stimulated cells was 7636 ± 2720 RLU. Significance of difference between TNF and TNF + tandem repeat form of $TNFRI_{209-211}$: * $p < 0.05$ (Dunnett's Multiple Comparisons test).

Table 5.1 Summary of biological activities of TNFRI-derived peptides

	$TNFRI_{209-211}$	**$TNFRI_{206-211}$**	**D-$TNFRI_{206-211}$**	**$TNFRI_{198-211}$**	**$TNFRI_{(209-211)}$ (TR)**
TNF-induced superoxide	123 μM*	171 μM*	51 μM	50 μM	50 μM
TNF-RM-induced superoxide	216.4 μM*	146.3 μM*	ND	ND	ND
TNF-induced CD11b	ND	200 μM	ND	ND	ND
TNF-induced p38 in PMN	200 μM	200 μM	ND	ND	ND
TNF-induced p38 in Mono Mac	ND	200 μM	ND	ND	ND
TNF-induced IL-1β production	ND	200 μM	ND	ND	ND
TNF-induced IL-8 production	ND	200 μM	ND	ND	ND

*Quoted as IC_{50}s.

Abbreviations: IL, interleukin; ND, not determined; PMN, polymorphonuclear cells; TNF-RM, TNF rich medium, TR, tandem repeat.

5.13 SUMMARY

The data show that the mimetic peptide TNF_{70-80} is able to activate p38 kinase activity by specifically ligating with a region of the extracellular portion of the TNFRI proximal to the transmembrane domain. Using truncated TNFRI mutants, we showed that the remnant 3 amino acids (GTT) are the minimum sequence required for the receptor to activate p38 activity. Peptides ($TNFRI_{209-211}$ and $TNFRI_{206-211}$) to this region of the receptor were able to block the neutrophil stimulating properties of TNF_{70-80} and TNF from stimulated leukocytes when examined at the level of the superoxide production. These effects were specific since scrambled control peptides did not affect TNF-induced response tested at similar concentrations. The specificity of these receptor derived peptides was further established by the lack of effect on fMLP-induced neutrophil activation.

Furthermore, these studies (Table 5.1) have shown that $TNFRI_{206-211}$ was able to block TNF-induced p38 activation in neutrophils and Mono Mac 6 cells. It inhibited TNF-induced CD11b upregulation in neutrophils. Additionally, $TNFRI_{206-211}$ was also able to block TNF-induced IL-1β and IL-8 mRNA production in neutrophils. Increased activity was achieved by further modification of the TNFR peptide. The D-amino form of $TNFRI_{206-211}$ was shown to have inhibitory activity at lower concentrations, compared to $TNFRI_{198-211}$ and a tandem repeat of $TNFR1_{209-211}$ exhibited inhibitory activity at lower concentrations than $TNFRI_{209-211}$ (Table 5.1). These data demonstrate that TNF_{70-80} activated p38 by binding to a specific site of the TNFRI. Thus, peptides to this region

were able to block p38 activation and p38-dependent functions such as superoxide production, CR3 upregulation and cytokine in production in neutrophils. These data identifies a novel way of targeting the TNF-TNFR-p38 pathway to inhibit specific actions. Lastly modifications can be made to the TNFRI mimetics to enhance their activity.

6.0 CHAPTER SIX: EFFECTS OF TNFRI-DERIVED PEPTIDES ON INFLAMMATION

6.1 INTRODUCTION

It is well established that TNF plays a major role in several inflammatory diseases such as RA. This central role of TNF in inflammation has made it an attractive therapeutic target (Aggarwal *et al.*, 2006). Studies using TNFRI or TNFRII knockout mice have shown that the neutrophil accumulation in response to LPS is mediated via the TNFRI (Calkins *et al.,* 2001). The use of anti-TNF antibodies has also shown the importance of this cytokine in the pathogenesis of inflammatory diseases (Andreakos *et al.,* 2002). Consistent with these findings, several anti-TNF strategies are now in clinical use to treat debilitating inflammatory conditions (Table 1.7).

Recently research has been focussing on small peptide inhibitors of TNF. An exocyclic peptide mimetic of a critical binding site on TNFRI was shown to inhibit bone destruction in murine collagen-induced arthritis model, to a similar extent as anti-TNF Ab (Saito *et al.,* 2007). Another peptide inhibitor of TNF based on the pre-ligand assembly domain (PLAD) of TNFRI has recently been shown to inhibit TNF signalling by binding to the PLAD of TNFRI thereby preventing TNF aggregation and TNF-TNFR signalling (Deng *et al.,* 2005). This peptide was similarly shown to inhibit murine CIA (Deng *et al.*, 2005).

The ability of the TNFRI mimetics to prevent TNF-induced activation of p38 and related cellular activation, suggest a singular approach to inhibiting inflammation and was

examined in three different types of (inflammatory models) LPS-induced peritonitis, carrageenan-induced paw swelling and delayed type hypersensitivity..

6.2 THE EFFECT OF $TNFRI_{206-211}$ ON A MURINE MODEL OF ACUTE LPS-INDUCED PERITONITIS

To investigate the effects of the TNFRI-derived peptides in acute inflammation BALB/c female mice were injected intraperitoneally with 50 µg of LPS or HBSS, 2 h later the mice received an injection of different concentrations of $TNFRI_{206-211}$ or HBSS. At 24 h, the animals were euthanized and the total leukocytes in the peritoneal exudates enumerated by haemocytometer chamber. Cytospins of the cellular infiltrates were prepared by centrifugation of 200 µl (vehicle challenge) or 100 µl (LPS challenge) aliquots. Slides were fixed and stained with a Giemsa stain. LPS caused a significant increase in total leukocytes infiltration into the peritoneum, in comparison to the vehicle or $TNFRI_{206-211}$ only treated animals (Figure 6.1). Treatment with $TNFRI_{206-211}$ significantly reduced the total number of infiltrating leukocytes compared to vehicle treated animals. At the highest dose of 40 mg/kg, $TNFRI_{206-211}$ caused a complete reduction in leukocyte numbers ($p < 0.001$). This effect is displayed in the photomicrographs of the Giemsa stained cytospins (Figure 6.2)

$TNFRI_{206-211}$ caused a decrease in total leukocytes which can be explained by differences observed in the neutrophils and macrophages. LPS caused a dramatic influx of

neutrophils into the peritoneal cavity, incresing from a baseline level of 1.2 x 10^5 to 2.35 x 10^6 cells. This number was significantly reduced by treating with of 40 mg/kg of $TNFRI_{206-211}$ to 1.5 x 10^6 cells ($p < 0.01$) (Figure 6.3). The peptide itself did not induce neutrophil influx. Interestingly, treatment with $TNFRI_{206-211}$ significantly reduced the number of peritoneal macrophages even at the lower doses of the peptide tested (Figure 6.4). Although the total macrophages in the peritoneal cavity did not change, it is likely that this level is a result of reduced resident peritoneal macrophages, from LPS-induced apoptosis (Xaus *et al.*, 2000, Soler *et al.*, 2001), and monocyte influx. The latter must likely being inhibited by $TNFRI_{206-211}$ leading to a lower level of macrophages in the peritoneal cavity. Indeed studies from our laboratory showed that within 4 h after injection of LPS, the number of mouse resident peritoneal macrophages was reduced by approximately 50 %.

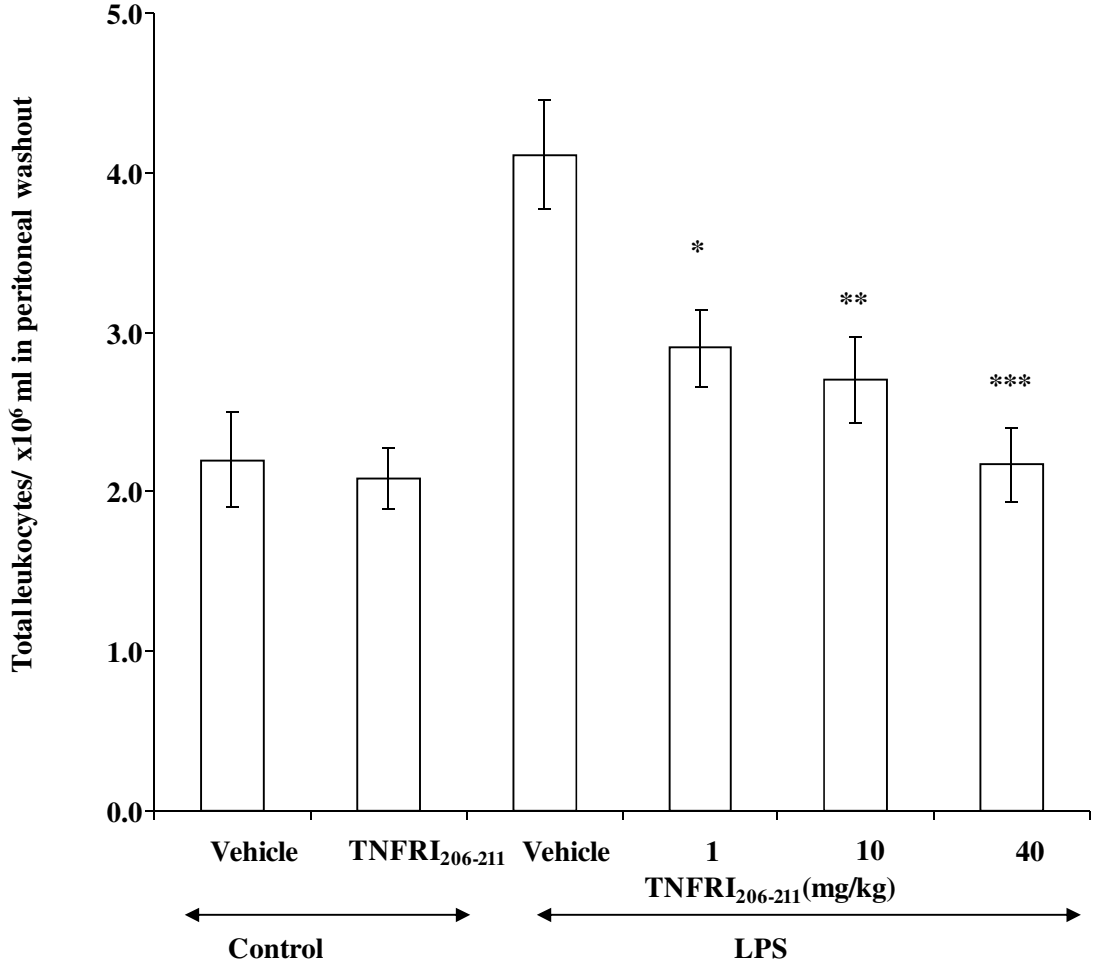

Figure 6.1 Effect of $TNFRI_{206-211}$ on total leukocyte infiltration in response to LPS. Mice were challenged intraperitoneally with LPS or HBSS (control) and then treated with different concentrations of $TNFRI_{206-211}$ or HBSS. At 24 h, the animals were euthanized and number of cells in peritoneal washouts enumerated. Data are presented as mean ± SEM. Control (HBSS) n= 4, $TNFRI_{206-211}$: n= 3; LPS: n=7; LPS + $TNFRI_{206-211}$: n= 7. Significance of difference between LPS challenge alone and LPS and $TNFRI_{206-211}$ treated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Dunnett's Multiple Comparisons test).

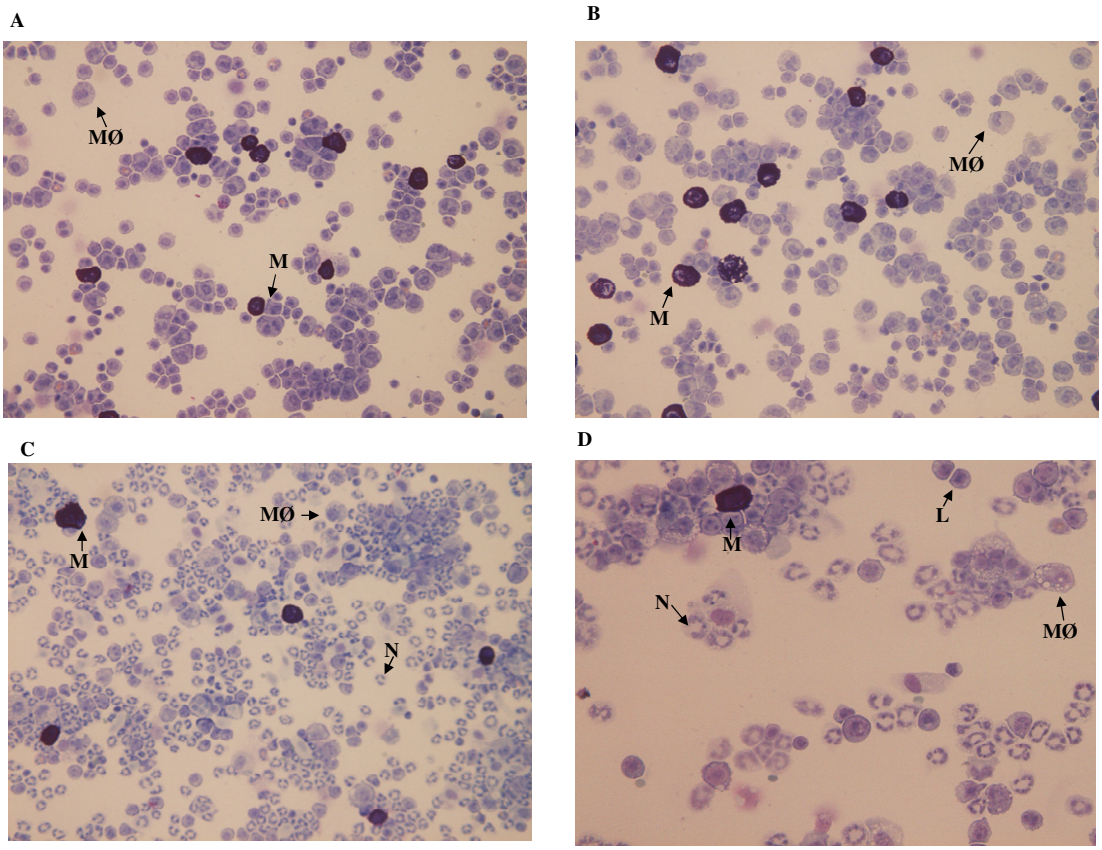

Figure 6.2 Photomicrographs of peritoneal exudate preparation for the effects $TNFRI_{206-211}$ on LPS-induced acute peritonitis. Mice received an intraperitoneal injection of either HBSS (A, B) or LPS (C, D), 2 h later the mice received HBSS (A, C) or $TNFRI_{206-211}$ 40 mg/kg body weight intraperitoneally. After 24 h animals were euthanized and cytospins of the cellular infiltrates were prepared by centrifugation of 200 µl (vehicle challenge) or 100 µl (LPS challenge) aliquots. Slides were fixed and stained with Giemsa (magnification x 40). Mast cell -M; macrophage- MØ; neutrophil- N; lymphocyte- L.

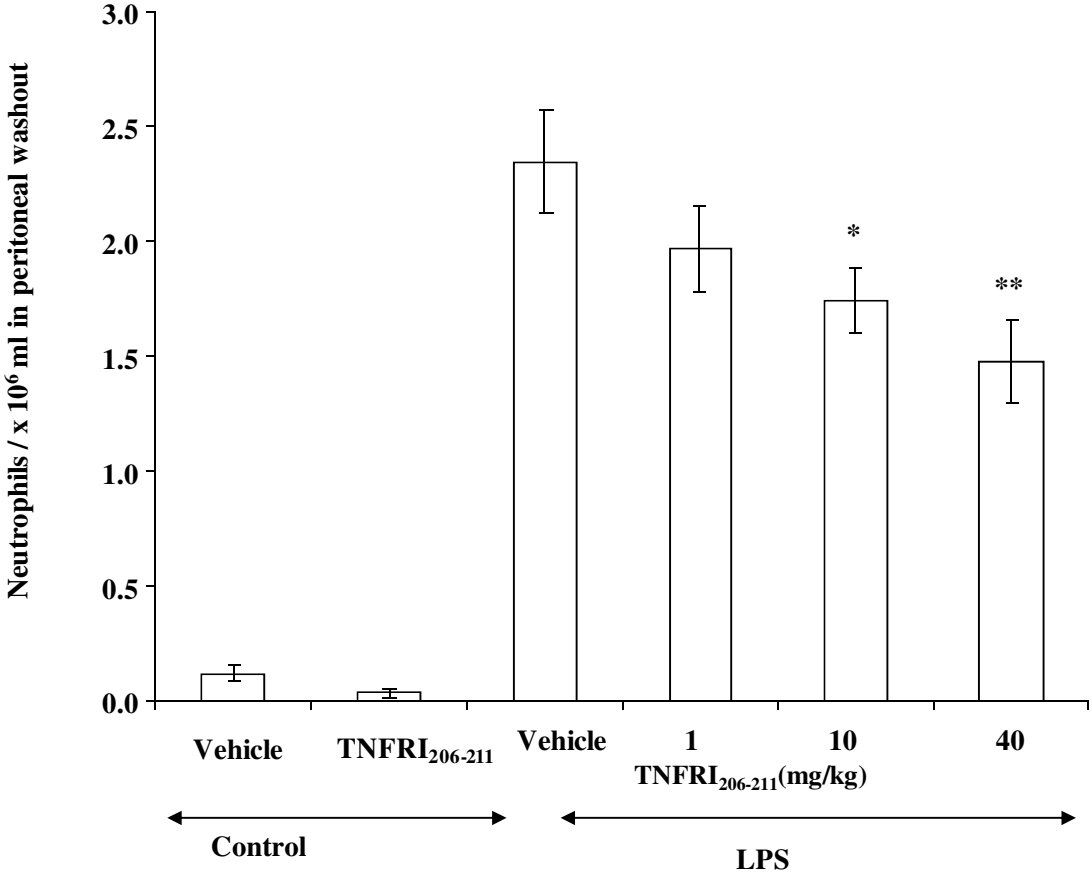

Figure 6.3 Effect of $TNFRI_{206-211}$ on the accumulation of neutrophils in the peritoneal cavity induced by LPS. Treatments were as per figure 6.1 legend. Slides were fixed and stained with Giemsa stain and the number of neutrophils in peritoneal exudate fluid was enumerated. Data are presented as mean ± SEM. Control (HBSS) n= 4, $TNFRI_{206-211}$: n= 3; LPS: n=7; LPS + $TNFRI_{206-211}$: n= 7. Significance of difference between LPS challenge alone and LPS and $TNFRI_{206-211}$ treated: * $p < 0.05$; * $p < 0.01$ (Dunnett's Multiple Comparisons test).

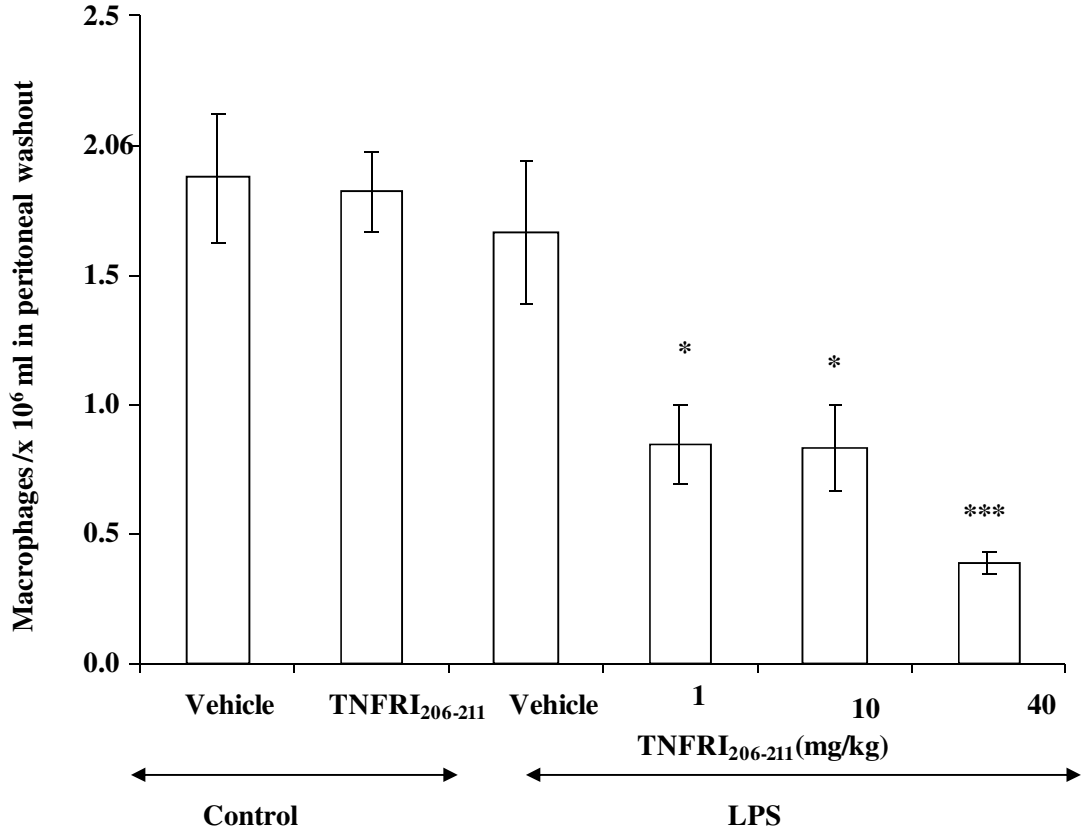

Figure 6.4 Effect of $TNFRI_{206-211}$ on the accumulation of macrophages induced by LPS. Treatments were as per figure 6.1 legend. Slides were fixed and stained with Giemsa stain and the number of macrophages in the peritoneal exudate was enumerated. Data are presented as mean ± SEM. Control (HBSS) n= 4, $TNFRI_{206-211}$: n= 3; LPS: n=7; LPS + $TNFRI_{206-211}$: n= 7. Significance of difference between LPS challenge alone and LPS and $TNFRI_{206-211}$ treated: * $p < 0.05$; *** $p < 0.001$ (Dunnett's Multiple Comparisons test).

6.3 THE EFFECT OF $TNFRI_{206-211}$ ON CARRAGEENAN-INDUCED PAW SWELLING

To investigate the effect of $TNFRI_{206-211}$ on carrageenan-induced paw swelling, female BALB/c mice received a subplantar injection of Type IV carrageenan (1 ml/kg of a 1 % solution) in the left hind paw. After 2 h mice were injected intraperitoneally with 40 mg/kg of $TNFRI_{206-211}$ or control peptide. Footpad thickness was assessed 24 h after challenge. Injection of carrageenan produced marked paw swelling. Treating mice with this dose of $TNFRI_{206-211}$ had no effect on the swelling (Figure 6.5).

One likely reason for the lack of effect of the TNFR mimetic is the limited amount of peptide which reached the inflammatory foci. Sekut *et al.,* (1994) demonstrated that in the carrageenan induced paw inflammation model in both mice and rats very little serum TNF was detected, in contrast to a marked increase in local TNF levels after carrageenan challenge in the paws. To overcome this potential problem, we took the alternative approach of injecting $TNFRI_{206-211}$ directly into the paw, simultaneously with the carrageenan. Thus in the modified approach mice received a 20 µl injection which contained a combination of carrageenan equivalent to (1 ml/kg of a 1 % solution) and 10 mg/kg of $TNFRI_{206-211}$ or control peptide. The footpad thickness was assessed 24 h after challenge. The results showed that carrageenan induced a marked increase in paw thickness of 0.9 ± 0.07 mm which was significantly ($p < 0.001$) reduced by the TNFRI mimetic but not by the control peptide (Figure 6.6).

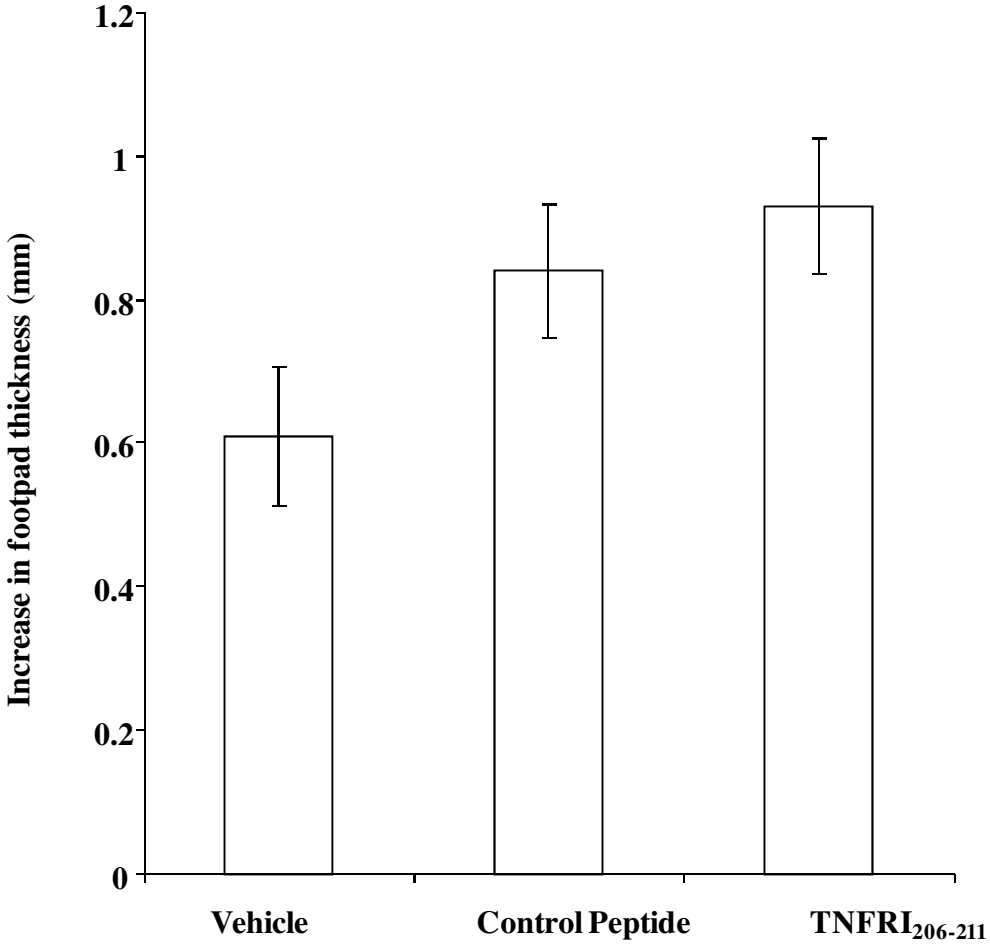

Figure 6.5 The effect of $TNFRI_{206-211}$ on carrageenan-induced paw inflammation. Mice were injected with carrageenan (1 ml/kg of a 1 % solution) into the hind footpad and 2 h later, the animals were treated intraperitoneally with 40 mg/kg of control peptide $TNFRI_{206-211}$. The reaction was assessed by measuring the difference in footpad thickness between challenged and unchallenged footpads 24 h after challenge. Data are presented as mean ± SEM of 5 mice in each group.

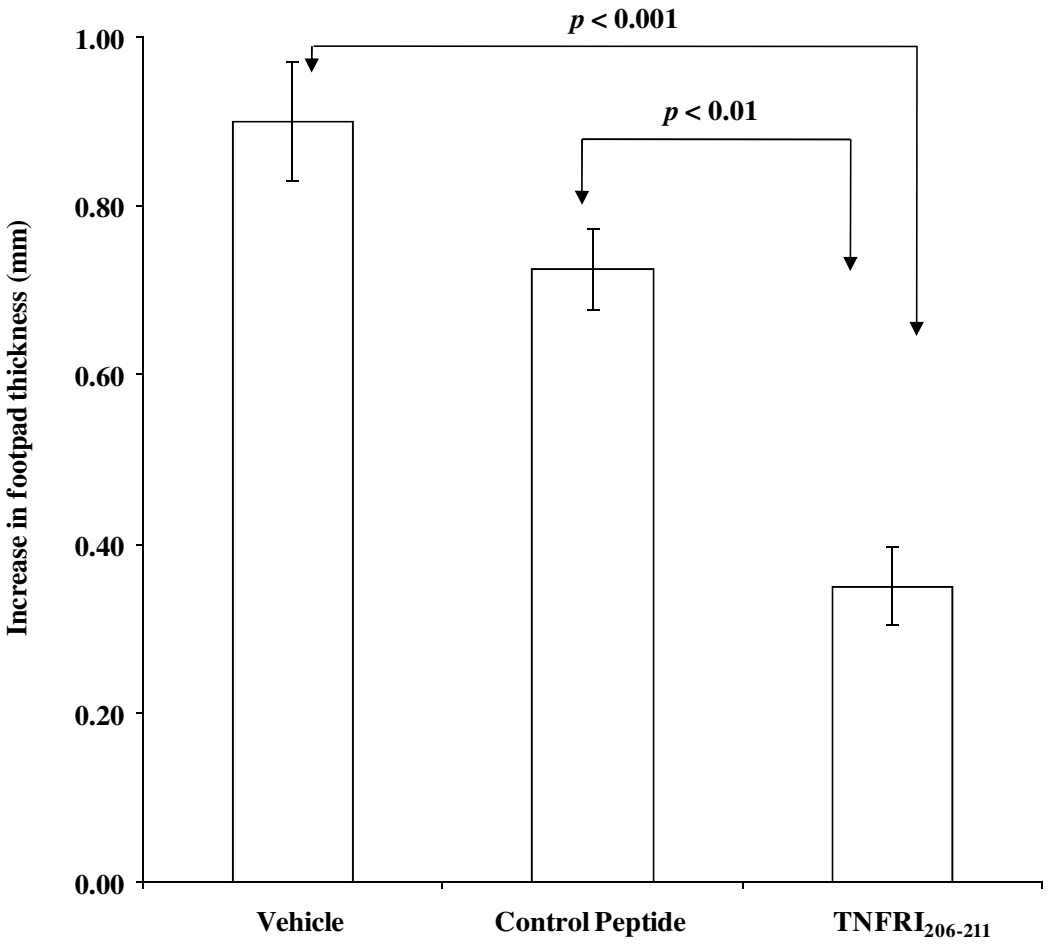

Figure 6.6 The effect of local application of $TNFRI_{206-211}$ on carrageenan-induced paw inflammation. Mice were injected with a 20 µl mixture of carrageenan (1 ml/kg of a 1 % solution) and control peptide or $TNFRI_{206-211}$ (10 mg/kg) or vehicle into the hind footpad. The reaction was assessed by measuring the difference in footpad thickness between challenged and unchallenged footpads 24 h after challenge. Data are presented as mean ± SEM of 4 (Vehicle) and 5 (control peptide and $TNFRI_{206-211}$). Significance of difference between carrageenan challenge + vehicle and carrageenan + $TNFRI_{206-211}$: *** $p < 0.001$ (Dunnett's Multiple Comparisons test). Significance of difference between control peptide and $TNFRI_{206-211}$ treated: ** $p < 0.01$ (Tukey's Multiple Comparison Test).

6.4 THE EFFECT OF $TNFRI_{206-211}$ ON ANTIGEN-INDUCED DELAYED TYPE HYPERSENSITIVITY

The delayed type hypersensitivity response to sheep red blood cell (SRBC) is an important *in vivo* manifestation of the cell-mediated immune response commonly seen against intracellular bacteria, several viral, parasitic pathogens and systemic fungal pathogens as well in chronic inflammatory diseases (Arvin, 2008, Bogdan, 2008, Mustafa *et al.*, 2008). To investigate the effects of $TNFRI_{206-211}$ on the DTH response, female BALB/c mice were primed with an injection of 10 % haemotocrit, SRBC. After six days, mice received a subplantar 20 µl injection which contained a combination of 40 % SRBC suspension and 12.5 mg/kg of $TNFRI_{206-211}$ or control peptide. The DTH response was assessed by measuring the difference between challenged and unchallenged footpad thickness 24 h after challenge. The results showed that a dose of 12.5 mg/kg of $TNFRI_{206-211}$ significantly inhibited the DTH response in mice by 33 % (Figure 6.7) while the control peptide had no effect.

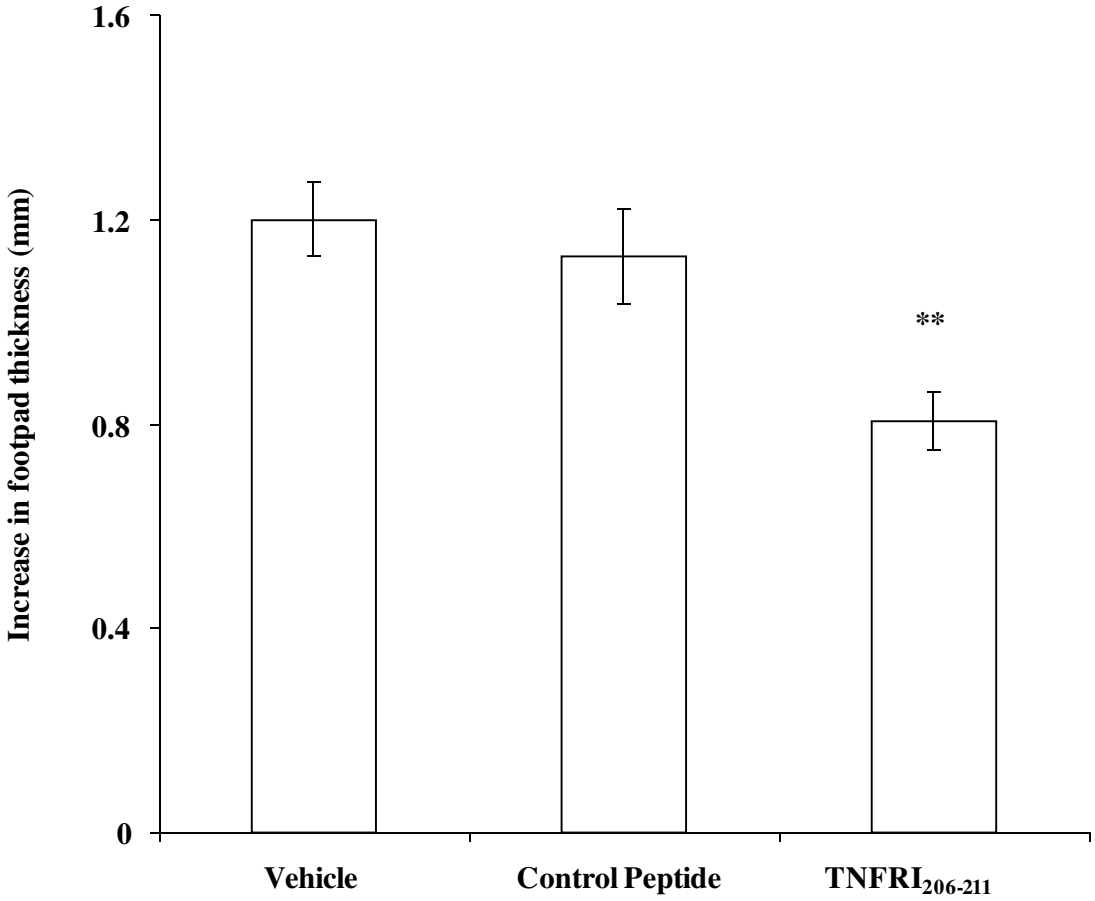

Figure 6.7 The effect of local application of $TNFRI_{206-211}$ on DTH response to SRBC. Mice were injected with 100 µl of a 10 % SRBC solution. Six days later the mice were challenged with the antigen containing either control peptide or $TNFRI_{206-211}$ (12.5 mg/kg) or vehicle in the hind footpad. The reaction was assessed by measuring the difference in footpad thickness between challenged and unchallenged footpads 24 h after challenge. Data are presented as mean ± SEM of the increase in footpad thickness. Vehicle n = 9, control peptide n = 9, $TNFRI_{206-211}$ n = 8. Significance of difference between SRBC challenge + vehicle and SRBC + $TNFRI_{206-211}$: ** $p < 0.01$ (Dunnett's Multiple Comparisons test). Significance of difference between control peptide and $TNFRI_{206-211}$ treated: * $p < 0.05$ (Tukey's Multiple Comparison Test).

6.5 SUMMARY

The data presented show that the *in vitro* anti-inflammatory properties of $TNFRI_{206-211}$ were also evident in these models of inflammation. $TNFRI_{206-211}$ inhibited the infiltration of leukocytes, namely neutrophils and macrophages into the peritoneal cavity following challenge with LPS. Studies with carrageenan induced acute inflammatory model similarly showed that $TNFRI_{206-211}$ was able to inhibit swelling. The effects on macrophages were confirmed in a chronic inflammation model where the peptide was also shown to inhibit inflammation in the DTH model when challenged with SRBC. A lack of effect after intraperitoneal administration of $TNFRI_{206-211}$ demonstrated that its inhibitory properties were more effective when administered directly into local sites of inflammation. Nevertheless, the data presented provide proof of principle/concept that peptides that block cytokine receptors can potentially be developed into therapeutics. The findings show that $TNFRI_{206-211}$ can inhibit acute and chronic inflammatory responses.

7.0 CHAPTER SEVEN: DISCUSSION

7.1 INTERACTION OF TNF_{70-80} WITH THE TNFRI

Previously the biological actions of TNF_{70-80} had not been shown to be directly mediated by the interaction of the peptide with the TNFR. Using labelled TNF_{70-80} in a competition assay, we demonstrate for the first time that the peptide is a ligand for TNFRI. The previously observed selective biological properties of TNF_{70-80} (Kumaratilake *et al.*, 1995) were due to its ability to activate only the p38 and NF-κB and not the JNK and ERK1/ERK2 pathways. Similarly the selective properties of $TNF_{132-150}$ were due to its ability to activate JNK and ERK1/ERK2 and not p38 pathways. The inability of TNF_{70-80} to activate all three MAPK pathways, activated by TNF was found to be due to the partial recruitment of adaptor proteins. TNF_{70-80} was able to couple TRAF2 and not GCK or ASK-1 to the TNFR. The incomplete activation of the MAPK could be due to the small size of the peptide and its inability to aggregate the TNFR into a functional trimer, leading to an inappropriate recruitment of adaptor proteins. By means of truncated mutant extracellular regions of TNFRI, we have demonstrated that TNF_{70-80} activates p38 by interacting with a specific site of the TNFR (GTT). Longer peptides to this region were able to inhibit TNF-induced p38 activation and p38-mediated effects *in vitro* and *in vivo*, and consequently could be potential therapeutics in targeting the TNF-TNFR-p38 axis.

The striking finding that TNF_{70-80} has selective TNF activity has raised the issue as to whether the peptide ligates the TNFRI in a manner similar to TNF. While previous findings showed that the activity of TNF_{70-80} could be neutralised by treatment with

soluble TNFRI and TNFRII or anti-TNF antibodies (Kumaratilake *et al.*, 1995, Britton *et al.*, 1998, Briscoe *et al.*, 2000), issues still remain as to whether the differential biological activity is through the receptor. We have provided direct evidence of the importance of the TNFRI for its biological activity. Thus TNF_{70-80} failed to stimulate activity (p38) in the pre-B cell line 70 Z/3 which lack TNFRs. Transfection of the TNFRI into these cells resulted in their responsiveness to TNF_{70-80}.

While these results show that TNFRI is essential for the activity of TNF_{70-80}, they do not prove that the mimetic is a ligand for the receptor. Results from further investigations demonstrated that labelled TNF_{70-80} could be specifically displaced from the TNFRI by unlabelled TNF_{70-80} using scatchard analysis. Previously the cytotoxic mimetic peptide $TNF_{132-150}$ was shown, to displace radiolabelled ^{125}I-TNF binding to TNFR on WEHI-164 cells (Rathjen *et al.*, 1993). These results therefore show that both are ligands for TNFR but transduce different signals from each other and TNF.

The hallmark of a true cytokine mimetic is its ability to interact with its putative receptor. One such is a recently described peptide of IL-2, corresponding to amino acids 1-30 which are essential for binding to the IL-2Rβ2 chain and are critical for signal transduction (Rose *et al.*, 2003). Using theoretical 3-D models of free peptide amino acid 1-30 (p1-30) and IL-2Rβ2, a model of p1-30•IL-2Rβ2 complex they proposed a mechanism by which signal transduction occurs through a conformational change of the COOH-terminal domains of the receptor, resulting in the transmembrane regions coming together facilitating the transmission of signal to the cytoplasm (Rose *et al.*, 2003). Thus

it is possible for a small peptide region such as TNF_{70-80} to interact specifically with a receptor allowing for signal transduction.

The previous findings that the biological properties of TNF_{70-80} could be blocked by soluble TNFRs and anti-TNF antibodies, together with our present findings show that not only is TNFRI required for activation of cellular signals, but that there is a specific interaction of the mimetic with TNFRI, strongly favouring a selective signalling of cells through interacting with a specific region of TNFRI.

7.2 SELECTIVE ACTIVATION OF MAPK PATHWAYS

In neutrophils, both TNF and TNF_{70-80} was shown to stimulate p38 but not JNK or ERK activity. In endothelial cells, however, there were striking differences in the ability to activate MAPK between TNF and TNF_{70-80}. Our results showed that while both TNF and TNF_{70-80} were able to stimulate p38 activity in HUVECs, only TNF stimulated JNK and ERK1/ERK2 activity. In contrast, while TNF was able to activate all three MAP kinases in adherent WEHI-64 cells, $TNF_{132-150}$ only stimulated the activity of JNK and ERK1/2 but not p38 at concentrations which were shown to cause cytotoxicity in WEHI-164 cells (Rathjen *et al.*, 1993). Our data demonstrated that $TNF_{132-150}$ stimulated JNK and ERK1/ERK2 activity to a similar extent to TNF. It is therefore likely that an inability of $TNF_{132-150}$ to activate neutrophils can be attributed to its inability to stimulate p38 kinase activity.

Binding of the trimeric TNF to the p55 kDa TNF receptor complexes initiates the recruitment of receptor-associated proteins such as TRAF-2, TRADD and FADD to the TNF receptors (Chen and Goeddel, 2002). One possible explanation for the restricted activation of the MAPK family by TNF_{70-80} is an inappropriate recruitment of these receptor-associated proteins compared to TNF, and in this manner having a major impact on the range of downstream signalling molecules stimulated. Studies have shown that TRAF2 is essential for TNF-induced activation of MAPK (Liu *et al.*, 1996, Reinhard *et al.*, 1997, Yeh *et al.*, 1997, Liu and Han, 2001). Interestingly transfection of the dominant negative form of TRAF2 blocked the activation of p38 by TNF_{70-80}. We conclude that TRAF2 was recruited to the TNF receptors and was required for the activation of p38 by TNF_{70-80}. TNF_{70-80} was found in comparison to TNF to be poor at stimulating GCK activity in adherent neutrophils. This implies that TNF_{70-80} is unable to cause the coupling of TRAF2 to an upstream regulator(s) of the JNK module (Figure 7.1).

The selective activation of the peptides could also be due to their inability to cause the TNFR to form a functional trimer, as the binding of a single TNF to single receptor is not sufficient to transduce a signal, rather the homotrimer of TNF is essential for intracellular domains of the TNFR to dimerise and trimerise for signal transduction to occur (Locksley *et al.*, 2001, Schottelius *et al.*, 2004). Thus the peptides existing as monomers might not be able to cause trimerisation of the TNFR resulting in partial recruitment of adaptor proteins and consequently partial activation.

The ability of TNF_{70-80} to prime neutrophils and activate microbicidal properties such as the respiratory burst is comparable to that of TNF, whose properties have been well established. (Kumaratilake *et al.*, 1995). The role p38 in TNF-induced chemiluminescence response is well established. While the p38 inhibitor SB203580 inhibited the TNF-induced respiratory burst activity, the ERK1/ERK2 inhibitor, PD98059 had no effect (Zu *et al.*, 1998). Thus the production of oxygen intermediates by neutrophils is primarily mediated by p38 MAPK and not by ERK. Thus it is likely that TNF_{70-80} activates neutrophils by stimulating p38 activity. The inhibitory effect of SB203580 on TNF_{70-80}-induced chemiluminescence response provides further evidence of TNF_{70-80} mediating its effects via the p38 pathway. p38 inhibitors have also been shown to reduce CD11b/CD18 upregulation in response to TNF; while treatment with ERK1/ERK2 inhibitors had no significant effect (Tandon *et al.*, 2000, Forsberg *et al.*, 2001, Bouaouina *et al.*, 2004).

Studies using cell lines expressing dominant negative mutants of MKK4 or JNK were protected from TNF-induced apoptosis, thus delineating the importance of JNK in promoting apoptosis (Roulston *et al.,* 1998). The fact that TNF_{70-80} was unable to stimulate JNK activity is not surprising as previous work has shown that TNF_{70-80} lacked cytotoxic effects via the p38 pathway both *in vitro* and *in vivo* against tumour cells (Rathjen *et al.*, 1993).

The findings have therefore revealed that is possible to exploit the TNFR-p38 signalling pathway to promote anti-microbial function of the immune system. When injected into mice the peptide $TNF_{70-80,}$ while enhancing the clearance of microbial pathogens, did not

show the usual TNF toxic properties and the upregulation of adhesion molecules expression in endothelial cells (Rathjen *et al.*) Presumably this was due to an inability or poor ability to stimulate JNK and ERK1/ERK2.

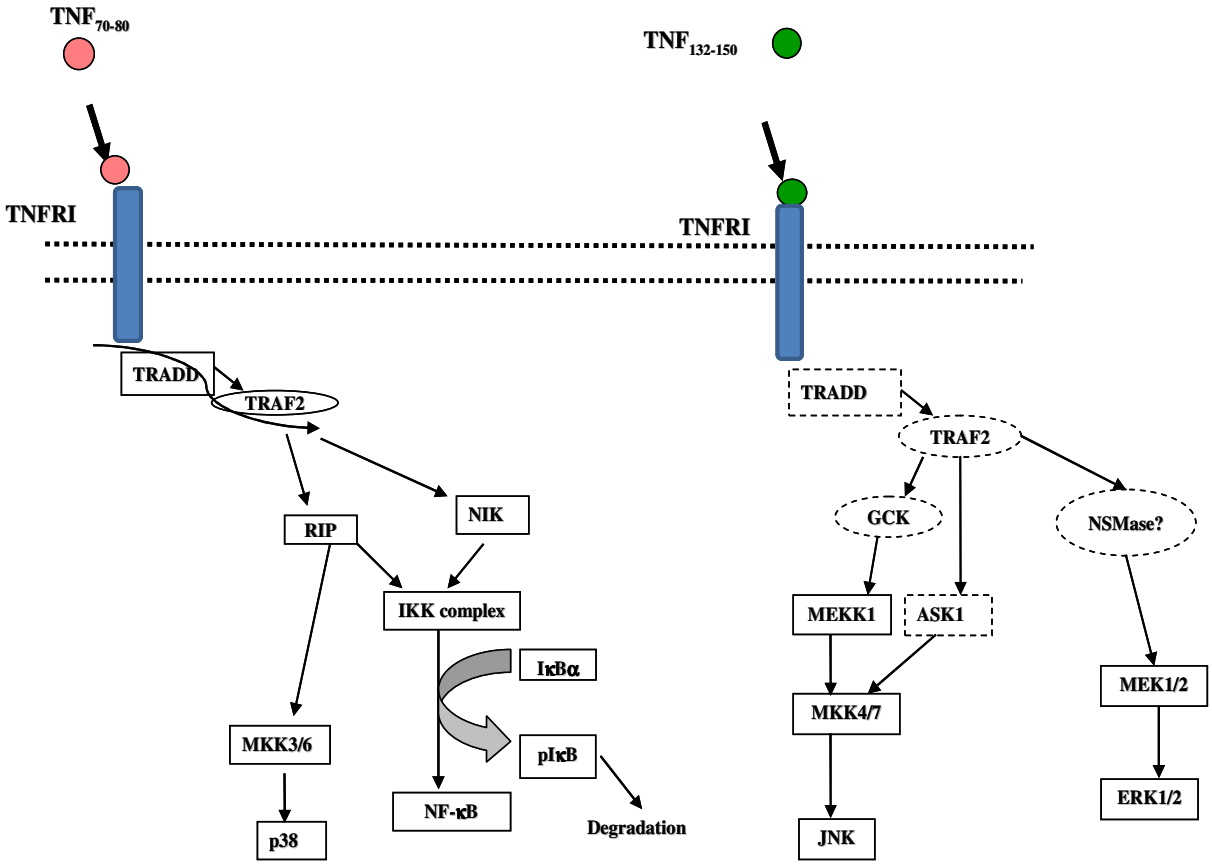

Figure 7.1 Diagrammatic representation of coupling of TNF_{70-80} to TNFRI and associated adaptor proteins and the inferred coupling of $TNF_{132-150}$. The __ represents the characterised coupling of adaptor proteins while the --- represent the inferred coupled proteins. Abbreviations: ASK1, apoptosis signal-regulating kinase; ERK1/2, extracellular-signal-regulated kinases; GCK, germinal centre kinase; IKK, IκB kinase; MEK1/2, MAPK/ERK kinase, MEKK3/6, MAPK kinase kinase 3/6; NFκB, nuclear factor kappa B; NIK, NF-κB inducing kinase; IκB, inhibitor kappa B; NSMase, neutral sphingomyelinase; RIP, receptor interacting protein; TRADD, TNFR associated DD; TRAF2, TNFR associated factor 2.

7.3 TNF_{70-80} BINDING REGION- IDENTIFICATION OF A TNF ANTAGONIST

Using truncated constructs of TNFRI we were able to localise the sites on the receptor involved in the TNF_{70-80} induced p38 activation. Constructs designated M1, M3 and M4 were made and transfected into the pre B 70Z/3 cell line. TNF_{70-80} was able to stimulate p38 activity in cells transfected with M1, M3 and M4 mutant receptors. Since the M4 region was common to all three mutants this region was the minimum region of the TNFR required by TNF_{70-80} to activate p38.

Based on the interaction of TNF_{70-80} and TNFRI, peptides derived from the remnant receptor sequence (GTT) were generated to determine whether it had the ability to block TNF stimulation of the p38 pathway. $TNFRI_{206-211}$ blocked TNF-induced p38 activation, superoxide production, CD11b/CD18 upregulation and cytokine production in neutrophils. Similar results were found with $TNFRI_{209-211}$. The specificity of the action of these TNFR mimetics was shown by the fact that these did not inhibit the fMLP-induced superoxide production in neutrophils and that control peptides of these mimetics were not inhibitory to the TNF-induced response in neutrophils. The inhibition of the TNFR-p38 signalling axis was not restricted to neutrophils; $TNFRI_{206-211}$ was also able to block p38 activation in Mono Mac 6 cells, a macrophage cell line. The fact that $TNFRI_{206-211}$ (EDSGTT) was also able to inhibit these functions is further evidence to this peptide blocking the TNF-p38 pathway.

TNF is a key cytokine in many types of inflammatory diseases. Neutrophils have also been reported to play an important role in pathogenesis of inflammation (Harris, 1990, Sewell and Trentham, 1993, Keshavarzian *et al.*, 1999, Witko-Sarsat *et al.*, 2000, Kasama *et al.*, 2005, Nathan, 2006). In RA, neutrophils are found in large numbers during the early stages and acute exacerbation of the disease. Neutrophils accumulate at the pannus-cartilage junction (site of early cartilage erosion), where they are thought to contribute directly to cartilage damage by production of reactive ROS and chloraminated oxidants. This activation of neutrophils is mediated by TNF and IL-1β (Witko-Sarsat *et al.*, 2000, Dayer, 2003, Aggarwal *et al.*, 2006). Here we demonstrate that the events stimulated by TNF can be controlled by the TNFRI mimetic. This includes oxygen radical generation, upregulation of CR3 receptors and IL-1β production. All were significantly depressed by $TNFRI_{206\text{-}211}$. It should be noted that under cytokine stimulation the conformational changes leading to change in affinity of the integrins are more important, as increased CD11b expression does not necessarily equate to augmented CD11b-adhesive capacity (Orr *et al.*, 2007). It was however evident that while there was complete prevention of TNF-induced p38 activation the biological functional responses were not inhibited to the same degree. This suggests that p38 is utilised to maximise the TNF response. Perhaps this property may be beneficial in enabling the maintenance of a level of microbial pathogen killing ability by phagocytic cells.

The identification of TNF inhibitors is the goal of research into this cytokine. Early structure function research identified two TNFRI derived synthetic peptides that inhibited TNF-induced cytotoxicity and binding to TNFRI (Hwang *et al.*, 1991, Lie *et al.*, 1992).

These peptides are in fact overlapping in their amino acid sequences and are located within the third and fourth cysteine rich domains of the extracellular portion of the TNFRI. The first peptide, corresponding to amino acids 175-194, inhibited TNF-induced cytolysis in L929 cells at a concentration of 200 μM by 50% (Hwang *et al.*, 1991). It is evident that the majority of the residues in this peptide (RENECVSCSNCKKSLESTKL) are hydrophilic in nature. Hydrophilic regions of proteins are known to be exposed compared to their hydrophobic counterparts and thus are thought to play an important role in the binding and interaction with their ligands (Lie *et al.*, 1992). The subsequent receptor peptide corresponded to amino acid residues, 159-178. This peptide, although found to inhibit TNF-induced cytolysis in L929 cells to a similar degree as $TNFRI_{175-194}$, required a concentration of 1000 μM in comparison to the former which inhibited at 200 μM. Interestingly, this peptide (QEKQNTVATAHAGFFLRENEG) contained more hydrophobic residues such as alanines which were used as substitutes for cysteine and phenylalanine (Lie *et al.*, 1992). The authors suggest that the difference in the activities of the two TNFR mimetics is due to the increased hydrophobicity of this region. The more hydrophilic peptide ($TNFRI_{175-194}$) could have improved binding between the peptide and TNF, but the specific sequence of the binding site must be taken into account. However, perhaps this region ($TNFRI_{159-178}$) is not a critical in the interaction with TNF and also the substitution of cysteines for alanines might have interfered with the specific interaction leading to lower inhibitory activity.

7.3.1 Structural modification to TNFRI derived peptides

Our studies demonstrated that the longer peptide of the region, $TNFRI_{198-211}$ (QIENVKGTEDSGTT) exhibited greater inhibitory activity, with an IC_{50} of 50 μM compared to 171 μM of the shorter $TNFRI_{206-211}$. This improved activity may be due to its longer length providing a greater binding surface for TNF (Table 7.1).

Although the tandem repeat form of $TNFRI_{209-211}$ did not show inhibitory properties at lower concentrations, it did cause a 45 % reduction at 50 μM which is greater than that observed at a similar concentration of $TNFRI_{209-211}$, suggesting that the repeat form was able to achieve a more stable interaction (Table 7.1). The use of tandem repeats of peptides to enhance activity has recently been used to increase the anti-viral activity of a mimetic peptide of lactoferrin. The peptide corresponds to residues 600-632, which are thought to be important in the binding to hepatitis c virus envelope protein, thus inhibiting the infection (Nozaki *et al.*, 2003). Tandem repeats (two and three repeats) of the 33-mer peptide, decreased the IC_{50} against viral infected cultured hepatocytes from 23 μM to 10 μM (Abe *et al.,* 2007). Thus making tandem repeats of a small peptide is a viable way to enhance activity.

Extending our studies to a D-amino acid substituted form of $TNFRI_{206-211}$ showed that, this form had enhanced inhibitory effect on TNF-induced respiratory burst in comparison to the natural L-form, most likely due to increased stability of the D-form. D-amino acid substitution has recently been applied to a mimetic peptide of granulysin- a cytolytic

protein localised to cytotoxic lymphocytes and natural killer cell granules (Hamamoto *et al.*, 2002). Different mimetic peptides of granulysin have been shown to have antimicrobial properties against various microorganisms such as: Escherichia coli and Staphylococcus aureus (Wang *et al.*, 2000, Hamamoto *et al.*, 2002). However, it was found that the natural L-form of the peptide (residues 23-42) was susceptible to proteolytic degradation by proteases present in serum. Partial or total substitution with D-amino acids resulted in decreased susceptibility to degradation by trypsin and foetal calf serum (FCS) compared to the natural L-form of the peptide, with the resistance to proteolytic degradation being greater in the wholly substituted peptide compared to the partially substituted one. When the FCS was pre-incubated with protease inhibitors such as aprotinin, the antimicrobial activity of the L-peptides was recovered (Hamamoto *et al.*, 2002).

Total D-amino acid substitution compared to single D-amino acid replacement was similarly proven to be advantageous with selectin based mimetic peptides that inhibit neutrophil infiltration into inflammatory sites (Briggs *et al.,* 1996). When all the residues in the heptapeptide were replaced with their D-amino acid forms, the *in vitro* IC_{50} was less than half that of the natural L-form. Interestingly, single substitutions at various positions resulted in loss of activity of the peptide, with IC_{50} increasing by 10 fold (Briggs *et al.*, 1996). The L-form of $TNFRI_{206-211}$ had an IC_{50} of 171 μM for TNF-induced chemiluminescence response while its D-amino acid form had an IC_{50} of 51 μM (Table 7.1).

Thus the finding that the D-amino form of $TNFRI_{206-211}$ was a more potent inhibitor than its natural L-amino acid form highlights the importance of making this L to D-form substitution to increase not only the *in vitro* but the *in vivo* stability of peptides in order to maximise their effects.

Table 7.1 Inhibition of TNF-induced superoxide production by TNFRI-derived peptides in neutrophils

Peptide	*Sequence*	*IC_{50} (μM)*
$TNFRI_{209-211}$	GTT	123
Tandem repeat of $TNFRI_{209-211}$	GTTGTTGTTGTT	50 (45 % inhibition)
$TNFRI_{206-211}$	EDSGTT	171
D-amino-$TNFRI_{206-211}$	EDSGTTa	51
$TNFRI_{198-211}$	QIENVKGTEDSGTT	50

[a]Italic letters denote D-amino acids.

7.4 ANTI-INFLAMMATORY PROPERTIES OF TNFRI DERIVED PEPTIDES

The *in vitro* inhibitory properties of the TNFRI peptides on leukocyte functions were associated with *in vivo* anti-inflammatory activities. The leukocyte infiltration induced by LPS injection into the peritoneal cavity of mice was inhibited by $TNFRI_{206-211}$. This was reflected both in a significant decrease in neutrophil and macrophages accumulation after 24 h. The inhibitory effects were likely a result of the prevention of TNF-induced p38 activation, as LPS-induced inflammation is dependent on TNF and TNFRI (Skerrett *et al.,* 1999). Previously, in mice challenged with LPS TNF production reached a peak 2 h after challenge (Calkins *et al.*, 2001), coinciding with the peak production of TNF. TNF is critical for normal neutrophil recruitment into inflammatory sites. TNFRI knockout (KO) mice, challenged with LPS exhibited a reduction in neutrophil accumulation and in particular a reduction in the chemokines MIP-2 and KC, compared to WT and TNFRII KO mice and most likely explain the inhibitory effects of our TNFRI mimetic (Calkins *et al.*, 2001). These findings highlight the importance of TNF in the production of the chemotactic chemokines which promote the large influx of leukocytes (Calkins *et al.*, 2001).

While the mechanisms of the inhibitory action of the receptor peptide were not investigated, these were likely to result from the blocking of p38 and NF-κB activation through TNFRI and downregulating cytokine and chemokines production from resident peritoneal macrophages. Inhibiting these pathways is also likely to be responsible for the

inhibition of phagocyte oxygen radical production, and upregulation of CR3 expression ultimately contributing to decreased leukocyte activation and infiltration.

The pro-inflammatory and destructive potential of TNF is mediated through activation of p38. There is evidence to support a key role in inflammation, such as its important in stimulating the production of pro-inflammatory mediators responsible for leukocyte recruitment and activation including IL-1β, TNF, IL-8, and IL-6. In a recent study, levels of phosphorylated MKK3/6 (an upstream regulator of p38) were found to be increased in the synovial membranes of RA patients, in comparison to those suffering from degenerative joint disease such osteoarthritis. MKK3/MKK6 was constitutively expressed in both patient groups (Chabaud-Riou and Firestein, 2004). The use of p38 inhibitors has also been profiled in several inflammatory models *in vivo*. Accordingly, its inhibitory activity has been displayed in a wide variety of TNF-mediated disease models such as arthritis, bone resorption and endotoxin shock (Badger *et al.*, 1996, Escott *et al.*, 2000, Haddad *et al.*, 2001, Nishikori *et al.*, 2002, Kumar *et al.*, 2003, Miwatashi *et al.*, 2005).

The p38 inhibitors have been specifically used in LPS-induced inflammation models to achieve the same goals as those involving anti-TNF or TNFRI KO mice application. Underwood et al., (2000) showed that when the p38 inhibitor was administered 1 h before and 4 h after LPS challenge (via inhalation), there was significant and dose dependent reduction in neutrophil and mononuclear cells found in the bronchoalveolar lavage (BAL) fluid (Underwood *et al.,* 2000). Similarly, another study also demonstrated the reduction in BAL neutrophil numbers following oral administration of p38 inhibitor

30 min prior to LPS challenge (Haddad *et al.*, 2001). The decreased number of infiltrating leukocytes observed in our study could also be due to $TNFRI_{206-211}$ inhibiting the p38-mediated TNF-induced expression of cell adhesion molecules by endothelial cells. LPS or TNF alone have been shown to induce upregulation of cell adhesion molecule on endothelial cells. Interestingly, together they act synergistically to increase cell adhesion molecules (CAM), via NF-κB and p38 pathways and JNK (Jersmann *et al.*, 2001). Thus the reduction in leukocyte infiltration in the peritoneal cavity observed in our studies could be due to decreased CAM expression resulting in a decreased number of infiltrating leukocytes due to reduced extravasation. TNF is known to induce IL-6 and chemokines, IL-8 and MCP-1 production in endothelial cell expression via the p38 pathway (Pober, 2002, Westra *et al.*, 2005). Thus, the reduction in macrophages and neutrophils observed in our studies could also be due to reduced chemokine expression resulting in reduced number of infiltrating leukocytes.

The inhibitory effects of the TNFRI peptides were not restricted to the LPS model. Local application of $TNFRI_{206-211}$ significantly inhibited the carrageenan-induced paw inflammation. The carrageen-induced acute paw inflammation has been linked to neutrophil infiltration and the production of neutrophil-derived oxygen free radicals (Salvemini *et al.*, 1996, Posadas *et al.*, 2004). The importance of TNF in this inflammatory response has recently been demonstrated. Using TNFRI KO mice, Rocha et al., 2006 showed that paw swelling was greatly reduced in TNFRI KO mice compared to WT mice. Interestingly, intravenous administration of murine neutralising anti-TNF antibody 5 min before carrageenan challenge reduced the initial paw oedema formation for up to 4 h. Treatment with the antibody 5 min before and 3 h after challenge reduced

paw oedema throughout the 24 h period (Rocha *et al.,* 2006). In our studies on carrageenan-induced paw inflammation when the $TNFRI_{206-211}$ was administered intraperitoneally no effect was observed suggesting a need to control/inhibit the action of TNF that was produced locally at the site of inflammation (Sekut *et al.,* 1994). Interestingly, co-administration of carrageenan and $TNFRI_{206-211}$ into the paw led to a significant reduction in the swelling formation. The lack of effect of $TNFRI_{206-211}$ when administered into the peritoneum, suggests that either the peptide, owing to its small size was highly labile or was rapidly removed by the kidneys or due to its natural L-amino acid confirmation, was susceptible to degradation by proteases present in serum before it reached the site of inflammation. Our data is thus in agreement with other studies that have used TNFRI knockout mice, anti-TNF therapy or anti-TNF synthesis agents (thalidomide) showing that TNF plays a major role in the carrageenan inflammation response (Rocha *et al.*, 2006, Mazzon *et al.*, 2008). Furthermore our findings suggest an important role for the p38 pathway in this disease model, which is also in agreement with studies which have utilised a specific p38 inhibitor in the same model (Nishikori *et al.*, 2002). However, with pharmacological inhibitors of p38, all responses requiring this signalling pathway will be affected.

The DTH response is a type IV hypersensitivity reaction which is characterised by a large cellular infiltrate into the affected sites on subsequent exposure to antigen, particularly macrophages, sensitised antigen specific T cells and neutrophils, as well as the production of a cascade of chemokines and cytokines namely IFNγ, TNF, LT and IL-8 (Li *et al.*, 1994, Buchanan and Murphy, 1997). Studies have shown that Th1 cytokines, IFNγ, IL-2 and LT, are important in eliciting the DTH response with IFNγ as the main

contributor, as treatment with anti-IFNγ antibodies led to a 50 % reduction of the response (Li *et al.*, 1994). In our studies, local application of $TNFRI_{206-211}$ caused a significant inhibition of the DTH response to SRBC. However, the inhibition was only 30 % compared to 60 % of the carrageenan response. This discrepancy could be due to the differences in cellular infiltrates and cytokine milieu in these two models. The carrageenan response has been shown to have a predominantly neutrophil influx with TNF playing an essential role in the development of the response (Sekut *et al.*, 1994, Rocha *et al.*, 2006, Mazzon *et al.*, 2008) . In contrast, although, TNF, is also produced in the DTH response, the amounts produced are significantly less than those of IFNγ (Buchanan and Murphy, 1997). In some DTH responses TNF has been shown to play a central role in the inflammation since treatment with anti-TNF antibodies abolished the response. For example, in the DTH model in response to the chemical irritant trinitrochlorobenzene, anti-TNF antibodies caused complete abolition of the response while anti-IFNγ antibodies only caused a 50 % reduction (Piguet *et al.,* 1991). Thus there is a differential use of TNF and IFNγ between different DTH models and attempts to block a DTH responses (a chronic inflammatory response) need to take this into consideration.

The kinetics of cytokine production in the DTH response compared to that of the carrageenan model should also be taken into consideration to explain the differences in the action of $TNFRI_{206-211}$. For example, in the DTH response to *Cryptococcus neoforms*, the local production of TNF was significantly increased between 18 – 24 h post challenge and thereafter (Buchanan and Murphy, 1997). However, it should be noted that their kinetic studies started 6 h after initial challenge and that the baseline levels of TNF were

200 pg/ml which is suggestive of an early surge in TNF production. The same group also reported that during the cellular infiltration stage, neutrophils were the first to appear followed by lymphocytes and monocytes (Buchanan and Murphy, 1997). The initial increase in local TNF production is often associated with an increase in infiltrating neutrophils in different inflammation models (Zhang *et al.,* 1992). Thus, it can be inferred that since their kinetic studies started 6 h after challenge, it might be possible that there is an initial surge in TNF production which leads to the influx of neutrophils. Since our peptide was injected along with the SRBC on challenge we would assume that it was acting on TNF released during the first few hours. Subsequent to this antigen challenge, antigen specific T cells arrive and begin to secrete large amounts of IFNγ, which results in activation of macrophages, promoting the secretion of additional inflammatory cytokines. However, if the levels of TNF in our DTH response to SRBC reached a peak as late and are as sustained as in the study by Buchanan and Murphy (1997), then it is possible that the decreased inhibitory effects were due to the consistently high levels of TNF during the course of the response (Buchanan and Murphy, 1997). Our studies thus show that targeting the TNF-TNFR-p38 pathway using $TNFRI_{206\text{-}211}$ is a viable and effective way to curb inflammation. We have shown that blocking one of the pathways by which TNF mediates its effects can lead to significant inhibitory effects.

7.5 THE THERAPEUTIC POTENTIAL OF TNFRI DERIVED PEPTIDES

The driving force behind anti-TNF therapy has come from our increasing knowledge of the pathogenesis of RA. Originally thought to be a cell-mediated disease, the discovery of inflammatory cytokines in the synovium and plasma of RA patients shifted the focus to attempts to develop neutralising agents of TNF (Feldmann and Maini, 2001, Taylor, 2003, Tracey *et al.*, 2008). Three protein-based drugs in the form of MAbs or soluble receptors are currently approved by FDA for use namely: etanercept, infliximab and adalimumab (Taylor, 2003, Via, 2007). Due to their protein nature all have to be administered via injection. Although these biologics have revolutionalised the treatment of RA, the most prevalent concerns with all three drugs has been an increased incidence of re-exacerbation of latent tuberculosis, an increased incidence of lymphoma and a potential for worsening congestive heart failure (Gabriel, 2008, Tracey *et al.*, 2008). This, coupled with the cost of manufacturing these large molecules resulted in a shift to develop small molecule inhibitors. However the specificity of these new compounds is paramount to ensure desired blockade of a specific target. A poor example of such an agent is CC-10004, an oral anti-inflammatory drug for use in psoriasis, which is currently in phase II clinical trials. Although results so far have been favourable, CC-10004 inhibits the production of TNF as well as IL-2, IFNγ, phosphodiesterase-4, leukotrienes and nitric oxide synthase (Via, 2007). It can be envisaged that there will be many considerable side effects due to the non-specific nature of the agent.

The evidence that many of the pathological effects associated with TNF are mediated by the p38 pathway, as well as p38 itself promoting the stability of inflammatory cytokine mRNA, has led research to focus on p38 inhibitors as a way to curb TNF's deleterious effects. In 2005, the combined global sales of etanercept, infliximab and adalimumab

were about US$7.5 billion. This figure includes sales for RA, Crohn's disease, ankylosing spondylitis and psoriatic arthritis (Via, 2007). Not surprisingly, many pharmaceutical companies have intensified their search for drugs that target p38. Many studies have demonstrated the efficacy of p38 inhibitors in animal models of diseases and inflammation. The arthritis model is the most common of animal models (Wadsworth *et al.*, 1999, Badger *et al.*, 2000, McLay *et al.*, 2001), though the p38 pathway has also proved to be important in lung inflammation, cerebral ischemia and burn wounds (Barone *et al.*, 2001, Kawashima *et al.*, 2001, Ipaktchi *et al.*, 2006). A few of these inhibitors have progressed to phase I and II clinical trials, such as the GSK-681323 and GSK-856553 for RA, Crohn's disease and psoriasis (Molfino, 2005), RWJ-67657 for Crohn's, RA, psoriasis and rhinitis (Fijen *et al.*, 2001) and VX-745 for RA, Crohn's and psoriasis (Stelmach *et al.*, 2003). The results of the phase I clinical trials for the former two are yet to be released, while trials had to be discontinued for the latter. p38 is expressed ubiquitously in all cell types, and in addition to its well known inflammatory functions, it is also involved in regulating other cellular responses such as apoptosis, cell cycle regulation and proliferation (Ono and Han, 2000). As a result of the wide range of cellular processes that p38 regulates, the possibility of adverse reactions from pharmacological blockade has been the bottleneck in development of anti-p38 therapeutics. The Vertex drug mentioned above (VX-745), was discontinued because of an undisclosed toxic effect in the central nervous system (Palladino *et al.,* 2003). Most other compounds have run into dose-limiting toxicities thus limiting their development, with unwanted side effects such as liver enzyme elevations with p38 inhibitor BIRB 796 which was in phase I trial for asthma, allergy and RA (Behr *et al.*, 2003, Dambach, 2005, Kuma *et al.*, 2005, Schindler *et al.*, 2007).

Our TNFRI derived peptide offers a "safer" alternative as it only affects the TNF-TNFR p38 pathway, thus eliminating the possibilities of unwanted adverse effects from the systemic blockade of the p38 pathway. $TNFRI_{206-211}$ has shown potential to have increased specificity without compromising the full immune enhancing effects of TNF, as seen with anti-TNF therapies such as antibodies and soluble receptors that block the entire action of this cytokine (Aggarwal *et al.*, 2006, Tracey *et al.*, 2008).

It should be noted nonetheless, that despite not being able to provide direct evidence of these TNFRI-derived peptides interfering with TNFRII; the following factors should be taken into account. Firstly, the neutrophil stimulatory properties of TNF_{70-80} were shown to be neutralised by mAbs to both TNFRI and TNFRII (Kumaratilake *et al.*, 1995). Accordingly, we can infer that TNF_{70-80} has a similar direct interaction with TNFRII as shown with TNFRI in our binding studies. Secondly, the initial peptides synthesised, were shown to inhibit TNF_{70-80} induced effects in neutrophils. The exact contribution of TNFRII signalling to TNF's biological effects remains a contentious and poorly understood area. While, the predominant evidence supports the accessory role of TNFRII, i.e. "ligand passing" of TNF to TNFRI, recent studies have elucidated an important role for TNFRII (Tartaglia *et al.*, 1993). It follows that, while soluble TNF (sTNF) and membrane bound (mTNF) ligands can bind to both TNFRI and TNFRII, there is a tendency for sTNF to couple to TNFRI and mTNF to TNFRII (Grell *et al.*, 1995, Tracey *et al.*, 2008). While, mTNF has been shown to be essential in host defence mechanisms against mycobaterical infection (Olleros *et al.*, 2002, Allie *et al.*, 2008), but it has also been implicated in the pathogenesis of cerebral malaria (CM) (Lou *et al.,*

2001). In contrast to most other infectious diseases in which TNF is involved that indicate a direct role for TNFRI, TNFRII KO mice were found to be protected from CM but not TNFRI KO mice (Lucas *et al.,* 1997). The same study also showed that mTNF, through binding to TNFRII is responsible for ICAM-1 upregulation on brain endothelial cells, which eventually leads to increased adhesiveness for parasitic RBC and leukocytes (Lucas *et al.*, 1997, Lou *et al.*, 2001). Therefore as mTNF contains the TNF_{70-80} region, it can be inferred that our TNFRI-derived peptides could interact with mTNF, under conditions where mTNF is expressed and thus prevent its interaction with TNFRII.

7.6 PEPTIDES AS VIABLE THERAPEUTICS

The development of peptides as viable therapeutics has steadily increased over the years. As cytokines and their cognate receptors exert their biological activity through comparatively small regions of their entire structure, which come into contact with specific sites on their receptors, it is possible to use these interactions to design smaller molecules that retain the ability to interact with these sites on the receptors. This approach for rational drug design has resulted in the interest in therapeutic peptides. Our TNF mimetic TNF_{70-80} was initially discovered and developed using this approach. Peptides have several advantages over antibodies as viable drug candidates, including cheaper manufacturing costs, higher activity per mass, less chance of interacting with the immune system and better organ/tumour penetration (Lien and Lowman, 2003, Ladner *et al.*, 2004). Our TNFRI mimetic peptide also appeared to have little or no toxicity and had

no observable effects on the well-being of the recipient mice under our treatment conditions. When $TNFRI_{206-211}$ was administered intraperitoneally alone, the mice did not show any visible signs of toxicity such as ruffled fur, hunched posture or touch sensitivity. This was also evident in the fact that peritoneal exudates from the mice that received the peptide alone were similar in cellular contents to those which had received vehicle only, i.e. the predominant cell types were the resident peritoneal macrophage and mast cells. Currently the vast majority of peptides available on the market are agonists, and if they are true agonists, only small quantities are required to activate the targeted receptor. In the treatment of cancer and inflammation as in our studies, antagonists are the most sought after therapeutic agents.

There has been extensive research into small peptide inhibitors of TNF with focus on developing agents with high specificity and a low IC_{50}. Different approaches have been utilised to design peptides. A recently described peptide that corresponds to the pre-ligand assembly domain (PLAD) of TNFRI was shown to inhibit TNF-signalling by binding to the PLAD region of TNFRI, thereby preventing receptor aggregation and consequently TNF-TNFR signalling (Deng *et al.*, 2005). This peptide was able to inhibit *in vitro* functions such as TNF-induced cytolysis and NF-κB activation and was also shown to block arthritis in animal models (Deng *et al.*, 2005). However, as this peptide was administered with as a GST fusion protein, the large size leads to Ab generation in the *in vivo* models (Deng *et al.*, 2005). Another approach that has been used was the interaction between TNFβ and TNFRI to identify critical binding sites on TNFR (Takasaki *et al.,* 1997). This led to the discovery of a cyclic mimetic peptide that corresponds to a critical binding site of TNFRI, which exhibited the same efficacy as

anti-TNF Ab in inhibit bone destruction (Saito *et al.*, 2007). Interestingly, *in vitro* 25 μM of the peptide was needed to inhibit TNF binding to TNFR to the same extent as 10 nM of anti-TNF Ab, whereas *in vivo* similar doses (4 mg/kg/day) of peptide and Ab gave similar effects on the arthritis model (Takasaki *et al.*, 1997, Saito *et al.*, 2007). This discrepancy suggests that the peptide might be inhibiting by different mechanisms and not solely as a TNF antagonist or perhaps the TNF has a greater affinity for the peptide than the Ab.

Recently a novel 12-mer peptide isolated from a phage-display library exhibited antagonistic properties to vascular endothelial growth factor and its receptor (Hetian *et al.,* 2002). *In vitro* studies showed that this peptide to have an IC_{50} of ~ 100 μM but *in vivo* 60 μl of 500 μM was sufficient to inhibit metastasis of carcinomas. Differences between *in vitro* and *in vivo* experiments could be due to the possibility that in the *in vitro* studies quantities of the peptide could be lost due to binding to plastic surfaces, whereas during the *in vivo* studies of breast carcinoma metastasis the peptide was injected directly into the tumour site. This action reduces the risk of the peptide undergoing rapid clearance by the renal or hepatic systems. In our studies, we found administering the peptide directly into the site of inflammation to have greater effect than at a distant site. Thus, when developing peptide based drugs, it is important to take into account factors such as degradation, clearance and dilution effect when a peptide is injected at a site different to the targeted local reaction.

7.7 CONCLUDING REMARKS

This study has demonstrated that the pleiotropic effects of TNF can be dissected by the use of synthetic peptides corresponding to different regions of TNF. For the first time we demonstrate that TNF_{70-80} interacts with TNFRI to modulate its effects. The TNF mimetic peptides' selective properties were reflected in their ability to differentially activate the MAPK pathways which can be explained by the inability of TNF_{70-80} to recruit all the necessary adaptor proteins to the TNF_{70-80}-TNFRI complex.

By use of the interaction of TNF_{70-80} and TNFRI we identified the minimum specific region required by TNF_{70-80} to activate the p38 pathway. Peptides to this region were designed and were shown to inhibit TNF_{70-80} induced respiratory burst in neutrophils. Modifications to this region led to TNFRI-based mimetics which were demonstrated to inhibit TNF-induced p38 activation, and p38-dependent effects of respiratory burst, CD11b/CD18 upregulation, and cytokine production in neutrophils. When a longer peptide, D-amino acid substitution or tandem repeats of this region (EDSGTT) were made their inhibitory properties were enhanced.

The anti-inflammatory effects of the TNFR mimetics were not limited to evidence from *in vitro* studies. When the natural L-form of $TNFRI_{206-211}$ was used in various models of inflammation in which the TNF-TNFR-p38 pathway plays an important role, the TNFRI mimetic inhibited acute leukocyte infiltration in response to LPS, inhibited carrageenan-induced paw swelling and inhibited chronic inflammation of DTH response to SRBC. In view of the limitation of therapeutics targeting either the TNF/TNFR or p38 signalling

pathway, our research has identified a potential improvement in targeting this receptor and intracellular signalling system in inflammatory disorders. We have identified a way of restricting the target to a TNFR-p38 signalling axis.

REFERENCES

Abe, K., A. Nozaki, K. Tamura, M. Ikeda, K. Naka, H. Dansako, H. O. Hoshino, K. Tanaka, and N. Kato. 2007. Tandem repeats of lactoferrin-derived anti-hepatitis C virus peptide enhance antiviral activity in cultured human hepatocytes. *Microbiol Immunol* 51: 117-25.

Adam-Klages, S., D. Adam, K. Wiegmann, S. Struve, W. Kolanus, J. Schneider-Mergener, and M. Kronke. 1996. FAN, a novel WD-repeat protein, couples the p55 TNF-receptor to neutral sphingomyelinase. *Cell* 86: 937-47.

Adam, D., K. Wiegmann, S. Adam-Klages, A. Ruff, and M. Kronke. 1996. A novel cytoplasmic domain of the p55 tumor necrosis factor receptor initiates the neutral sphingomyelinase pathway. *J Biol Chem* 271: 14617-22.

Aggarwal, B. B., S. Shishodia, Y. Takada, D. Jackson-Bernitsas, K. S. Ahn, G. Sethi, and H. Ichikawa. 2006. TNF blockade: an inflammatory issue. *Ernst Schering Res Found Workshop*: 161-86.

Aguirre, V., T. Uchida, L. Yenush, R. Davis, and M. F. White. 2000. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 275: 9047-54.

Alexander, J., and D. G. Russell. 1985. Parasite antigens, their role in protection, diagnosis and escape: the leishmaniases. *Curr Top Microbiol Immunol* 120: 43-67.

Allie, N., L. Alexopoulou, V. J. Quesniaux, L. Fick, K. Kranidioti, G. Kollias, B. Ryffel, and M. Jacobs. 2008. Protective role of membrane tumour necrosis factor in the host's resistance to mycobacterial infection. *Immunology*.

Altstaedt, J., H. Kirchner, and L. Rink. 1996. Cytokine production of neutrophils is limited to interleukin-8. *Immunology* 89: 563-8.

Anderson, C. F., and D. M. Mosser. 2002. A novel phenotype for an activated macrophage: the type 2 activated macrophage. *J Leukoc Biol* 72: 101-6.

Andreakos, E. T., B. M. Foxwell, F. M. Brennan, R. N. Maini, and M. Feldmann. 2002. Cytokines and anti-cytokine biologicals in autoimmunity: present and future. *Cytokine Growth Factor Rev* 13: 299-313.

Anker, S. D., and A. J. Coats. 2002. How to RECOVER from RENAISSANCE? The significance of the results of RECOVER, RENAISSANCE, RENEWAL and ATTACH. *Int J Cardiol* 86: 123-30.

Antoni, C., and J. R. Kalden. 1999. Combination therapy of the chimeric monoclonal anti-tumor necrosis factor alpha antibody (infliximab) with methotrexate in patients with rheumatoid arthritis. *Clin Exp Rheumatol* 17: S73-7.

Antonyak, M. A., D. K. Moscatello, and A. J. Wong. 1998. Constitutive activation of c-Jun N-terminal kinase by a mutant epidermal growth factor receptor. *J Biol Chem* 273: 2817-22.

Araki, H., N. Katayama, Y. Yamashita, H. Mano, A. Fujieda, E. Usui, H. Mitani, K. Ohishi, K. Nishii, M. Masuya, N. Minami, T. Nobori, and H. Shiku. 2004. Reprogramming of human postmitotic neutrophils into macrophages by growth factors. *Blood* 103: 2973-80.

Arvin, A. M. 2008. Humoral and cellular immunity to varicella-zoster virus: an overview. *J Infect Dis* 197 Suppl 2: S58-60.

Badger, A. M., J. N. Bradbeer, B. Votta, J. C. Lee, J. L. Adams, and D. E. Griswold. 1996. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone

resorption, endotoxin shock and immune function. *J Pharmacol Exp Ther* 279: 1453-61.

Badger, A. M., D. E. Griswold, R. Kapadia, S. Blake, B. A. Swift, S. J. Hoffman, G. B. Stroup, E. Webb, D. J. Rieman, M. Gowen, J. C. Boehm, J. L. Adams, and J. C. Lee. 2000. Disease-modifying activity of SB 242235, a selective inhibitor of p38 mitogen-activated protein kinase, in rat adjuvant-induced arthritis. *Arthritis Rheum* 43: 175-83.

Baeuerle, P. A., and T. Henkel. 1994. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol* 12: 141-79.

Barone, F. C., E. A. Irving, A. M. Ray, J. C. Lee, S. Kassis, S. Kumar, A. M. Badger, R. F. White, M. J. McVey, J. J. Legos, J. A. Erhardt, A. H. Nelson, E. H. Ohlstein, A. J. Hunter, K. Ward, B. R. Smith, J. L. Adams, and A. A. Parsons. 2001. SB 239063, a second-generation p38 mitogen-activated protein kinase inhibitor, reduces brain injury and neurological deficits in cerebral focal ischemia. *J Pharmacol Exp Ther* 296: 312-21.

Bateman, R. M., M. D. Sharpe, and C. G. Ellis. 2003. Bench-to-bedside review: microvascular dysfunction in sepsis--hemodynamics, oxygen transport, and nitric oxide. *Crit Care* 7: 359-73.

Bates, E. J., A. Ferrante, and L. J. Beard. 1991. Characterization of the major neutrophil-stimulating activity present in culture medium conditioned by Staphylococcus aureus-stimulated mononuclear leucocytes. *Immunology* 72: 448-50.

Bathoorn, E., H. Kerstjens, D. Postma, W. Timens, and W. MacNee. 2008. Airways inflammation and treatment during acute exacerbations of COPD. *Int J Chron Obstruct Pulmon Dis* 3: 217-29.

Baud, V., and M. Karin. 2001. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 11: 372-7.

Bazzoni, F., and B. Beutler. 1996. The tumor necrosis factor ligand and receptor families. *N Engl J Med* 334: 1717-25.

Beg, A. A., W. C. Sha, R. T. Bronson, S. Ghosh, and D. Baltimore. 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376: 167-70.

Behr, T. M., M. Berova, C. P. Doe, H. Ju, C. E. Angermann, J. Boehm, and R. N. Willette. 2003. p38 mitogen-activated protein kinase inhibitors for the treatment of chronic cardiovascular disease. *Curr Opin Investig Drugs* 4: 1059-64.

Bennouna, S., S. K. Bliss, T. J. Curiel, and E. Y. Denkers. 2003. Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. *J Immunol* 171: 6052-8.

Beutler, B. 1992. Application of transcriptional and posttranscriptional reporter constructs to the analysis of tumor necrosis factor gene regulation. *Am J Med Sci* 303: 129-33.

Beutler, B., and T. Brown. 1993. Polymorphism of the mouse TNF-alpha locus: sequence studies of the 3'-untranslated region and first intron. *Gene* 129: 279-83.

Beutler, B., and A. Cerami. 1986. Cachectin/tumor necrosis factor: an endogenous mediator of shock and inflammation. *Immunol Res* 5: 281-93.

Beutler, B., D. Greenwald, J. D. Hulmes, M. Chang, Y. C. Pan, J. Mathison, R. Ulevitch, and A. Cerami. 1985. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. *Nature* 316: 552-4.

Bevilacqua, M. P., J. S. Pober, G. R. Majeau, W. Fiers, R. S. Cotran, and M. A. Gimbrone, Jr. 1986. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc Natl Acad Sci U S A* 83: 4533-7.

Bogdan, C. 2008. Mechanisms and consequences of persistence of intracellular pathogens: leishmaniasis as an example. *Cell Microbiol* 10: 1221-34.

Bokoch, G. M. 2002. Microbial killing: hold the bleach and pass the salt! *Nat Immunol* 3: 340-2.

Boldt, S., and W. Kolch. 2004. Targeting MAPK signalling: Prometheus' fire or Pandora's box? *Curr Pharm Des* 10: 1885-905.

Bouaouina, M., E. Blouin, L. Halbwachs-Mecarelli, P. Lesavre, and P. Rieu. 2004. TNF-induced beta2 integrin activation involves Src kinases and a redox-regulated activation of p38 MAPK. *J Immunol* 173: 1313-20.

Boulton, T. G., S. H. Nye, D. J. Robbins, N. Y. Ip, E. Radziejewska, S. D. Morgenbesser, R. A. DePinho, N. Panayotatos, M. H. Cobb, and G. D. Yancopoulos. 1991. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65: 663-75.

Boulton, T. G., G. D. Yancopoulos, J. S. Gregory, C. Slaughter, C. Moomaw, J. Hsu, and M. H. Cobb. 1990. An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* 249: 64-7.

Breedveld, F. C. 2000. Therapeutic monoclonal antibodies. *Lancet* 355: 735-40.

Brennan, F. M., D. Chantry, A. Jackson, R. Maini, and M. Feldmann. 1989. Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet* 2: 244-7.

Brenner, D. A. 1998. Signal transduction during liver regeneration. *J Gastroenterol Hepatol* 13 Suppl: S93-5.

Briggs, J. B., R. A. Larsen, R. B. Harris, K. V. Sekar, and B. A. Macher. 1996. Structure/activity studies of anti-inflammatory peptides based on a conserved peptide region of the lectin domain of E-, L- and P-selectin. *Glycobiology* 6: 831-6.

Briscoe, H., D. R. Roach, N. Meadows, D. Rathjen, and W. J. Britton. 2000. A novel tumor necrosis factor (TNF) mimetic peptide prevents recrudescence of Mycobacterium bovis bacillus Calmette-Guerin (BCG) infection in CD4+ T cell-depleted mice. *J Leukoc Biol* 68: 538-44.

Britigan, B. E., G. M. Rosen, Y. Chai, and M. S. Cohen. 1986. Do human neutrophils make hydroxyl radical? Determination of free radicals generated by human neutrophils activated with a soluble or particulate stimulus using electron paramagnetic resonance spectrometry. *J Biol Chem* 261: 4426-31.

Britton, W. J., N. Meadows, D. A. Rathjen, D. R. Roach, and H. Briscoe. 1998. A tumor necrosis factor mimetic peptide activates a murine macrophage cell line to inhibit mycobacterial growth in a nitric oxide-dependent fashion. *Infect Immun* 66: 2122-7.

Brockhaus, M., H. J. Schoenfeld, E. J. Schlaeger, W. Hunziker, W. Lesslauer, and H. Loetscher. 1990. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc Natl Acad Sci U S A* 87: 3127-31.

Brown, G. D., and S. Gordon. 2005. Phagocytes part 1: Macrophages. Pages 21-32 in S. Kaufmann and M. Steward, eds. *Topley and Wilson Microbiology and Microbial Infections (Immunology Volume)*. Arnold Publisher, London.

Buchanan, K. L., and J. W. Murphy. 1997. Kinetics of cellular infiltration and cytokine production during the efferent phase of a delayed-type hypersensitivity reaction. *Immunology* 90: 189-97.

Burg, N. D., and M. H. Pillinger. 2001. The neutrophil: function and regulation in innate and humoral immunity. *Clin Immunol* 99: 7-17.

Calkins, C. M., J. K. Heimbach, D. D. Bensard, Y. Song, C. D. Raeburn, X. Meng, and R. C. McIntyre, Jr. 2001. TNF receptor I mediates chemokine production and neutrophil accumulation in the lung following systemic lipopolysaccharide. *J Surg Res* 101: 232-7.

Cameron, M. J., and D. J. Kelvin. 2003. Cytokines and chemokines--their receptors and their genes: an overview. *Adv Exp Med Biol* 520: 8-32.

Caput, D., B. Beutler, K. Hartog, R. Thayer, S. Brown-Shimer, and A. Cerami. 1986. Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc Natl Acad Sci U S A* 83: 1670-4.

Carlos, T. M., and J. M. Harlan. 1990. Membrane proteins involved in phagocyte adherence to endothelium. *Immunol Rev* 114: 5-28.

—. 1994. Leukocyte-endothelial adhesion molecules. *Blood* 84: 2068-101.

Carpinteiro, A., C. Dumitru, M. Schenck, and E. Gulbins. 2008. Ceramide-induced cell death in malignant cells. *Cancer Lett* 264: 1-10.

Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* 72: 3666-70.

Cassatella, M. A. 1999. Production of cytokines by polymorphonuclear neutrophils. Pages 151-229 in D. M. Gabrilovich, ed. *The Neutrophils: New Outlook for Old Cells*. Imperial College Press, London.

Chabaud-Riou, M., and G. S. Firestein. 2004. Expression and activation of mitogen-activated protein kinase kinases-3 and -6 in rheumatoid arthritis. *Am J Pathol* 164: 177-84.

Chadee, D. N., T. Yuasa, and J. M. Kyriakis. 2002. Direct activation of mitogen-activated protein kinase kinase kinase MEKK1 by the Ste20p homologue GCK and the adapter protein TRAF2. *Mol Cell Biol* 22: 737-49.

Chaplin, D. D. 2003. 1. Overview of the immune response. *J Allergy Clin Immunol* 111: S442-59.

Chaudhari, U., P. Romano, L. D. Mulcahy, L. T. Dooley, D. G. Baker, and A. B. Gottlieb. 2001. Efficacy and safety of infliximab monotherapy for plaque-type psoriasis: a randomised trial. *Lancet* 357: 1842-7.

Chen, C. Y., and A. B. Shyu. 1995. AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* 20: 465-70.

Chen, G., and D. V. Goeddel. 2002. TNF-R1 signaling: a beautiful pathway. *Science* 296: 1634-5.

Chertov, O., H. Ueda, L. L. Xu, K. Tani, W. J. Murphy, J. M. Wang, O. M. Howard, T. J. Sayers, and J. J. Oppenheim. 1997. Identification of human neutrophil-derived

cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *J Exp Med* 186: 739-47.

Chin, A. I., J. Shu, C. Shan Shi, Z. Yao, J. H. Kehrl, and G. Cheng. 1999. TANK potentiates tumor necrosis factor receptor-associated factor-mediated c-Jun N-terminal kinase/stress-activated protein kinase activation through the germinal center kinase pathway. *Mol Cell Biol* 19: 6665-72.

Chu, C. Q., M. Field, M. Feldmann, and R. N. Maini. 1991. Localization of tumor necrosis factor alpha in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis. *Arthritis Rheum* 34: 1125-32.

Chung, E. S., M. Packer, K. H. Lo, A. A. Fasanmade, and J. T. Willerson. 2003. Randomized, double-blind, placebo-controlled, pilot trial of infliximab, a chimeric monoclonal antibody to tumor necrosis factor-alpha, in patients with moderate-to-severe heart failure: results of the anti-TNF Therapy Against Congestive Heart Failure (ATTACH) trial. *Circulation* 107: 3133-40.

Clark, I. A. 2007. How TNF was recognized as a key mechanism of disease. *Cytokine Growth Factor Rev* 18: 335-43.

Clauss, M., C. Sunderkotter, B. Sveinbjornsson, S. Hippenstiel, A. Willuweit, M. Marino, E. Haas, R. Seljelid, P. Scheurich, N. Suttorp, M. Grell, and W. Risau. 2001. A permissive role for tumor necrosis factor in vascular endothelial growth factor-induced vascular permeability. *Blood* 97: 1321-9.

Cloutier, A., T. Ear, E. Blais-Charron, C. M. Dubois, and P. P. McDonald. 2007. Differential involvement of NF-kappaB and MAP kinase pathways in the generation of inflammatory cytokines by human neutrophils. *J Leukoc Biol* 81: 567-77.

Coletta, A. P., A. L. Clark, P. Banarjee, and J. G. Cleland. 2002. Clinical trials update: RENEWAL (RENAISSANCE and RECOVER) and ATTACH. *Eur J Heart Fail* 4: 559-61.

Cools, N., P. Ponsaerts, V. F. Van Tendeloo, and Z. N. Berneman. 2007. Regulatory T cells and human disease. *Clin Dev Immunol* 2007: 89195.

Cornelius, P., S. Enerback, G. Bjursell, T. Olivecrona, and P. H. Pekala. 1988. Regulation of lipoprotein lipase mRNA content in 3T3-L1 cells by tumour necrosis factor. *Biochem J* 249: 765-9.

Cossart, P., and J. Mengaud. 1989. Listeria monocytogenes. A model system for the molecular study of intracellular parasitism. *Mol Biol Med* 6: 463-74.

Costabile, M., C. S. Hii, B. S. Robinson, D. A. Rathjen, M. Pitt, C. Easton, R. C. Miller, A. Poulos, A. W. Murray, and A. Ferrante. 2001. A novel long chain polyunsaturated fatty acid, beta-Oxa 21:3n-3, inhibits T lymphocyte proliferation, cytokine production, delayed-type hypersensitivity, and carrageenan-induced paw reaction and selectively targets intracellular signals. *J Immunol* 167: 3980-7.

Cross, A. R., and A. W. Segal. 2004. The NADPH oxidase of professional phagocytes--prototype of the NOX electron transport chain systems. *Biochim Biophys Acta* 1657: 1-22.

Cuenda, A., and S. Rousseau. 2007. p38 MAP-Kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta*.

Cui, J., M. Holgado-Madruga, W. Su, H. Tsuiki, P. Wedegaertner, and A. J. Wong. 2005. Identification of a specific domain responsible for JNK2alpha2 autophosphorylation. *J Biol Chem* 280: 9913-20.

Czuprynski, C. J., J. F. Brown, N. Maroushek, R. D. Wagner, and H. Steinberg. 1994. Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to Listeria monocytogenes infection. *J Immunol* 152: 1836-46.

Dambach, D. M. 2005. Potential adverse effects associated with inhibition of p38alpha/beta MAP kinases. *Curr Top Med Chem* 5: 929-39.

Davis, R. J. 1999. Signal transduction by the c-Jun N-terminal kinase. *Biochem Soc Symp* 64: 1-12.

Dayer, J. M. 2003. The pivotal role of interleukin-1 in the clinical manifestations of rheumatoid arthritis. *Rheumatology (Oxford)* 42 Suppl 2: ii3-10.

Deiss, L. P., H. Galinka, H. Berissi, O. Cohen, and A. Kimchi. 1996. Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. *Embo J* 15: 3861-70.

Deleuran, B. W., C. Q. Chu, M. Field, F. M. Brennan, T. Mitchell, M. Feldmann, and R. N. Maini. 1992. Localization of tumor necrosis factor receptors in the synovial tissue and cartilage-pannus junction in patients with rheumatoid arthritis. Implications for local actions of tumor necrosis factor alpha. *Arthritis Rheum* 35: 1170-8.

Delhase, M., M. Hayakawa, Y. Chen, and M. Karin. 1999. Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. *Science* 284: 309-13.

Deng, G. M., L. Zheng, F. K. Chan, and M. Lenardo. 2005. Amelioration of inflammatory arthritis by targeting the pre-ligand assembly domain of tumor necrosis factor receptors. *Nat Med* 11: 1066-72.

Detmers, P. A., D. Zhou, E. Polizzi, R. Thieringer, W. A. Hanlon, S. Vaidya, and V. Bansal. 1998. Role of stress-activated mitogen-activated protein kinase (p38) in beta 2-integrin-dependent neutrophil adhesion and the adhesion-dependent oxidative burst. *J Immunol* 161: 1921-9.

Deveraux, Q. L., H. R. Stennicke, G. S. Salvesen, and J. C. Reed. 1999. Endogenous inhibitors of caspases. *J Clin Immunol* 19: 388-98.

Devin, A., Y. Lin, and Z. G. Liu. 2003. The role of the death-domain kinase RIP in tumour-necrosis-factor-induced activation of mitogen-activated protein kinases. *EMBO Rep* 4: 623-7.

Dhillon, A. S., and W. Kolch. 2002. Untying the regulation of the Raf-1 kinase. *Arch Biochem Biophys* 404: 3-9.

Dixon, W. G., K. Watson, M. Lunt, K. L. Hyrich, A. J. Silman, and D. P. Symmons. 2006. Rates of serious infection, including site-specific and bacterial intracellular infection, in rheumatoid arthritis patients receiving anti-tumor necrosis factor therapy: results from the British Society for Rheumatology Biologics Register. *Arthritis Rheum* 54: 2368-76.

Dong, C., D. D. Yang, C. Tournier, A. J. Whitmarsh, J. Xu, R. J. Davis, and R. A. Flavell. 2000. JNK is required for effector T-cell function but not for T-cell activation. *Nature* 405: 91-4.

Donovan, N., E. B. Becker, Y. Konishi, and A. Bonni. 2002. JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery. *J Biol Chem* 277: 40944-9.

Dreskin, S. C., G. W. Thomas, S. N. Dale, and L. E. Heasley. 2001. Isoforms of Jun kinase are differentially expressed and activated in human monocyte/macrophage (THP-1) cells. *J Immunol* 166: 5646-53.

Ehlers, S. 2003. Role of tumour necrosis factor (TNF) in host defence against tuberculosis: implications for immunotherapies targeting TNF. *Ann Rheum Dis* 62 Suppl 2: ii37-42.

—. 2005. Tumor necrosis factor and its blockade in granulomatous infections: differential modes of action of infliximab and etanercept? *Clin Infect Dis* 41 Suppl 3: S199-203.

Eliason, J. L., K. K. Hannawa, G. Ailawadi, I. Sinha, J. W. Ford, M. P. Deogracias, K. J. Roelofs, D. T. Woodrum, T. L. Ennis, P. K. Henke, J. C. Stanley, R. W. Thompson, and G. R. Upchurch, Jr. 2005. Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation. *Circulation* 112: 232-40.

Elliott, M. J., R. N. Maini, M. Feldmann, J. R. Kalden, C. Antoni, J. S. Smolen, B. Leeb, F. C. Breedveld, J. D. Macfarlane, H. Bijl, and et al. 1994a. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis. *Lancet* 344: 1105-10.

Elliott, M. J., R. N. Maini, M. Feldmann, A. Long-Fox, P. Charles, H. Bijl, and J. N. Woody. 1994b. Repeated therapy with monoclonal antibody to tumour necrosis factor alpha (cA2) in patients with rheumatoid arthritis. *Lancet* 344: 1125-7.

Endres, R., A. Luz, H. Schulze, H. Neubauer, A. Futterer, S. M. Holland, H. Wagner, and K. Pfeffer. 1997. Listeriosis in p47(phox-/-) and TRp55-/- mice: protection despite absence of ROI and susceptibility despite presence of RNI. *Immunity* 7: 419-32.

England, B. P., P. Balasubramanian, I. Uings, S. Bethell, M. J. Chen, P. J. Schatz, Q. Yin, Y. F. Chen, E. A. Whitehorn, A. Tsavaler, C. L. Martens, R. W. Barrett, and M. McKinnon. 2000. A potent dimeric peptide antagonist of interleukin-5 that binds two interleukin-5 receptor alpha chains. *Proc Natl Acad Sci U S A* 97: 6862-7.

English, D., J. G. Garcia, and D. N. Brindley. 2001. Platelet-released phospholipids link haemostasis and angiogenesis. *Cardiovasc Res* 49: 588-99.

English, D., Z. Welch, A. T. Kovala, K. Harvey, O. V. Volpert, D. N. Brindley, and J. G. Garcia. 2000. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. *Faseb J* 14: 2255-65.

Erickson, S. L., F. J. de Sauvage, K. Kikly, K. Carver-Moore, S. Pitts-Meek, N. Gillett, K. C. Sheehan, R. D. Schreiber, D. V. Goeddel, and M. W. Moore. 1994. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* 372: 560-3.

Escott, K. J., M. G. Belvisi, M. A. Birrell, S. E. Webber, M. L. Foster, and C. A. Sargent. 2000. Effect of the p38 kinase inhibitor, SB 203580, on allergic airway inflammation in the rat. *Br J Pharmacol* 131: 173-6.

Espevik, T., and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J Immunol Methods* 95: 99-105.

Ethuin, F., Chollet-Martin, S. 2005. Cytokine production by neutrophils. Pages 229-251 in D. Gabrilovich, ed. *The Neutrophils. New Outlook for Old Cells*. Imperial College Press, London.

Fairlie, D. P., M. L. West, and A. K. Wong. 1998. Towards protein surface mimetics. *Curr Med Chem* 5: 29-62.

Faurschou, M., and N. Borregaard. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 5: 1317-27.

Feldmann, M., F. M. Brennan, and R. N. Maini. 1996. Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* 14: 397-440.

Feldmann, M., and R. N. Maini. 2001. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annu Rev Immunol* 19: 163-96.

Ferrante, C. Hii, and D. Hume. 2007. Neutrophilic schizophrenia: breaching the barrier between innate and adaptive immunity. *Immunol Cell Biol* 85: 265-6.

Ferrante, A. 1992. Activation of neutrophils by interleukins-1 and -2 and tumor necrosis factors. Pages 417-36 in R. G. Coffey, ed. *Granulocyte responses to cytokines, basic and clinical research.* Marcel Dekker Inc, Tampa, FL.

—. 2005. Phagocytes Part 2: Neutrophils. . Pages 35-54 in S. Kaufmann and M. Steward, eds. *Topley and Wilson Microbiology and Microbial Infections (Immunology Volume)*. Arnold Publisher, London.

Ferrante, A., C. S. T. Hii, and D. A. Rathjen. 1995. Positive perspectives on TNFs: promise versus popular purpose. *Today's Life Sci.*: 40–47.

Ferrante, A., A. J. Martin, E. J. Bates, D. H. Goh, D. P. Harvey, D. Parsons, D. A. Rathjen, G. Russ, and J. M. Dayer. 1993. Killing of Staphylococcus aureus by tumor necrosis factor-alpha-activated neutrophils. The role of serum opsonins, integrin receptors, respiratory burst, and degranulation. *J Immunol* 151: 4821-8.

Ferrante, A., M. Nandoskar, E. J. Bates, and D. H. Goh. 1987. Staphylococcus aureus-stimulated human mononuclear leucocyte-conditioned medium augments the

basal and stimuli-induced neutrophil respiratory burst and degranulation. *Immunology* 60: 431-8.

Ferrante, A., M. Nandoskar, A. Walz, D. H. Goh, and I. C. Kowanko. 1988. Effects of tumour necrosis factor alpha and interleukin-1 alpha and beta on human neutrophil migration, respiratory burst and degranulation. *Int Arch Allergy Appl Immunol* 86: 82-91.

Ferrante, A., B. S. Robinson, H. Singh, H. P. Jersmann, J. V. Ferrante, Z. H. Huang, N. A. Trout, M. J. Pitt, D. A. Rathjen, C. J. Easton, A. Poulos, R. H. Prager, F. S. Lee, and C. S. Hii. 2006. A novel beta-oxa polyunsaturated fatty acid downregulates the activation of the IkappaB kinase/nuclear factor kappaB pathway, inhibits expression of endothelial cell adhesion molecules, and depresses inflammation. *Circ Res* 99: 34-41.

Ferrante, A., R. E. Staugas, B. Rowan-Kelly, S. Bresatz, L. M. Kumaratilake, C. M. Rzepczyk, and G. R. Adolf. 1990. Production of tumor necrosis factors alpha and beta by human mononuclear leukocytes stimulated with mitogens, bacteria, and malarial parasites. *Infect Immun* 58: 3996-4003.

Ferrante, A., and Y. H. Thong. 1982. Separation of mononuclear and polymorphonuclear leucocytes from human blood by the one-step Hypaque-Ficoll method is dependent on blood column height. *J Immunol Methods* 48: 81-5.

Ferrante, J. V., and A. Ferrante. 2005. Novel role of lipoxygenases in the inflammatory response: promotion of TNF mRNA decay by 15-hydroperoxyeicosatetraenoic acid in a monocytic cell line. *J Immunol* 174: 3169-72.

Ferrante, J. V., Z. H. Huang, M. Nandoskar, C. S. Hii, B. S. Robinson, D. A. Rathjen, A. Poulos, C. P. Morris, and A. Ferrante. 1997. Altered responses of human

macrophages to lipopolysaccharide by hydroperoxy eicosatetraenoic acid, hydroxy eicosatetraenoic acid, and arachidonic acid. Inhibition of tumor necrosis factor production. *J Clin Invest* 99: 1445-52.

Fialkow, L., Y. Wang, and G. P. Downey. 2007. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radic Biol Med* 42: 153-64.

Figari, I. S., N. A. Mori, and M. A. Palladino, Jr. 1987. Regulation of neutrophil migration and superoxide production by recombinant tumor necrosis factors-alpha and -beta: comparison to recombinant interferon-gamma and interleukin-1 alpha. *Blood* 70: 979-84.

Fijen, J. W., J. G. Zijlstra, P. De Boer, R. Spanjersberg, J. W. Tervaert, T. S. Van Der Werf, J. J. Ligtenberg, and J. E. Tulleken. 2001. Suppression of the clinical and cytokine response to endotoxin by RWJ-67657, a p38 mitogen-activated protein-kinase inhibitor, in healthy human volunteers. *Clin Exp Immunol* 124: 16-20.

Fischer, P. M. 2003. The design, synthesis and application of stereochemical and directional peptide isomers: a critical review. *Curr Protein Pept Sci* 4: 339-56.

Forsberg, M., R. Lofgren, L. Zheng, and O. Stendahl. 2001. Tumour necrosis factor-alpha potentiates CR3-induced respiratory burst by activating p38 MAP kinase in human neutrophils. *Immunology* 103: 465-72.

Freshney, N. W., L. Rawlinson, F. Guesdon, E. Jones, S. Cowley, J. Hsuan, and J. Saklatvala. 1994. Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* 78: 1039-49.

Fujiwara, N., and K. Kobayashi. 2005. Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy* 4: 281-6.

Gabay, C., N. Cakir, F. Moral, P. Roux-Lombard, O. Meyer, J. M. Dayer, T. Vischer, H. Yazici, and P. A. Guerne. 1997. Circulating levels of tumor necrosis factor soluble receptors in systemic lupus erythematosus are significantly higher than in other rheumatic diseases and correlate with disease activity. *J Rheumatol* 24: 303-8.

Gabriel, S. E. 2008. Tumor necrosis factor inhibition: a part of the solution or a part of the problem of heart failure in rheumatoid arthritis? *Arthritis Rheum* 58: 637-40.

Gahmberg, C. G. 1997. Leukocyte adhesion: CD11/CD18 integrins and intercellular adhesion molecules. *Curr Opin Cell Biol* 9: 643-50.

Gardnerova, M., R. Blanque, and C. R. Gardner. 2000. The use of TNF family ligands and receptors and agents which modify their interaction as therapeutic agents. *Curr Drug Targets* 1: 327-64.

Garrington, T. P., and G. L. Johnson. 1999. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol* 11: 211-8.

Gasque, P. 2004. Complement: a unique innate immune sensor for danger signals. *Mol Immunol* 41: 1089-98.

Gelderman, M. P., R. Stuart, D. Vigerust, S. Fuhrmann, D. L. Lefkowitz, R. C. Allen, S. S. Lefkowitz, and S. Graham. 1998. Perpetuation of inflammation associated with experimental arthritis: the role of macrophage activation by neutrophilic myeloperoxidase. *Mediators Inflamm* 7: 381-9.

Geletka, R. C., and E. W. St Clair. 2005. Infliximab for the treatment of early rheumatoid arthritis. *Expert Opin Biol Ther* 5: 405-17.

Getz, G. S. 2005. Thematic review series: the immune system and atherogenesis. Bridging the innate and adaptive immune systems. *J Lipid Res* 46: 619-22.

Ghosh, S., and M. Karin. 2002. Missing pieces in the NF-kappaB puzzle. *Cell* 109 Suppl: S81-96.

Goetz, M., X. Schiel, H. Heussel, T. Steinmetz, W. Hiddemann, and M. Weiss. 2002. Elevation of soluble tumor necrosis factor receptor II in non-febrile patients with acute myeloid leukemia. *Eur J Med Res* 7: 487-90.

Goldblatt, D., and A. J. Thrasher. 2000. Chronic granulomatous disease. *Clin Exp Immunol* 122: 1-9.

Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3: 23-35.

Grau, G. E., P. F. Piguet, P. Vassalli, and P. H. Lambert. 1989. Tumor-necrosis factor and other cytokines in cerebral malaria: experimental and clinical data. *Immunol Rev* 112: 49-70.

Greenberg, S., P. Chang, D. C. Wang, R. Xavier, and B. Seed. 1996. Clustered syk tyrosine kinase domains trigger phagocytosis. *Proc Natl Acad Sci U S A* 93: 1103-7.

Greenberg, S., and S. Grinstein. 2002. Phagocytosis and innate immunity. *Curr Opin Immunol* 14: 136-45.

Grell, M., E. Douni, H. Wajant, M. Lohden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, and P. Scheurich. 1995. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 83: 793-802.

Guo, X., R. E. Gerl, and J. W. Schrader. 2003. Defining the involvement of p38alpha MAPK in the production of anti- and proinflammatory cytokines using an SB 203580-resistant form of the kinase. *J Biol Chem* 278: 22237-42.

Guo, Y. L., B. Kang, and J. R. Williamson. 1998. Inhibition of the expression of mitogen-activated protein phosphatase-1 potentiates apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. *J Biol Chem* 273: 10362-6.

Gupta, S. 2002. A decision between life and death during TNF-alpha-induced signaling. *J Clin Immunol* 22: 185-94.

Gupta, S., T. Barrett, A. J. Whitmarsh, J. Cavanagh, H. K. Sluss, B. Derijard, and R. J. Davis. 1996. Selective interaction of JNK protein kinase isoforms with transcription factors. *Embo J* 15: 2760-70.

Gyllenhammar, H. 1987. Lucigenin chemiluminescence in the assessment of neutrophil superoxide production. *J Immunol Methods* 97: 209-13.

Haddad, C. 1998. Characteristics of the intracellular signalling response induced by a tumor necrosis factor mimetic peptide *Department of Physiology*. University of Adelaide, Adelaide.

Haddad, E. B., M. Birrell, K. McCluskie, A. Ling, S. E. Webber, M. L. Foster, and M. G. Belvisi. 2001. Role of p38 MAP kinase in LPS-induced airway inflammation in the rat. *Br J Pharmacol* 132: 1715-24.

Hamamoto, K., Y. Kida, Y. Zhang, T. Shimizu, and K. Kuwano. 2002. Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions. *Microbiol Immunol* 46: 741-9.

Han, J., and B. Beutler. 1990. The essential role of the UA-rich sequence in endotoxin-induced cachectin/TNF synthesis. *Eur Cytokine Netw* 1: 71-5.

Haranaka, K., E. A. Carswell, B. D. Williamson, J. S. Prendergast, N. Satomi, and L. J. Old. 1986. Purification, characterization, and antitumor activity of

nonrecombinant mouse tumor necrosis factor. *Proc Natl Acad Sci U S A* 83: 3949-53.

Haraoka, M., L. Hang, B. Frendeus, G. Godaly, M. Burdick, R. Strieter, and C. Svanborg. 1999. Neutrophil recruitment and resistance to urinary tract infection. *J Infect Dis* 180: 1220-9.

Hardy, S. J., B. S. Robinson, A. Ferrante, C. S. Hii, D. W. Johnson, A. Poulos, and A. W. Murray. 1995. Polyenoic very-long-chain fatty acids mobilize intracellular calcium from a thapsigargin-insensitive pool in human neutrophils. The relationship between Ca2+ mobilization and superoxide production induced by long- and very-long-chain fatty acids. *Biochem J* 311 (Pt 2): 689-97.

Harrington, M. G. 1990. Elution of protein from gels. *Methods Enzymol* 182: 488-95.

Harris, E. D., Jr. 1990. Rheumatoid arthritis. Pathophysiology and implications for therapy. *N Engl J Med* 322: 1277-89.

Harris, S. G., J. Padilla, L. Koumas, D. Ray, and R. P. Phipps. 2002. Prostaglandins as modulators of immunity. *Trends Immunol* 23: 144-50.

Hart, P. H., R. L. Cooper, and J. J. Finlay-Jones. 1991. IL-4 suppresses IL-1 beta, TNF-alpha and PGE2 production by human peritoneal macrophages. *Immunology* 72: 344-9.

Hauser, T., K. Frei, R. M. Zinkernagel, and T. P. Leist. 1990. Role of tumor necrosis factor in Listeria resistance of nude mice. *Med Microbiol Immunol (Berl)* 179: 95-104.

Hayashi, F., T. K. Means, and A. D. Luster. 2003. Toll-like receptors stimulate human neutrophil function. *Blood* 102: 2660-9.

Helmy, K. Y., K. J. Katschke, Jr., N. N. Gorgani, N. M. Kljavin, J. M. Elliott, L. Diehl, S. J. Scales, N. Ghilardi, and M. van Lookeren Campagne. 2006. CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell* 124: 915-27.

Herbein, G., and K. A. Khan. 2008. Is HIV infection a TNF receptor signalling-driven disease? *Trends Immunol* 29: 61-7.

Herbein, G., and W. A. O'Brien. 2000. Tumor necrosis factor (TNF)-alpha and TNF receptors in viral pathogenesis. *Proc Soc Exp Biol Med* 223: 241-57.

Hetian, L., A. Ping, S. Shumei, L. Xiaoying, H. Luowen, W. Jian, M. Lin, L. Meisheng, Y. Junshan, and S. Chengchao. 2002. A novel peptide isolated from a phage display library inhibits tumor growth and metastasis by blocking the binding of vascular endothelial growth factor to its kinase domain receptor. *J Biol Chem* 277: 43137-42.

Heyworth, P. G., A. R. Cross, and J. T. Curnutte. 2003. Chronic granulomatous disease. *Curr Opin Immunol* 15: 578-84.

Heyworth, P. G., J. T. Curnutte, and J. A. Badwey. 1998. Structure and regulation of NADPH oxidase of phagocytic leukocytes: insights from chronic granulomatous disease in C. N. Serrhan and P. A. Ward, eds. *Molecular and Cellular Basis of Inflammation*. Humana Press Inc., Totowa, NJ, USA.

Hii, C. S., Z. H. Huang, A. Bilney, M. Costabile, A. W. Murray, D. A. Rathjen, C. J. Der, and A. Ferrante. 1998. Stimulation of p38 phosphorylation and activity by arachidonic acid in HeLa cells, HL60 promyelocytic leukemic cells, and human neutrophils. Evidence for cell type-specific activation of mitogen-activated protein kinases. *J Biol Chem* 273: 19277-82.

Hirata, Y., K. Adachi, and K. Kiuchi. 1998. Activation of JNK pathway and induction of apoptosis by manganese in PC12 cells. *J Neurochem* 71: 1607-15.

Hohlfeld, R. 1996. Inhibitors of tumor necrosis factor-alpha: promising agents for the treatment of multiple sclerosis? *Mult Scler* 1: 376-8.

Hohmann, H. P., R. Remy, M. Brockhaus, and A. P. van Loon. 1989. Two different cell types have different major receptors for human tumor necrosis factor (TNF alpha). *J Biol Chem* 264: 14927-34.

Hong, S. Y., J. E. Oh, and K. H. Lee. 1999. Effect of D-amino acid substitution on the stability, the secondary structure, and the activity of membrane-active peptide. *Biochem Pharmacol* 58: 1775-80.

Hume, D. A. 2006. The mononuclear phagocyte system. *Curr Opin Immunol* 18: 49-53.

Hume, D. A., I. L. Ross, S. R. Himes, R. T. Sasmono, C. A. Wells, and T. Ravasi. 2002. The mononuclear phagocyte system revisited. *J Leukoc Biol* 72: 621-7.

Hunt, N. H., and G. E. Grau. 2003. Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends Immunol* 24: 491-9.

Hwang, C. D., M. Gatanaga, E. K. Innins, R. S. Yamamoto, G. A. Granger, and T. Gatanaga. 1991. A 20 amino acid synthetic peptide of a region from the 55 kDa human TNF receptor inhibits cytolytic and binding activities of recombinant human tumour necrosis factor in vitro. *Proc Biol Sci* 245: 115-9.

Ipaktchi, K., A. Mattar, A. D. Niederbichler, L. M. Hoesel, M. R. Hemmila, G. L. Su, D. G. Remick, S. C. Wang, and S. Arbabi. 2006. Topical p38MAPK inhibition reduces dermal inflammation and epithelial apoptosis in burn wounds. *Shock* 26: 201-9.

Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 52: 2745-56.

Jersmann, H. P., C. S. Hii, J. V. Ferrante, and A. Ferrante. 2001. Bacterial lipopolysaccharide and tumor necrosis factor alpha synergistically increase expression of human endothelial adhesion molecules through activation of NF-kappaB and p38 mitogen-activated protein kinase signaling pathways. *Infect Immun* 69: 1273-9.

Jupin, C., M. Parant, and L. Chedid. 1989. Involvement of reactive oxygen metabolites in the candidacidal activity of human neutrophils stimulated by muramyl dipeptide or tumor necrosis factor. *Immunobiology* 180: 68-79.

Jurewicz, A. M., A. K. Walczak, and K. W. Selmaj. 1999. Shedding of TNF receptors in multiple sclerosis patients. *Neurology* 53: 1409-14.

Kapadia, S., G. Torre-Amione, T. Yokoyama, and D. L. Mann. 1995. Soluble TNF binding proteins modulate the negative inotropic properties of TNF-alpha in vitro. *Am J Physiol* 268: H517-25.

Kapadia, S. R., H. Oral, J. Lee, M. Nakano, G. E. Taffet, and D. L. Mann. 1997. Hemodynamic regulation of tumor necrosis factor-alpha gene and protein expression in adult feline myocardium. *Circ Res* 81: 187-95.

Karin, M. 1998. Mitogen-activated protein kinase cascades as regulators of stress responses. *Ann N Y Acad Sci* 851: 139-46.

—. 2006. Nuclear factor-kappaB in cancer development and progression. *Nature* 441: 431-6.

Karin, M., and A. Lin. 2002. NF-kappaB at the crossroads of life and death. *Nat Immunol* 3: 221-7.

Kasama, T., Y. Miwa, T. Isozaki, T. Odai, M. Adachi, and S. L. Kunkel. 2005. Neutrophil-derived cytokines: potential therapeutic targets in inflammation. *Curr Drug Targets Inflamm Allergy* 4: 273-9.

Katz, S. D., R. Rao, J. W. Berman, M. Schwarz, L. Demopoulos, R. Bijou, and T. H. LeJemtel. 1994. Pathophysiological correlates of increased serum tumor necrosis factor in patients with congestive heart failure. Relation to nitric oxide-dependent vasodilation in the forearm circulation. *Circulation* 90: 12-6.

Kawashima, Y., I. Takeyoshi, Y. Otani, Y. Koibuchi, D. Yoshinari, T. Koyama, M. Kobayashi, K. Matsumoto, and Y. Morishita. 2001. FR167653 attenuates ischemia and reperfusion injury of the rat lung with suppressing p38 mitogen-activated protein kinase. *J Heart Lung Transplant* 20: 568-74.

Keane, J. 2005. TNF-blocking agents and tuberculosis: new drugs illuminate an old topic. *Rheumatology (Oxford)* 44: 714-20.

Kee, T. H., P. Vit, and A. J. Melendez. 2005. Sphingosine kinase signalling in immune cells. *Clin Exp Pharmacol Physiol* 32: 153-61.

Kelliher, M. A., S. Grimm, Y. Ishida, F. Kuo, B. Z. Stanger, and P. Leder. 1998. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity* 8: 297-303.

Keshavarzian, A., R. D. Fusunyan, M. Jacyno, D. Winship, R. P. MacDermott, and I. R. Sanderson. 1999. Increased interleukin-8 (IL-8) in rectal dialysate from patients with ulcerative colitis: evidence for a biological role for IL-8 in inflammation of the colon. *Am J Gastroenterol* 94: 704-12.

Kmiec, Z. 1998. Cytokines in inflammatory bowel disease. *Arch Immunol Ther Exp (Warsz)* 46: 143-55.

Kolch, W. 2002. Ras/Raf signalling and emerging pharmacotherapeutic targets. *Expert Opin Pharmacother* 3: 709-18.

Kowanko, I. C., and A. Ferrante. 1996. Adhesion and TNF priming in neutrophil-mediated cartilage damage. *Clin Immunol Immunopathol* 79: 36-42.

Kozik, D. J., and J. S. Tweddell. 2006. Characterizing the inflammatory response to cardiopulmonary bypass in children. *Ann Thorac Surg* 81: S2347-54.

Kriegler, M., C. Perez, K. DeFay, I. Albert, and S. D. Lu. 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell* 53: 45-53.

Kronke, M. 1999. Involvement of sphingomyelinases in TNF signaling pathways. *Chem Phys Lipids* 102: 157-66.

Kruppa, G., B. Thoma, T. Machleidt, K. Wiegmann, and M. Kronke. 1992. Inhibition of tumor necrosis factor (TNF)-mediated NF-kappa B activation by selective blockade of the human 55-kDa TNF receptor. *J Immunol* 148: 3152-7.

Kubes, P., I. Kurose, and D. N. Granger. 1994. NO donors prevent integrin-induced leukocyte adhesion but not P-selectin-dependent rolling in postischemic venules. *Am J Physiol* 267: H931-7.

Kubota, T., G. S. Bounoutas, M. Miyagishima, T. Kadokami, V. J. Sanders, C. Bruton, P. D. Robbins, C. F. McTiernan, and A. M. Feldman. 2000. Soluble tumor necrosis factor receptor abrogates myocardial inflammation but not hypertrophy in cytokine-induced cardiomyopathy. *Circulation* 101: 2518-25.

Kuma, Y., G. Sabio, J. Bain, N. Shpiro, R. Marquez, and A. Cuenda. 2005. BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *J Biol Chem* 280: 19472-9.

Kumar, S., J. Boehm, and J. C. Lee. 2003. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov* 2: 717-26.

Kumaratilake, L. M., and A. Ferrante. 1988. Purification of human monocytes/macrophages by adherence to cytodex microcarriers. *J Immunol Methods* 112: 183-90.

—. 1992. IL-4 inhibits macrophage-mediated killing of Plasmodium falciparum in vitro. A possible parasite-immune evasion mechanism. *J Immunol* 149: 194-9.

Kumaratilake, L. M., D. A. Rathjen, P. Mack, F. Widmer, V. Prasertsiriroj, and A. Ferrante. 1995. A synthetic tumor necrosis factor-alpha agonist peptide enhances human polymorphonuclear leukocyte-mediated killing of Plasmodium falciparum in vitro and suppresses Plasmodium chabaudi infection in mice. *J Clin Invest* 95: 2315-23.

Kurien, B. T., and R. H. Scofield. 2008. Autoimmunity and oxidatively modified autoantigens. *Autoimmun Rev* 7: 567-73.

Kurrelmeyer, K. M., L. H. Michael, G. Baumgarten, G. E. Taffet, J. J. Peschon, N. Sivasubramanian, M. L. Entman, and D. L. Mann. 2000. Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction. *Proc Natl Acad Sci U S A* 97: 5456-61.

Kwiatkowski, D., M. E. Molyneux, S. Stephens, N. Curtis, N. Klein, P. Pointaire, M. Smit, R. Allan, D. R. Brewster, G. E. Grau, and et al. 1993. Anti-TNF therapy inhibits fever in cerebral malaria. *Q J Med* 86: 91-8.

Kwon, H. J., T. R. Cote, M. S. Cuffe, J. M. Kramer, and M. M. Braun. 2003. Case reports of heart failure after therapy with a tumor necrosis factor antagonist. *Ann Intern Med* 138: 807-11.

Kyriakis, J. M., and J. Avruch. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 81: 807-69.

Ladner, R. C., A. K. Sato, J. Gorzelany, and M. de Souza. 2004. Phage display-derived peptides as therapeutic alternatives to antibodies. *Drug Discov Today* 9: 525-9.

LaDuca, J. R., and A. A. Gaspari. 2001. Targeting tumor necrosis factor alpha. New drugs used to modulate inflammatory diseases. *Dermatol Clin* 19: 617-35.

Le, Y., P. M. Murphy, and J. M. Wang. 2002. Formyl-peptide receptors revisited. *Trends Immunol* 23: 541-8.

Lee, C. S., K. H. Chen, and P. C. Wang. 1997. Soluble tumor necrosis factor receptor in serum of patients with arthritis. *J Formos Med Assoc* 96: 573-8.

Lee, J. C., S. Kumar, D. E. Griswold, D. C. Underwood, B. J. Votta, and J. L. Adams. 2000. Inhibition of p38 MAP kinase as a therapeutic strategy. *Immunopharmacology* 47: 185-201.

Lee, J. C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, and et al. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 372: 739-46.

Lefkowitz, D. L., and S. S. Lefkowitz. 2001. Macrophage-neutrophil interaction: a paradigm for chronic inflammation revisited. *Immunol Cell Biol* 79: 502-6.

Lei, K., and R. J. Davis. 2003. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc Natl Acad Sci U S A* 100: 2432-7.

Ley, K. 2002. Integration of inflammatory signals by rolling neutrophils. *Immunol Rev* 186: 8-18.

Li, L., J. F. Elliott, and T. R. Mosmann. 1994. IL-10 inhibits cytokine production, vascular leakage, and swelling during T helper 1 cell-induced delayed-type hypersensitivity. *J Immunol* 153: 3967-78.

Li, Z. W., W. Chu, Y. Hu, M. Delhase, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin. 1999. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med* 189: 1839-45.

Lie, B. L., D. Tunemoto, H. Hemmi, Y. Mizukami, H. Fukuda, H. Kikuchi, S. Kato, and N. Numao. 1992. Identification of the binding site of 55kDa tumor necrosis factor receptor by synthetic peptides. *Biochem Biophys Res Commun* 188: 503-9.

Lien, S., and H. B. Lowman. 2003. Therapeutic peptides. *Trends Biotechnol* 21: 556-62.

Lindemann, S. W., C. C. Yost, M. M. Denis, T. M. McIntyre, A. S. Weyrich, and G. A. Zimmerman. 2004. Neutrophils alter the inflammatory milieu by signal-dependent translation of constitutive messenger RNAs. *Proc Natl Acad Sci U S A* 101: 7076-81.

Listing, J., A. Strangfeld, J. Kekow, M. Schneider, A. Kapelle, S. Wassenberg, and A. Zink. 2008. Does tumor necrosis factor alpha inhibition promote or prevent heart failure in patients with rheumatoid arthritis? *Arthritis Rheum* 58: 667-77.

Liu, J., and A. Lin. 2005. Role of JNK activation in apoptosis: a double-edged sword. *Cell Res* 15: 36-42.

Liu, P., and R. G. Anderson. 1995. Compartmentalized production of ceramide at the cell surface. *J Biol Chem* 270: 27179-85.

Liu, Z. G., and J. Han. 2001. Cellular responses to tumor necrosis factor. *Curr Issues Mol Biol* 3: 79-90.

Liu, Z. G., H. Hsu, D. V. Goeddel, and M. Karin. 1996. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. *Cell* 87: 565-76.

Locksley, R. M., N. Killeen, and M. J. Lenardo. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104: 487-501.

Lou, J., R. Lucas, and G. E. Grau. 2001. Pathogenesis of cerebral malaria: recent experimental data and possible applications for humans. *Clin Microbiol Rev* 14: 810-20, table of contents.

Lucas, R., J. N. Lou, P. Juillard, M. Moore, H. Bluethmann, and G. E. Grau. 1997. Respective role of TNF receptors in the development of experimental cerebral malaria. *J Neuroimmunol* 72: 143-8.

Ludwig, I. S., T. B. Geijtenbeek, and Y. van Kooyk. 2006. Two way communication between neutrophils and dendritic cells. *Curr Opin Pharmacol* 6: 408-13.

MacEwan, D. J. 2002. TNF receptor subtype signalling: differences and cellular consequences. *Cell Signal* 14: 477-92.

MacKinnon, A. C., A. Buckley, E. R. Chilvers, A. G. Rossi, C. Haslett, and T. Sethi. 2002. Sphingosine kinase: a point of convergence in the action of diverse neutrophil priming agents. *J Immunol* 169: 6394-400.

Maganto-Garcia, E., C. Punzon, C. Terhorst, and M. Fresno. 2008. Rab5 activation by Toll-like receptor 2 is required for Trypanosoma cruzi internalization and replication in macrophages. *Traffic* 9: 1299-315.

Mann, D. L. 2003. Stress-activated cytokines and the heart: from adaptation to maladaptation. *Annu Rev Physiol* 65: 81-101.

Mann, D. L., J. J. McMurray, M. Packer, K. Swedberg, J. S. Borer, W. S. Colucci, J. Djian, H. Drexler, A. Feldman, L. Kober, H. Krum, P. Liu, M. Nieminen, L. Tavazzi, D. J. van Veldhuisen, A. Waldenstrom, M. Warren, A. Westheim, F. Zannad, and T. Fleming. 2004. Targeted anticytokine therapy in patients with chronic heart failure: results of the Randomized Etanercept Worldwide Evaluation (RENEWAL). *Circulation* 109: 1594-602.

Manna, S. K., and B. B. Aggarwal. 1998. IL-13 suppresses TNF-induced activation of nuclear factor-kappa B, activation protein-1, and apoptosis. *J Immunol* 161: 2863-72.

Manning, A. M., and R. J. Davis. 2003. Targeting JNK for therapeutic benefit: from junk to gold? *Nat Rev Drug Discov* 2: 554-65.

Massol, P., P. Montcourrier, J. C. Guillemot, and P. Chavrier. 1998. Fc receptor-mediated phagocytosis requires CDC42 and Rac1. *Embo J* 17: 6219-29.

Mathias, S., K. A. Dressler, and R. N. Kolesnick. 1991. Characterization of a ceramide-activated protein kinase: stimulation by tumor necrosis factor alpha. *Proc Natl Acad Sci U S A* 88: 10009-13.

Mazzon, E., E. Esposito, R. Di Paola, C. Muia, C. Crisafulli, T. Genovese, R. Caminiti, R. Meli, P. Bramanti, and S. Cuzzocrea. 2008. Effect of tumour necrosis factor-

alpha receptor 1 genetic deletion on carrageenan-induced acute inflammation: a comparison with etanercept. *Clin Exp Immunol* 153: 136-49.

McLay, L. M., F. Halley, J. E. Souness, J. McKenna, V. Benning, M. Birrell, B. Burton, M. Belvisi, A. Collis, A. Constan, M. Foster, D. Hele, Z. Jayyosi, M. Kelley, C. Maslen, G. Miller, M. C. Ouldelhkim, K. Page, S. Phipps, K. Pollock, B. Porter, A. J. Ratcliffe, E. J. Redford, S. Webber, B. Slater, V. Thybaud, and N. Wilsher. 2001. The discovery of RPR 200765A, a p38 MAP kinase inhibitor displaying a good oral anti-arthritic efficacy. *Bioorg Med Chem* 9: 537-54.

Medzhitov, R., and C. A. Janeway, Jr. 1997. Innate immunity: impact on the adaptive immune response. *Curr Opin Immunol* 9: 4-9.

Megiovanni, A. M., F. Sanchez, M. Robledo-Sarmiento, C. Morel, J. C. Gluckman, and S. Boudaly. 2006. Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J Leukoc Biol* 79: 977-88.

Melendez, A., R. A. Floto, D. J. Gillooly, M. M. Harnett, and J. M. Allen. 1998. FcgammaRI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. *J Biol Chem* 273: 9393-402.

Melendez, A. J. 2008. Sphingosine kinase signalling in immune cells: potential as novel therapeutic targets. *Biochim Biophys Acta* 1784: 66-75.

Mellman, I., T. Koch, G. Healey, W. Hunziker, V. Lewis, H. Plutner, H. Miettinen, D. Vaux, K. Moore, and S. Stuart. 1988. Structure and function of Fc receptors on macrophages and lymphocytes. *J Cell Sci Suppl* 9: 45-65.

Memon, R. A., K. R. Feingold, A. H. Moser, J. Fuller, and C. Grunfeld. 1998. Regulation of fatty acid transport protein and fatty acid translocase mRNA levels by endotoxin and cytokines. *Am J Physiol* 274: E210-7.

Mestan, J., W. Digel, S. Mittnacht, H. Hillen, D. Blohm, A. Moller, H. Jacobsen, and H. Kirchner. 1986. Antiviral effects of recombinant tumour necrosis factor in vitro. *Nature* 323: 816-9.

Michaud, J., M. Kohno, R. L. Proia, and T. Hla. 2006. Normal acute and chronic inflammatory responses in sphingosine kinase 1 knockout mice. *FEBS Lett* 580: 4607-12.

Ming, X. F., G. Stoecklin, M. Lu, R. Looser, and C. Moroni. 2001. Parallel and independent regulation of interleukin-3 mRNA turnover by phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase. *Mol Cell Biol* 21: 5778-89.

Miwatashi, S., Y. Arikawa, E. Kotani, M. Miyamoto, K. Naruo, H. Kimura, T. Tanaka, S. Asahi, and S. Ohkawa. 2005. Novel inhibitor of p38 MAP kinase as an anti-TNF-alpha drug: discovery of N-[4-[2-ethyl-4-(3-methylphenyl)-1,3-thiazol-5-yl]-2-pyridyl]benzamide (TAK-715) as a potent and orally active anti-rheumatoid arthritis agent. *J Med Chem* 48: 5966-79.

Modur, V., G. A. Zimmerman, S. M. Prescott, and T. M. McIntyre. 1996. Endothelial cell inflammatory responses to tumor necrosis factor alpha. Ceramide-dependent and -independent mitogen-activated protein kinase cascades. *J Biol Chem* 271: 13094-102.

Moghaddami, N., M. Costabile, P. K. Grover, H. P. Jersmann, Z. H. Huang, C. S. Hii, and A. Ferrante. 2003. Unique effect of arachidonic acid on human neutrophil

TNF receptor expression: up-regulation involving protein kinase C, extracellular signal-regulated kinase, and phospholipase A2. *J Immunol* 171: 2616-24.

Molfino, N. A. 2005. Drugs in clinical development for chronic obstructive pulmonary disease. *Respiration* 72: 105-12.

Moller, A. S., R. Ovstebo, K. B. Haug, G. B. Joo, A. B. Westvik, and P. Kierulf. 2005. Chemokine production and pattern recognition receptor (PRR) expression in whole blood stimulated with pathogen-associated molecular patterns (PAMPs). *Cytokine* 32: 304-15.

Morgan, B. P., and C. L. Harris. 2003. Complement therapeutics; history and current progress. *Mol Immunol* 40: 159-70.

Moss, M. L., S. L. Jin, J. D. Becherer, D. M. Bickett, W. Burkhart, W. J. Chen, D. Hassler, M. T. Leesnitzer, G. McGeehan, M. Milla, M. Moyer, W. Rocque, T. Seaton, F. Schoenen, J. Warner, and D. Willard. 1997. Structural features and biochemical properties of TNF-alpha converting enzyme (TACE). *J Neuroimmunol* 72: 127-9.

Mustafa, A. S., A. M. El-Shamy, N. M. Madi, H. A. Amoudy, and R. Al-Attiyah. 2008. Cell-mediated immune responses to complex and single mycobacterial antigens in tuberculosis patients with diabetes. *Med Princ Pract* 17: 325-30.

Nakane, A., T. Minagawa, and K. Kato. 1988. Endogenous tumor necrosis factor (cachectin) is essential to host resistance against Listeria monocytogenes infection. *Infect Immun* 56: 2563-9.

Nakano, M., A. A. Knowlton, Z. Dibbs, and D. L. Mann. 1998. Tumor necrosis factor-alpha confers resistance to hypoxic injury in the adult mammalian cardiac myocyte. *Circulation* 97: 1392-400.

Nambi, V. 2005. The use of myeloperoxidase as a risk marker for atherosclerosis. *Curr Atheroscler Rep* 7: 127-31.

Nathan, C. 2006. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 6: 173-82.

Nathan, C. F. 1987. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *J Clin Invest* 80: 1550-60.

Nauta, A. J., A. Roos, and M. R. Daha. 2004. A regulatory role for complement in innate immunity and autoimmunity. *Int Arch Allergy Immunol* 134: 310-23.

Nguyen, J., J. Gogusev, P. Knapnougel, and B. Bauvois. 2006. Protein tyrosine kinase and p38 MAP kinase pathways are involved in stimulation of matrix metalloproteinase-9 by TNF-alpha in human monocytes. *Immunol Lett* 106: 34-41.

Niederberger, E., and G. Geisslinger. 2008. The IKK-NF-{kappa}B pathway: a source for novel molecular drug targets in pain therapy? *Faseb J*.

Nikas, S. N., and A. A. Drosos. 2004. SCIO-469 Scios Inc. *Curr Opin Investig Drugs* 5: 1205-12.

Nishikori, T., K. Irie, T. Suganuma, M. Ozaki, and T. Yoshioka. 2002. Anti-inflammatory potency of FR167653, a p38 mitogen-activated protein kinase inhibitor, in mouse models of acute inflammation. *Eur J Pharmacol* 451: 327-33.

Nishina, H., C. Vaz, P. Billia, M. Nghiem, T. Sasaki, J. L. De la Pompa, K. Furlonger, C. Paige, C. Hui, K. D. Fischer, H. Kishimoto, T. Iwatsubo, T. Katada, J. R. Woodgett, and J. M. Penninger. 1999. Defective liver formation and liver cell

apoptosis in mice lacking the stress signaling kinase SEK1/MKK4. *Development* 126: 505-16.

Niwa, M., O. Kozawa, H. Matsuno, Y. Kanamori, A. Hara, and T. Uematsu. 2000. Tumor necrosis factor-alpha-mediated signal transduction in human neutrophils: involvement of sphingomyelin metabolites in the priming effect of TNF-alpha on the fMLP-stimulated superoxide production. *Life Sci* 66: 245-56.

Nozaki, A., M. Ikeda, A. Naganuma, T. Nakamura, M. Inudoh, K. Tanaka, and N. Kato. 2003. Identification of a lactoferrin-derived peptide possessing binding activity to hepatitis C virus E2 envelope protein. *J Biol Chem* 278: 10162-73.

Nozaki, N., S. Yamaguchi, M. Shirakabe, H. Nakamura, and H. Tomoike. 1997. Soluble tumor necrosis factor receptors are elevated in relation to severity of congestive heart failure. *Jpn Circ J* 61: 657-64.

Nutchey, B. K., J. S. Kaplan, P. P. Dwivedi, J. L. Omdahl, A. Ferrante, B. K. May, and C. S. Hii. 2005. Molecular action of 1,25-dihydroxyvitamin D 3 and phorbol ester on the activation of the rat cytochrome P450C24 (CYP24)promoter: role of MAP kinase activities and identification of an important transcription factor binding site. *Biochem J*.

O'Toole, A., S. K. Moule, P. J. Lockyer, and A. P. Halestrap. 2001. Tumour necrosis factor-alpha activation of protein kinase B in WEHI-164 cells is accompanied by increased phosphorylation of Ser473, but not Thr308. *Biochem J* 359: 119-27.

Ogilvie, A. L., C. Antoni, C. Dechant, B. Manger, J. R. Kalden, G. Schuler, and M. Luftl. 2001. Treatment of psoriatic arthritis with antitumour necrosis factor-alpha antibody clears skin lesions of psoriasis resistant to treatment with methotrexate. *Br J Dermatol* 144: 587-9.

Ohl, M. E., and S. I. Miller. 2001. Salmonella: a model for bacterial pathogenesis. *Annu Rev Med* 52: 259-74.

Ohnuma, T., L. Szrajer, J. F. Holland, M. Kurimoto, and J. Minowada. 1993. Effects of natural interferon alpha, natural tumor necrosis factor alpha and their combination on human mesothelioma xenografts in nude mice. *Cancer Immunol Immunother* 36: 31-6.

Olleros, M. L., R. Guler, N. Corazza, D. Vesin, H. P. Eugster, G. Marchal, P. Chavarot, C. Mueller, and I. Garcia. 2002. Transmembrane TNF induces an efficient cell-mediated immunity and resistance to Mycobacterium bovis bacillus Calmette-Guerin infection in the absence of secreted TNF and lymphotoxin-alpha. *J Immunol* 168: 3394-401.

Ono, K., and J. Han. 2000. The p38 signal transduction pathway: activation and function. *Cell Signal* 12: 1-13.

Orr, Y., J. M. Taylor, S. Cartland, P. G. Bannon, C. Geczy, and L. Kritharides. 2007. Conformational activation of CD11b without shedding of L-selectin on circulating human neutrophils. *J Leukoc Biol* 82: 1115-25.

Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 401: 82-5.

Pai, R. K., M. Convery, T. A. Hamilton, W. H. Boom, and C. V. Harding. 2003. Inhibition of IFN-gamma-induced class II transactivator expression by a 19-kDa lipoprotein from Mycobacterium tuberculosis: a potential mechanism for immune evasion. *J Immunol* 171: 175-84.

Palladino, M. A., F. R. Bahjat, E. A. Theodorakis, and L. L. Moldawer. 2003. Anti-TNF-alpha therapies: the next generation. *Nat Rev Drug Discov* 2: 736-46.

Pearson, G., F. Robinson, T. Beers Gibson, B. E. Xu, M. Karandikar, K. Berman, and M. H. Cobb. 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 22: 153-83.

Peschon, J. J., D. S. Torrance, K. L. Stocking, M. B. Glaccum, C. Otten, C. R. Willis, K. Charrier, P. J. Morrissey, C. B. Ware, and K. M. Mohler. 1998. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J Immunol* 160: 943-52.

Pfeffer, K., T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P. S. Ohashi, M. Kronke, and T. W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell* 73: 457-67.

Piguet, P. F., G. E. Grau, C. Hauser, and P. Vassalli. 1991. Tumor necrosis factor is a critical mediator in hapten induced irritant and contact hypersensitivity reactions. *J Exp Med* 173: 673-9.

Piguet, P. F., G. E. Grau, C. Vesin, H. Loetscher, R. Gentz, and W. Lesslauer. 1992. Evolution of collagen arthritis in mice is arrested by treatment with anti-tumour necrosis factor (TNF) antibody or a recombinant soluble TNF receptor. *Immunology* 77: 510-4.

Pimentel-Muinos, F. X., and B. Seed. 1999. Regulated commitment of TNF receptor signaling: a molecular switch for death or activation. *Immunity* 11: 783-93.

Pober, J. S. 2002. Endothelial activation: intracellular signaling pathways. *Arthritis Res* 4 Suppl 3: S109-16.

Pohlman, T. H., and J. M. Harlan. 1989. Human endothelial cell response to lipopolysaccharide, interleukin-1, and tumor necrosis factor is regulated by protein synthesis. *Cell Immunol* 119: 41-52.

Posadas, I., M. Bucci, F. Roviezzo, A. Rossi, L. Parente, L. Sautebin, and G. Cirino. 2004. Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. *Br J Pharmacol* 142: 331-8.

Puellmann, K., W. E. Kaminski, M. Vogel, C. T. Nebe, J. Schroeder, H. Wolf, and A. W. Beham. 2006. A variable immunoreceptor in a subpopulation of human neutrophils. *Proc Natl Acad Sci U S A* 103: 14441-6.

Pushkareva, M., L. M. Obeid, and Y. A. Hannun. 1995. Ceramide: an endogenous regulator of apoptosis and growth suppression. *Immunol Today* 16: 294-7.

Rahman, M. M., and G. McFadden. 2006. Modulation of tumor necrosis factor by microbial pathogens. *PLoS Pathog* 2: e4.

Rajasingh, J., E. Bord, C. Luedemann, J. Asai, H. Hamada, T. Thorne, G. Qin, D. Goukassian, Y. Zhu, D. W. Losordo, and R. Kishore. 2006. IL-10-induced TNF-alpha mRNA destabilization is mediated via IL-10 suppression of p38 MAP kinase activation and inhibition of HuR expression. *Faseb J* 20: 2112-4.

Rathjen, D., D. Parsons, and A. Ferrante. Resolution of murine Pseudomonas aeruginosa lung infection by enhancement of bacterial clearance and neutrophil function by a non-toxic synthetic TNF alpha peptide.

Rathjen, D. A., K. Cowan, L. J. Furphy, and R. Aston. 1991. Antigenic structure of human tumour necrosis factor: recognition of distinct regions of TNF alpha by different tumour cell receptors. *Mol Immunol* 28: 79-86.

Rathjen, D. A., A. Ferrante, and R. Aston. 1993. Differential effects of small tumour necrosis factor-alpha peptides on tumour cell cytotoxicity, neutrophil activation and endothelial cell procoagulant activity. *Immunology* 80: 293-9.

Read, M. A., M. Z. Whitley, S. Gupta, J. W. Pierce, J. Best, R. J. Davis, and T. Collins. 1997. Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. *J Biol Chem* 272: 2753-61.

Reeves, E. P., H. Lu, H. L. Jacobs, C. G. Messina, S. Bolsover, G. Gabella, E. O. Potma, A. Warley, J. Roes, and A. W. Segal. 2002. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature* 416: 291-7.

Reinhard, C., B. Shamoon, V. Shyamala, and L. T. Williams. 1997. Tumor necrosis factor alpha-induced activation of c-jun N-terminal kinase is mediated by TRAF2. *Embo J* 16: 1080-92.

Roach, D. R., A. G. Bean, C. Demangel, M. P. France, H. Briscoe, and W. J. Britton. 2002. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. *J Immunol* 168: 4620-7.

Robinson, B. S., C. S. Hii, A. Poulos, and A. Ferrante. 1996. Effect of tumor necrosis factor-alpha on the metabolism of arachidonic acid in human neutrophils. *J Lipid Res* 37: 1234-45.

Rocha, A. C., E. S. Fernandes, N. L. Quintao, M. M. Campos, and J. B. Calixto. 2006. Relevance of tumour necrosis factor-alpha for the inflammatory and nociceptive responses evoked by carrageenan in the mouse paw. *Br J Pharmacol* 148: 688-95.

Roilides, E., A. Dimitriadou-Georgiadou, T. Sein, I. Kadiltsoglou, and T. J. Walsh. 1998. Tumor necrosis factor alpha enhances antifungal activities of polymorphonuclear and mononuclear phagocytes against Aspergillus fumigatus. *Infect Immun* 66: 5999-6003.

Romagnani, S. 2005. Cytokines. Pages 273-299 in S. Kaufmann and M. Steward, eds. *Topley and Wilson Microbiology and Microbial Infections (Immunology Volume)*. Edward Arnold Ltd, London.

Roos, D., M. de Boer, F. Kuribayashi, C. Meischl, R. S. Weening, A. W. Segal, A. Ahlin, K. Nemet, J. P. Hossle, E. Bernatowska-Matuszkiewicz, and H. Middleton-Price. 1996. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. *Blood* 87: 1663-81.

Rose, T., J. L. Moreau, R. Eckenberg, and J. Theze. 2003. Structural analysis and modeling of a synthetic interleukin-2 mimetic and its interleukin-2Rbeta2 receptor. *J Biol Chem* 278: 22868-76.

Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362: 801-9.

Rothe, J., W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Kontgen, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann. 1993. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. *Nature* 364: 798-802.

Roulston, A., C. Reinhard, P. Amiri, and L. T. Williams. 1998. Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. *J Biol Chem* 273: 10232-9.

Sabroe, I., E. C. Jones, M. K. Whyte, and S. K. Dower. 2005. Regulation of human neutrophil chemokine receptor expression and function by activation of Toll-like receptors 2 and 4. *Immunology* 115: 90-8.

Saccani, S., S. Pantano, and G. Natoli. 2002. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat Immunol* 3: 69-75.

Saito, H., T. Kojima, M. Takahashi, W. C. Horne, R. Baron, T. Amagasa, K. Ohya, and K. Aoki. 2007. A tumor necrosis factor receptor loop peptide mimic inhibits bone destruction to the same extent as anti-tumor necrosis factor monoclonal antibody in murine collagen-induced arthritis. *Arthritis Rheum* 56: 1164-74.

Saklatvala, J. 2004. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Curr Opin Pharmacol* 4: 372-7.

Salaun, B., P. Romero, and S. Lebecque. 2007. Toll-like receptors' two-edged sword: when immunity meets apoptosis. *Eur J Immunol* 37: 3311-8.

Salvemini, D., Z. Q. Wang, P. S. Wyatt, D. M. Bourdon, M. H. Marino, P. T. Manning, and M. G. Currie. 1996. Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br J Pharmacol* 118: 829-38.

Sarzi-Puttini, P., F. Atzeni, A. Doria, L. Iaccarino, and M. Turiel. 2005. Tumor necrosis factor-alpha, biologic agents and cardiovascular risk. *Lupus* 14: 780-4.

Sato, A., and S. Sone. 2003. A peptide mimetic of human interferon (IFN)-beta. *Biochem J* 371: 603-8.

Schindler, J. F., J. B. Monahan, and W. G. Smith. 2007. p38 pathway kinases as anti-inflammatory drug targets. *J Dent Res* 86: 800-11.

Schmid-Schonbein, G. W. 2006. Analysis of inflammation. *Annu Rev Biomed Eng* 8: 93-131.

Schneider, K., K. G. Potter, and C. F. Ware. 2004. Lymphotoxin and LIGHT signaling pathways and target genes. *Immunol Rev* 202: 49-66.

Schottelius, A. J., L. L. Moldawer, C. A. Dinarello, K. Asadullah, W. Sterry, and C. K. Edwards, 3rd. 2004. Biology of tumor necrosis factor-alpha- implications for psoriasis. *Exp Dermatol* 13: 193-222.

Schwandner, R., K. Wiegmann, K. Bernardo, D. Kreder, and M. Kronke. 1998. TNF receptor death domain-associated proteins TRADD and FADD signal activation of acid sphingomyelinase. *J Biol Chem* 273: 5916-22.

Schwinger, W., V. Klass, M. Benesch, H. Lackner, H. J. Dornbusch, P. Sovinz, A. Moser, G. Schwantzer, and C. Urban. 2005. Feasibility of high-dose interleukin-2 in heavily pretreated pediatric cancer patients. *Ann Oncol*.

Sedmak, J. J., P. Jameson, and S. E. Grossberg. 1981. Procedures for stabilization of interferons. *Methods Enzymol* 78: 591-5.

Seger, R., and E. G. Krebs. 1995. The MAPK signaling cascade. *Faseb J* 9: 726-35.

Sekut, L., and K. Connolly. 1998. AntiTNF-alpha agents in the treatment of inflammation. *Expert Opin Investig Drugs* 7: 1825-39.

Sekut, L., J. A. Menius, Jr., M. F. Brackeen, and K. M. Connolly. 1994. Evaluation of the significance of elevated levels of systemic and localized tumor necrosis factor in different animal models of inflammation. *J Lab Clin Med* 124: 813-20.

Sethi, J. K., and G. S. Hotamisligil. 1999. The role of TNF alpha in adipocyte metabolism. *Semin Cell Dev Biol* 10: 19-29.

Sewell, K. L., and D. E. Trentham. 1993. Pathogenesis of rheumatoid arthritis. *Lancet* 341: 283-6.

Shaik, Z. P., E. K. Fifer, and G. Nowak. 2007. Protein kinase B/Akt modulates nephrotoxicant-induced necrosis in renal cells. *Am J Physiol Renal Physiol* 292: F292-303.

Shaw, G., and R. Kamen. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 46: 659-67.

Sim, R. B., and S. A. Tsiftsoglou. 2004. Proteases of the complement system. *Biochem Soc Trans* 32: 21-7.

Skerrett, S. J., G. J. Bagby, R. A. Schmidt, and S. Nelson. 1997. Antibody-mediated depletion of tumor necrosis factor-alpha impairs pulmonary host defenses to Legionella pneumophila. *J Infect Dis* 176: 1019-28.

Skerrett, S. J., T. R. Martin, E. Y. Chi, J. J. Peschon, K. M. Mohler, and C. B. Wilson. 1999. Role of the type 1 TNF receptor in lung inflammation after inhalation of endotoxin or Pseudomonas aeruginosa. *Am J Physiol* 276: L715-27.

Smith, J. A. 1994. Neutrophils, host defense, and inflammation: a double-edged sword. *J Leukoc Biol* 56: 672-86.

Soler, C., R. Valdes, J. Garcia-Manteiga, J. Xaus, M. Comalada, F. J. Casado, M. Modolell, B. Nicholson, C. MacLeod, A. Felipe, A. Celada, and M. Pastor-Anglada. 2001. Lipopolysaccharide-induced apoptosis of macrophages determines the up-regulation of concentrative nucleoside transporters Cnt1 and Cnt2 through tumor necrosis factor-alpha-dependent and -independent mechanisms. *J Biol Chem* 276: 30043-9.

Spiegel, S., D. Foster, and R. Kolesnick. 1996. Signal transduction through lipid second messengers. *Curr Opin Cell Biol* 8: 159-67.

Spriggs, D. R., M. L. Sherman, H. Michie, K. A. Arthur, K. Imamura, D. Wilmore, E. Frei, 3rd, and D. W. Kufe. 1988. Recombinant human tumor necrosis factor administered as a 24-hour intravenous infusion. A phase I and pharmacologic study. *J Natl Cancer Inst* 80: 1039-44.

Stein, B., M. X. Yang, D. B. Young, R. Janknecht, T. Hunter, B. W. Murray, and M. S. Barbosa. 1997. p38-2, a novel mitogen-activated protein kinase with distinct properties. *J Biol Chem* 272: 19509-17.

Stelmach, J. E., L. Liu, S. B. Patel, J. V. Pivnichny, G. Scapin, S. Singh, C. E. Hop, Z. Wang, J. R. Strauss, P. M. Cameron, E. A. Nichols, S. J. O'Keefe, E. A. O'Neill, D. M. Schmatz, C. D. Schwartz, C. M. Thompson, D. M. Zaller, and J. B. Doherty. 2003. Design and synthesis of potent, orally bioavailable dihydroquinazolinone inhibitors of p38 MAP kinase. *Bioorg Med Chem Lett* 13: 277-80.

Sugamori, T., Y. Ishibashi, T. Shimada, N. Takahashi, T. Sakane, S. Ohata, Y. Kunizawa, S. Inoue, K. Nakamura, Y. Ohta, H. Shimizu, H. Katoh, N. Oyake, Y. Murakami, and M. Hashimoto. 2002. Increased nitric oxide in proportion to the severity of heart failure in patients with dilated cardiomyopathy: close correlation of tumor necrosis factor-alpha with systemic and local production of nitric oxide. *Circ J* 66: 627-32.

Tahan, F., E. Jazrawi, T. Moodley, G. E. Rovati, and I. M. Adcock. 2008. Montelukast inhibits tumour necrosis factor-alpha-mediated interleukin-8 expression through inhibition of nuclear factor-kappaB p65-associated histone acetyltransferase activity. *Clin Exp Allergy* 38: 805-11.

Takasaki, W., Y. Kajino, K. Kajino, R. Murali, and M. I. Greene. 1997. Structure-based design and characterization of exocyclic peptidomimetics that inhibit TNF alpha binding to its receptor. *Nat Biotechnol* 15: 1266-70.

Tanaka, D., T. Kagari, H. Doi, and T. Shimozato. 2006. Essential role of neutrophils in anti-type II collagen antibody and lipopolysaccharide-induced arthritis. *Immunology* 119: 195-202.

Tandon, R., R. I. Sha'afi, and R. S. Thrall. 2000. Neutrophil beta2-integrin upregulation is blocked by a p38 MAP kinase inhibitor. *Biochem Biophys Res Commun* 270: 858-62.

Tarlowe, M. H., A. Duffy, K. B. Kannan, K. Itagaki, R. F. Lavery, D. H. Livingston, P. Bankey, and C. J. Hauser. 2005. Prospective study of neutrophil chemokine responses in trauma patients at risk for pneumonia. *Am J Respir Crit Care Med* 171: 753-9.

Tartaglia, L. A., and D. V. Goeddel. 1992. Two TNF receptors. *Immunol Today* 13: 151-3.

Tartaglia, L. A., D. Pennica, and D. V. Goeddel. 1993. Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J Biol Chem* 268: 18542-8.

Taylor, P. C. 2003. Anti-TNFalpha therapy for rheumatoid arthritis: an update. *Intern Med* 42: 15-20.

Theis, V. S., and J. M. Rhodes. 2008. Review article: minimizing tuberculosis during anti-tumour necrosis factor-alpha treatment of inflammatory bowel disease. *Aliment Pharmacol Ther* 27: 19-30.

Tilley, S. L., T. M. Coffman, and B. H. Koller. 2001. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest* 108: 15-23.

Tobiume, K., A. Matsuzawa, T. Takahashi, H. Nishitoh, K. Morita, K. Takeda, O. Minowa, K. Miyazono, T. Noda, and H. Ichijo. 2001. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* 2: 222-8.

Torre-Amione, G., B. Bozkurt, A. Deswal, and D. L. Mann. 1999. An overview of tumor necrosis factor alpha and the failing human heart. *Curr Opin Cardiol* 14: 206-10.

Tracey, D., L. Klareskog, E. H. Sasso, J. G. Salfeld, and P. P. Tak. 2008. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther* 117: 244-79.

Tracey, K. J., S. F. Lowry, and A. Cerami. 1988. The pathophysiologic role of cachectin/TNF in septic shock and cachexia. *Ann Inst Pasteur Immunol* 139: 311-7.

Trebble, T., N. K. Arden, M. A. Stroud, S. A. Wootton, G. C. Burdge, E. A. Miles, A. B. Ballinger, R. L. Thompson, and P. C. Calder. 2003. Inhibition of tumour necrosis factor-alpha and interleukin 6 production by mononuclear cells following dietary fish-oil supplementation in healthy men and response to antioxidant co-supplementation. *Br J Nutr* 90: 405-12.

Tugwell, P. 2000. Pharmacoeconomics of drug therapy for rheumatoid arthritis. *Rheumatology (Oxford)* 39 Suppl 1: 43-7.

Underwood, D. C., R. R. Osborn, S. Bochnowicz, E. F. Webb, D. J. Rieman, J. C. Lee, A. M. Romanic, J. L. Adams, D. W. Hay, and D. E. Griswold. 2000. SB 239063,

a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. *Am J Physiol Lung Cell Mol Physiol* 279: L895-902.

Uysal, K. T., S. M. Wiesbrock, M. W. Marino, and G. S. Hotamisligil. 1997. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389: 610-4.

Vaday, G. G., and O. Lider. 2000. Extracellular matrix moieties, cytokines, and enzymes: dynamic effects on immune cell behavior and inflammation. *J Leukoc Biol* 67: 149-59.

van der Bij, G. J., S. J. Oosterling, S. Meijer, R. H. Beelen, and M. van Egmond. 2005. The role of macrophages in tumor development. *Cell Oncol* 27: 203-13.

Van Ginderachter, J. A., K. Movahedi, J. Van den Bossche, and P. De Baetselier. 2008. Macrophages, PPARs, and Cancer. *PPAR Res* 2008: 169414.

van Lookeren Campagne, M., C. Wiesmann, and E. J. Brown. 2007. Macrophage complement receptors and pathogen clearance. *Cell Microbiol* 9: 2095-102.

Van Ostade, X., P. Vandenabeele, B. Everaerdt, H. Loetscher, R. Gentz, M. Brockhaus, W. Lesslauer, J. Tavernier, P. Brouckaert, and W. Fiers. 1993. Human TNF mutants with selective activity on the p55 receptor. *Nature* 361: 266-9.

Vanhaesebroeck, B., and D. R. Alessi. 2000. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J* 346 Pt 3: 561-76.

Varfolomeev, E. E., and A. Ashkenazi. 2004. Tumor necrosis factor: an apoptosis JuNKie? *Cell* 116: 491-7.

Via, M. C. 2007. *Cytokine Therapies and Inhibitors: A vibrant pipeline and active approved market*. Cambridge Healthtech Institute, Needham.

Wadsworth, S. A., D. E. Cavender, S. A. Beers, P. Lalan, P. H. Schafer, E. A. Malloy, W. Wu, B. Fahmy, G. C. Olini, J. E. Davis, J. L. Pellegrino-Gensey, M. P. Wachter, and J. J. Siekierka. 1999. RWJ 67657, a potent, orally active inhibitor of p38 mitogen-activated protein kinase. *J Pharmacol Exp Ther* 291: 680-7.

Wang, X., P. Yue, Y. A. Kim, H. Fu, F. R. Khuri, and S. Y. Sun. 2008. Enhancing mammalian target of rapamycin (mTOR)-targeted cancer therapy by preventing mTOR/raptor inhibition-initiated, mTOR/rictor-independent Akt activation. *Cancer Res* 68: 7409-18.

Wang, X. S., K. Diener, C. L. Manthey, S. Wang, B. Rosenzweig, J. Bray, J. Delaney, C. N. Cole, P. Y. Chan-Hui, N. Mantlo, H. S. Lichenstein, M. Zukowski, and Z. Yao. 1997. Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. *J Biol Chem* 272: 23668-74.

Wang, Z., E. Choice, A. Kaspar, D. Hanson, S. Okada, S. C. Lyu, A. M. Krensky, and C. Clayberger. 2000. Bactericidal and tumoricidal activities of synthetic peptides derived from granulysin. *J Immunol* 165: 1486-90.

Warren, D. J., L. Slordal, and M. A. Moore. 1990. Tumor-necrosis factor induces cell cycle arrest in multipotential hematopoietic stem cells: a possible radioprotective mechanism. *Eur J Haematol* 45: 158-63.

Waterman, W. H., T. F. Molski, C. K. Huang, J. L. Adams, and R. I. Sha'afi. 1996. Tumour necrosis factor-alpha-induced phosphorylation and activation of cytosolic phospholipase A2 are abrogated by an inhibitor of the p38 mitogen-activated protein kinase cascade in human neutrophils. *Biochem J* 319 (Pt 1): 17-20.

Weiss, T., M. Grell, K. Siemienski, F. Muhlenbeck, H. Durkop, K. Pfizenmaier, P. Scheurich, and H. Wajant. 1998. TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J Immunol* 161: 3136-42.

Weston, C. R., and R. J. Davis. 2002. The JNK signal transduction pathway. *Curr Opin Genet Dev* 12: 14-21.

Westra, J., J. M. Kuldo, M. H. van Rijswijk, G. Molema, and P. C. Limburg. 2005. Chemokine production and E-selectin expression in activated endothelial cells are inhibited by p38 MAPK (mitogen activated protein kinase) inhibitor RWJ 67657. *Int Immunopharmacol* 5: 1259-69.

Wiegmann, K., S. Schutze, T. Machleidt, D. Witte, and M. Kronke. 1994. Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell* 78: 1005-15.

Winzen, R., M. Kracht, B. Ritter, A. Wilhelm, C. Y. Chen, A. B. Shyu, M. Muller, M. Gaestel, K. Resch, and H. Holtmann. 1999. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *Embo J* 18: 4969-80.

Wisdom, R., R. S. Johnson, and C. Moore. 1999. c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. *Embo J* 18: 188-97.

Witko-Sarsat, V., P. Rieu, B. Descamps-Latscha, P. Lesavre, and L. Halbwachs-Mecarelli. 2000. Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* 80: 617-53.

Wooley, P. H., J. Dutcher, M. B. Widmer, and S. Gillis. 1993. Influence of a recombinant human soluble tumor necrosis factor receptor FC fusion protein on type II collagen-induced arthritis in mice. *J Immunol* 151: 6602-7.

Xaus, J., M. Comalada, A. F. Valledor, J. Lloberas, F. Lopez-Soriano, J. M. Argiles, C. Bogdan, and A. Celada. 2000. LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-alpha. *Blood* 95: 3823-31.

Xia, P., L. Wang, P. A. Moretti, N. Albanese, F. Chai, S. M. Pitson, R. J. D'Andrea, J. R. Gamble, and M. A. Vadas. 2002. Sphingosine kinase interacts with TRAF2 and dissects tumor necrosis factor-alpha signaling. *J Biol Chem* 277: 7996-8003.

Xing, L., and D. G. Remick. 2003. Relative cytokine and cytokine inhibitor production by mononuclear cells and neutrophils. *Shock* 20: 10-6.

Yan, M., T. Dai, J. C. Deak, J. M. Kyriakis, L. I. Zon, J. R. Woodgett, and D. J. Templeton. 1994. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* 372: 798-800.

Yao, B., Y. Zhang, S. Delikat, S. Mathias, S. Basu, and R. Kolesnick. 1995. Phosphorylation of Raf by ceramide-activated protein kinase. *Nature* 378: 307-10.

Yarovinsky, F. 2008. Toll-like receptors and their role in host resistance to Toxoplasma gondii. *Immunol Lett* 119: 17-21.

Yeh, W. C., A. Shahinian, D. Speiser, J. Kraunus, F. Billia, A. Wakeham, J. L. de la Pompa, D. Ferrick, B. Hum, N. Iscove, P. Ohashi, M. Rothe, D. V. Goeddel, and T. W. Mak. 1997. Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* 7: 715-25.

Younes, A., and M. E. Kadin. 2003. Emerging applications of the tumor necrosis factor family of ligands and receptors in cancer therapy. *J Clin Oncol* 21: 3526-34.

Young, P. R., M. M. McLaughlin, S. Kumar, S. Kassis, M. L. Doyle, D. McNulty, T. F. Gallagher, S. Fisher, P. C. McDonnell, S. A. Carr, M. J. Huddleston, G. Seibel, T. G. Porter, G. P. Livi, J. L. Adams, and J. C. Lee. 1997. Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *J Biol Chem* 272: 12116-21.

Zamkoff, K. W., N. B. Newman, A. R. Rudolph, J. Young, and B. J. Poiesz. 1989. A phase I trial of subcutaneously administered recombination tumor necrosis factor to patients with advanced malignancy. *J Biol Response Mod* 8: 539-52.

Zamora, R., Y. Vodovotz, and T. R. Billiar. 2000. Inducible nitric oxide synthase and inflammatory diseases. *Mol Med* 6: 347-73.

Zemann, B., N. Urtz, R. Reuschel, D. Mechtcheriakova, F. Bornancin, R. Badegruber, T. Baumruker, and A. Billich. 2007. Normal neutrophil functions in sphingosine kinase type 1 and 2 knockout mice. *Immunol Lett* 109: 56-63.

Zernecke, A., I. Bot, Y. Djalali-Talab, E. Shagdarsuren, K. Bidzhekov, S. Meiler, R. Krohn, A. Schober, M. Sperandio, O. Soehnlein, J. Bornemann, F. Tacke, E. A. Biessen, and C. Weber. 2008. Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. *Circ Res* 102: 209-17.

Zhang, Y., B. F. Ramos, and B. A. Jakschik. 1992. Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. *Science* 258: 1957-9.

Zhang, Y., B. Yao, S. Delikat, S. Bayoumy, X. H. Lin, S. Basu, M. McGinley, P. Y. Chan-Hui, H. Lichenstein, and R. Kolesnick. 1997. Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell* 89: 63-72.

Zhou, Z., P. Gengaro, W. Wang, X. Q. Wang, C. Li, S. Faubel, C. Rivard, and R. W. Schrier. 2008. Role of NF-{kappa}B and PI 3-kinase/Akt in TNF-{alpha}-induced cytotoxicity in microvascular endothelial cells. *Am J Physiol Renal Physiol*.

Zu, Y. L., J. Qi, A. Gilchrist, G. A. Fernandez, D. Vazquez-Abad, D. L. Kreutzer, C. K. Huang, and R. I. Sha'afi. 1998. p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or FMLP stimulation. *J Immunol* 160: 1982-9.