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The role of tumour necrosis factor alpha in lung inflammation

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The role of tumour necrosis factor α in lung inflammation

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To Mum and Dad

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Contents

Abbreviations

$[Ca^{2+}]_i$	intracellular free calcium concentration
5-HPETE	5-hydroperoxyeicosatetraenoic acid
5-HT	5-hydroxytryptamine
A-SMase	acidic sphingomyelinase
AA	arachidonic acid
Amino acid residues:	
A, C, D, E, F,	alanine, cysteine, aspartic acid, glutamic acid, phenylalanine,
G, H, I, K, L, M,	glycine, histidine, isoleucine, lysine, leucine, methionine,
N, P, Q, R, S, T,	asparagine, proline,glutamine, arginine, serine, threonine,
V, W, Y	valine, tryptophan, tyrosine
AP-1	activator protein-1
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bromophenol blue	3',3",5',5"-tetrabromophenolsulphonephthalein
BSA	bovine serum albumin (low endotoxin)
C5a	complement fragment 5
Ca^{2+}	calcium
CAPK	ceramide activated protein kinase
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CSPD	disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phoshphate
DAB	diaminobenzidine
DAG	diacylglycerol
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cystisine-5'-triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate
DIG	digoxigenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	thymidine-5'-triphosphate
dUTP	2'-deoxy-uridine-5'-triphosphate

EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethylether) N,N,N',N'- tetraacetic acid
ELAM-	endothelial leukocyte adhesion molecule-
ELISA	enzyme linked immunoassay
EPO	eosinophil peroxidase
ERK	extracellular signal-regulated kinases
FcεRI/II	IgE receptor I (high affinity) /II (low affinity)
FCS	foetal calf serum
Fura-2AM	{1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2(2'-amino-5'-methylphenoxy)-ethane-N,N,N', N'-tetraacetic acid, pentaacetoxymethyl ester}
GM-CSF	granulocyte-macrophage colony-stimulating factor
gpTNFα	guinea-pig tumour necrosis factorα
HBSS	Hank's balanced salt solution pH 7.2-7.4 without Ca^{2+} Mg^{2+} or phenol red
Heparin	heparin sodium
HTAB	hexadecyltrimethylammonium bromide
I-κB	inhibitor of NF-κB
ICAM-	intercellular adhesion molecule-
IFN-	interferon-
Ig	immunoglobulin
IL-	interleukin-
IL-1ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
kDa	kilo Daltons
LPS	lipopolysaccharide
LT	leukotrienes
MAP	mitogen-activated protein kinase
MBP	maltose-binding protein
MCP-	monocyte chemotactic protein-
Mg^{2+}	magnesium
MHC	major histocompatibility complex
MIP-	macrophage inflammatory peptide-
mRNA	messenger ribonucleic acid

N-SMase	neutral sphingomyelinase
NAP-	neutrophil activating peptide-
NBT	nitro blue tetrazolium
NF-κB	nuclear factor -κB
OA	ovalbumin (antigen)
OD	optical density
OPD	o-phenylenediamine
p50+p65	proteins of NF-κB complex
p55	p55 TNF receptor
p55-sR / p75-sR	p55 soluble TNFα receptor / p75 soluble TNFα receptor
p75	p75 TNF receptor
PAF	platelet activating factor (1-o-hexadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine
PC-PLC	phosphatidylcholine-specific phospholipase C
PCR	polymerase chain reaction
PG	prostaglandin
PKC	protein kinase C
PL	phospholipid
PLA_2	phospholipase A_2
Protogel	30%(w/v) acrylamide, 0.8%(w/v) bisacrylamide stock solution 37.5:1
RANTES	regulated upon activation in normal T cells expressed and secreted
RNA	ribonucleic acid
s-TNFαR-Fc	soluble TNFα receptor : Ig construct
SDS	lauryl sulphate (sodium dodecyl sulphate)
Selectins: E-, P-, L-	endothelial-, platelet-, leukocyte-selectin
SM	sphingomyelin
TACE	TNFα converting enzyme
TBE	tris-borate EDTA
TBS	tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine

TNF	tumour necrosis factor
TNFα	tumour necrosis factor α / cachectin
TNFβ	tumour necrosis factor β / lymphotoxin, two forms $TNF\beta_{\alpha}$ and $TNF\beta_{\beta}$
TNFα-BP	TNFα binding protein
TNFRrp	TNF receptor related protein
TRADD	TNF receptor associated death domain
TRAF-1, -2	TNF receptor associated factor-1, -2
Triton X-100	octylphenoxyolyethoxyethanol
Tween-20	polyoxyethylene-sorbitan monolaurate
Tx	thromboxane
VCAM-	vascular cell adhesion molecule-
VLA-	very late activation antigen-
WEHI	Walter and Eliza Hall Institute (in Melbourne, Australia)

Abstract

Recombinant guinea-pig TNFα (gpTNFα) has been cloned, expressed and purified, and its *in vivo* biological activity in the guinea-pig lung examined. cDNA corresponding to the secreted form of gpTNFα was amplified by the polymerase chain reaction, cloned into the pMAL-p2 vector and expressed in *E. coli*. This system results in the expression of a maltose binding protein-gpTNFα fusion protein incorporating a factor Xa cleavage site. The fusion protein was extracted from the *E. coli* lysate by affinity chromatography. Factor Xa cleavage releases gpTNFα from the fusion protein, resulting in a 17kDa protein detectable with anti murine TNFα antibodies in western blotting. Hydroxyapatite, amylose and polymyxin B-bound agarose columns were used for further purification. gpTNFα exhibited a cytotolytic activity 4 fold greater than recombinant human TNFα standards in a murine TNFα bioassay.

The *in vivo* bioactivity of gpTNFα in the lung was characterised in guinea-pigs. gpTNFα induced a pronounced and protracted airway eosinophilia which was both time-related (peaking at 24 hours) and dose dependent. Pre-incubating gpTNFα with anti TNFα antibody or heat inactivating the protein, prior to tracheal instillation inhibited gpTNFα induced leukocyte accumulation. The instillation of human TNFα elicited a weaker inflammatory cell recruitment with different cellular characteristics than that induced by gpTNFα indicating species selectivity.

The role of TNFα in a guinea-pig model of allergic airway inflammation was also examined. Following antigen challenge temporal changes in lung leukocyte infiltration and chemokine-like activity in the airways were observed. The administration of anti TNFα antibody partially inhibited antigen-induced eosinophil accumulation. In addition, treatment with human IL-13, a cytokine which exhibits anti-inflammatory activities, inhibited both gpTNFα- and antigen-induced airway eosinophilia, and inhibited the generation of chemokine-like bioactivity in the airways in response to antigen.

This thesis indicates the importance of TNFα as mediator of airway eosinophil accumulation *in vivo*.

1. Introduction

1.1 Tumour necrosis factor α (TNFα)

Cell to cell communication is a complex set of events involving receptor-ligand interactions, intracellular signaling pathways, gene expression and protein synthesis. In order to exert an effect on a biological system these events must occur in a coordinated manner. One system which is highly regulated by cell to cell communication is the immune/inflammatory system. The controlled modulation of immune-cell derived protein mediators is essential since these mediators not only orchestrate the inflammatory response, but are also involved in normal cellular growth and differentiation. One such mediator is tumour necrosis factor (TNF).

1.1.1 History, isolation, characterisation and cloning

The phenomenon of bacterial induced tumour necrosis was reported at the turn of the century by William Coley (Coley, 1893; Coley, 1906) and later further investigated by Shear and colleagues (Shear *et al.* 1943). This was probably the first report of the concept of TNF. Shear isolated a gram-negative bacterial factor responsible for the induced necrosis, now known as lipopolysaccharide (LPS), and then showed that LPS did not directly cause tumour necrosis (O'Malley *et al.* 1962). A decade later, 1975, Carswell *et al.* 1975 reported similar and more extensive findings that the serum derived from mice, rats and rabbits treated with *Bacillus Calmette-Guérin* (BCG) prior to injection of endotoxin contained a necrotising factor capable of acting upon tumours implanted in the skin of recipient mice with no apparent effect on the recipient. This activity of the serum was named TNF. Initial *in vitro* studies in 1975, using the supernatant from endotoxin stimulated rat peritoneal macrophages provided evidence that TNF was cytotoxic to benzpyrene-induced sarcoma cells but not normal rat kidney or diaphragm cells (Currie & Basham, 1975). This cytotoxic selectivity was also observed by Helson (Helson, 1975) who induced lysis of human melanoma cells with partially purified murine TNF.

The nature and sequence of the protein TNF was not identified until the mid-80's when two forms were isolated from human sources and subsequently their cDNA was cloned (Aggarwal *et al.* 1985b; Pennica *et al.* 1984; Gray *et al.* 1984; Wang *et al.* 1985; Shirai *et al.* 1985). The 2 forms of TNF defined as TNFα and lymphotoxin (TNFβ) exhibited 34% amino acid sequence homology, with TNFα believed to be produced by monocytes/ macrophages and TNFβ by lymphocytes. Originally it was generally perceived that these 2 proteins which exhibited structural homology and bound common receptors functioned similarly, but it is now becoming clear that TNFβ is not a redundant aspect of TNFα biology. Indications that TNFβ could induce separate biological functions from TNFα were implicated by the identification of a TNFβ-specific receptor (Crowe *et al.* 1994) named TNF receptor related protein (TNFRrp). TNFβ ($TNF\beta_{\alpha}$) binds to TNFR-rp as a heterotrimer produced by binding with a third member of the TNF ligand family $TNF\beta_{\beta}$, forming a $TNF\beta_{\alpha 2}$:$TNF\beta_{\beta 1}$ complex (Browning *et al.* 1993).

At a similar time a report described a factor, in a murine system, which was involved in the suppression of lipoprotein lipase and the induction of cachexia (Kawakami *et al.* 1982). Cachexia is a state of malnutrition which often complicates illness as the host continues to catabolise vital lipid and protein stores despite reducing food intake. This factor termed cachectin possessed similar biological activity and NH_2-terminal sequence homology to TNFα. Following the purification of cachectin it was found to be identical to TNFα (Beutler *et al.* 1985a). By the end of the eighties the sequencing and cloning of rabbit (Ito *et al.* 1986), murine (Pennica *et al.* 1985) and rat (Shirai *et al.* 1989) TNFα confirmed TNFα to be a 17kDa protein under denaturing conditions and 45-51kDa under native conditions, suggesting activity as a trimer. TNFα was subsequently classified as a cytokine, a large family of host-derived proteins which are involved in cell to cell communication.

1.1.2 Regulation of TNFα biosynthesis

TNFα is produced primarily by activated macrophages, but also by a wide variety of inflammatory and tissue cell types of which an abridged list is shown in table 1.1. The protein is also produced by a range of tumour cells (Cordingley *et al.* 1988), and it is currently speculated that under the appropriate conditions all nucleated cell types are capable of producing TNFα. Although it was first thought that TNFα was solely synthesised *de novo* following cell activation it has been shown that in mast cells TNFα can exist in a stored form (Gordon & Galli, 1990). The biosynthesis of TNFα is highly regulated at the transcriptional, translational, and post-translational levels. The involvement of transcriptional regulation was noted in peritoneal murine macrophages by Beutler *et al* (Beutler *et al.* 1986a). In resting cells the TNFα gene was transcriptionally active and TNFα mRNA detected but TNFα protein was not measurable, indicating that the mRNA was not being translated. Cellular activation by LPS was postulated to mobilise the sequestered mRNA for translation and to stimulate the biosynthesis of additional message. Following stimulation the rate of transcription was increased approximately threefold whilst TNFα mRNA levels increased 50-100 times and protein secretion 10,000 times (Beutler *et al.* 1986a; Chung *et al.* 1992), suggesting the involvement of transcriptional and translational regulation. Dexamethasone inhibits both gene transcription and mRNA translation, but dexamethasone will only suppress the latter if added prior to LPS stimulation (Beutler *et al.* 1986a). Once induction of the translation phase had occurred dexamethasone was incapable of regulating TNFα production, hence dexamethasone may only be an effective suppresser of TNFα biosynthesis in a prophylactic manner.

The use of protein synthesis inhibitors has demonstrated that post-translational mechanisms also regulate TNFα biosynthesis. The addition of cycloheximide increased mRNA levels and the gene transcription rate in phorbol ester stimulated

Table 1.1: An abridged list of different cell types which produce TNFα.

Cell type	Reference
monocytes	(Beutler *et al.* 1985a)
neutrophils	(Dubravec *et al.* 1990)
eosinophils	(Costa *et al.* 1993)
lymphocytes	(Cuturi *et al.* 1987)
mast cells	(Gordon & Galli, 1990)
smooth muscle cells	(Warner & Libby, 1989)
endothelial cells	(Shanahan *et al.* 1989)
epithelial cells	(Spriggs *et al.* 1988)
fibroblasts	(Havell & Rogerson, 1993)
Kupffer cells	(Karck *et al.* 1988)
mesangial cells	(Baud *et al.* 1989)
cardiac myocytes	(Kapadia *et al.* 1995)
astrocytes	(Robbins *et al.* 1987)

monocytes. As mRNA levels were also increased in the presence of a combination of actinomycin D (an inhibitor of transcription) and cycloheximide this suggests that labile proteins may reduce the stability of TNFα mRNA and that gene activation can occur in the absence of protein synthesis presumably via modification of pre-existing cellular factors and the inhibition of repressor protein synthesis (Sariban *et al.* 1988; Chung *et al.* 1992; Collart *et al.* 1986). Such factors may include NF-κB which is activated by the phosphorylation and subsequent cleavage of an inhibitory factor I-κB. The TNFα gene promoter region contains NF-κB binding motifs which on activation by active NF-κB induces gene transcription (Collart *et al.* 1990). Another factor controlling gene expression is the regulation of mRNA stability. A region of 3'-untranslated TNFα mRNA contains a long U + A exclusive region bearing overlapping and repeating octameric elements (UUAUUUAU) which is similar to 3'-untranslated sequences in other cytokine mRNAs. Using gene constructs it has been shown that the A U region is involved in mRNA destabilisation and enhanced

translation efficiency (Shaw & Kamen, 1986; Caput *et al.* 1986; Han *et al.* 1990). Due to TNFα biosynthesis being regulated at several levels it is perhaps not surprising that the agents capable of altering TNFα production are highly diverse, an abridged list is shown in table 1.2.

Table 1.2: An abridged list of agents capable of modulating TNFα production.

	Induction of TNFα production	
Agent type	**example**	**reference**
Cytokines	TNFα	(Philip & Epstein, 1986)
	interferon-γ (IFNγ)	(Philip & Epstein, 1986)
	IL-1	(Philip & Epstein, 1986)
	IL-2	(Nedwin *et al.* 1985)
Drugs and biologically active lipids	inducers of PKC (phorbol esters)	(Sariban *et al.* 1988)
	leukotrienes	(Dubois *et al.* 1989)
	platelet activating factor (PAF)	(Dubois *et al.* 1989)
Parasite products	malaria exoantigens	(Bate *et al.* 1988)
Viruses	influenza virus	(Beutler *et al.* 1986b)
Bacterial products	LPS	(Beutler *et al.* 1986a)
	Borrelia burgdorferi	(Radolf *et al.* 1991)

	Suppression of TNFα production	
Agent type	**example**	**reference**
Cytokines	IL-4	(Hart *et al.* 1991)
	IL-6	(Aderka *et al.* 1989)
	TGF-β	(Chantry *et al.* 1989)
Drugs and biologically active lipids	lipooxygenase inhibitors	(Dubois *et al.* 1989)
	dexamethasone	(Beutler *et al.* 1986a)
	phosphodiesterase inhibitors e.g. pentoxifylline	(Doherty *et al.* 1991)
	inhibitors of NF-κB	(Takasuka *et al.* 1995)
	cyclosporin A	(Espevik *et al.* 1987)
Viruses	Epstein-Barr	(Gosselin *et al.* 1991)

1.1.3 Biosynthesis and processing of TNFα

TNFα is synthesised as a 26kDa, type II cell-surface associated, propeptide anchored to the cell membrane by an NH_2-terminal hydrophobic domain (Kriegler *et al.* 1988). In human cells mature TNFα is produced following the proteolytic cleavage of the membrane bound 233 amino acid protein resulting in the secretion of a 157 amino acid 17kDa peptide (Jue *et al.* 1990). The enzyme or enzymes responsible for this process, termed TNFα converting enzyne (TACE) have yet to be fully characterised but have recently been cloned (Black *et al.* 1997; Moss *et al.* 1997). Both the transmembrane and mature protein are biologically active. Elegant studies by Perez *et al* (Perez *et al.* 1990) using transmembrane TNFα mutants which were resistant to proteolytic processing showed that the membrane form mediated tumour and virus-infected cell cytotoxicity by cell to cell contact. Further studies have implicated the membrane bound form of TNFα as a mediator of cell-cell interaction in co-stimulatory signalling for B lymphocyte activation by T lymphocytes (Aversa *et al.* 1993). Secreted mature TNFα is active as a noncovalently associated homotrimer, the lack of biological activity induced by the monomeric form has been ascribed to the decreased affinity of a monomer for the receptor (Smith & Baglioni, 1987). Crystal structure resolution suggests that each monomer contains an elongated, antiparallel β-pleated sheet which displays the topology of the classical viral "jelly-roll" (Jones *et al.* 1989). A diagrammatic sketch of the homotrimer is shown in figure 1.1.

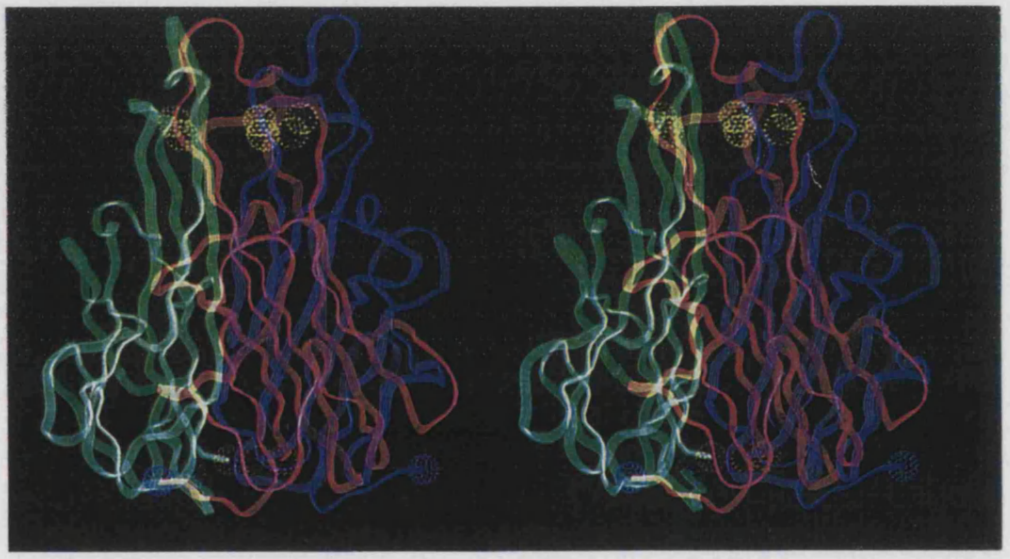


Figure 1.1: Ribbon representation of the trimeric form of human TNFα. Individual subunits are coloured green, red and blue. The NH_2-termini are marked by blue spheres and the COOH-termini by red spheres. The yellow spheres (near the top of the molecule) represent disulphide bridges. (From Jones *et al.* 1989).

1.1.4 TNFα receptors

TNFα binds to at least two types of receptor, the p55 receptor (also referred to as the p60 receptor, TNF receptor I, high affinity receptor and the TNF receptor type B) and to the p75 receptor (also referred to as the p80 receptor, TNF receptor II low affinity receptor and the TNF receptor type A) these having molecular weights of approximately 55kDa and 75kDa respectively (Loetscher *et al.* 1990; Lewis *et al.* 1991; Gray *et al.* 1990). The two types of receptor mediate both overlapping and nonoverlapping functions (Tartaglia *et al.* 1991; Tartaglia & Goeddel, 1992b). The distribution of TNFα receptors is believed to be wide spread, being present to varying extents on virtually all cells except red blood cells. These receptors are members of large family of receptors which are all characterised by common cysteine-rich domains (3-6 domains/molecule) in the extracellular portion of the receptor and include; Fas, CD27, nerve growth factor receptor and TNFR-rp (Smith *et al.* 1990). Typical of all the members of this family p55 and p75 have distinct cytoplasmic domains. One exception is a short section of the p55 cytoplasmic domain which has homology with the Fas receptor (28% homology over 65 amino acids) (Tartaglia *et al.* 1993). Mutations of both the Fas and p55 receptor have indicated that this region is involved in the signal mediation of target cell apoptosis and cytotoxicity, respectively, and is referred to as the death domain (Tartaglia & Goeddel, 1992a; Tartaglia *et al.* 1993).

Results from crystal structure resolution of homotrimeric TNFβ binding to the p55 receptor (Banner *et al.* 1993) and multivalent anti-TNF receptor antibodies mediating TNFα cytotoxicity (Engelmann *et al.* 1990) support a putative mechanism that on the binding of secreted TNFα homotrimer to the receptor, receptor clustering occurs with the trimer binding to 3 receptors and stabilising the complex (Banner *et al.* 1993). If receptor clustering is required for optimum receptor signalling it can be postulated that this would render the monomeric form, which is unable to induce receptor clustering, less active than the oligomeric forms of TNFα. This would be in accordance with the findings of Smith & Baglioni, 1987 that monomeric TNFα lacks

biological activity. The signalling events which occur following receptor activation are complex and not at present fully understood. Unravelling this complex signalling pathway to establish how the stimulation of a TNFα receptor can induce such diverse cellular responses is an expanding area of TNFα biology. TNFα receptor stimulation has been shown to recruit TNF receptor associated factors (e.g. TRAF-1, -2) to the receptor complex and subsequently induce the activation of various signalling pathways including serine/threonine kinases (e.g. protein kinase C (PKC) and extracellular signal-regulated kinases (ERK)), protein tyrosine kinases (e.g. mitogen-activated protein kinase (MAP)), acidic and neutral sphingomyelinase, phospholipase A_2, transcription factors (e.g. NF-κB, AP-1). Although signalling will not be described in detail in this report a possible signalling pathway is shown in figure 1.2 and reference should be made to reviews and references in Beutler & Van Huffel, 1994; Warzocha *et al.* 1994 and Aggarwal & Natarajan, 1996.

Like TNFα, receptor expression has been shown to be regulated by a wide variety of agents with the modulation being cell type dependent. For example IFNγ is capable of either upregulating and downmodulating receptor expression in different cell types (Aggarwal *et al.* 1985a; Drapier & Wietzerbin, 1991). The mechanisms for inducing up- and down-regulation are unclear, although both appear to involve protein phosphorylation. Receptor shedding is a major pathway for the downregulation of receptor expression and although the proteases responsible have not yet been identified metalloproteases have recently been implicated (Crowe *et al.* 1995; Mullberg *et al.* 1995). Interestingly, TNFα can regulate expression of its own receptor. (Higuchi & Aggarwal, 1994) showed that in histiocytic lymphoma U-937 cells TNFα induced the internalisation of the p55 receptor whereas the p75 form was secreted into the external environment. Conversely, in a human epithelial cell line it was demonstrated that TNFα could upregulate the expression of the p75 but not the p55 receptor (Kalthoff *et al.* 1993).

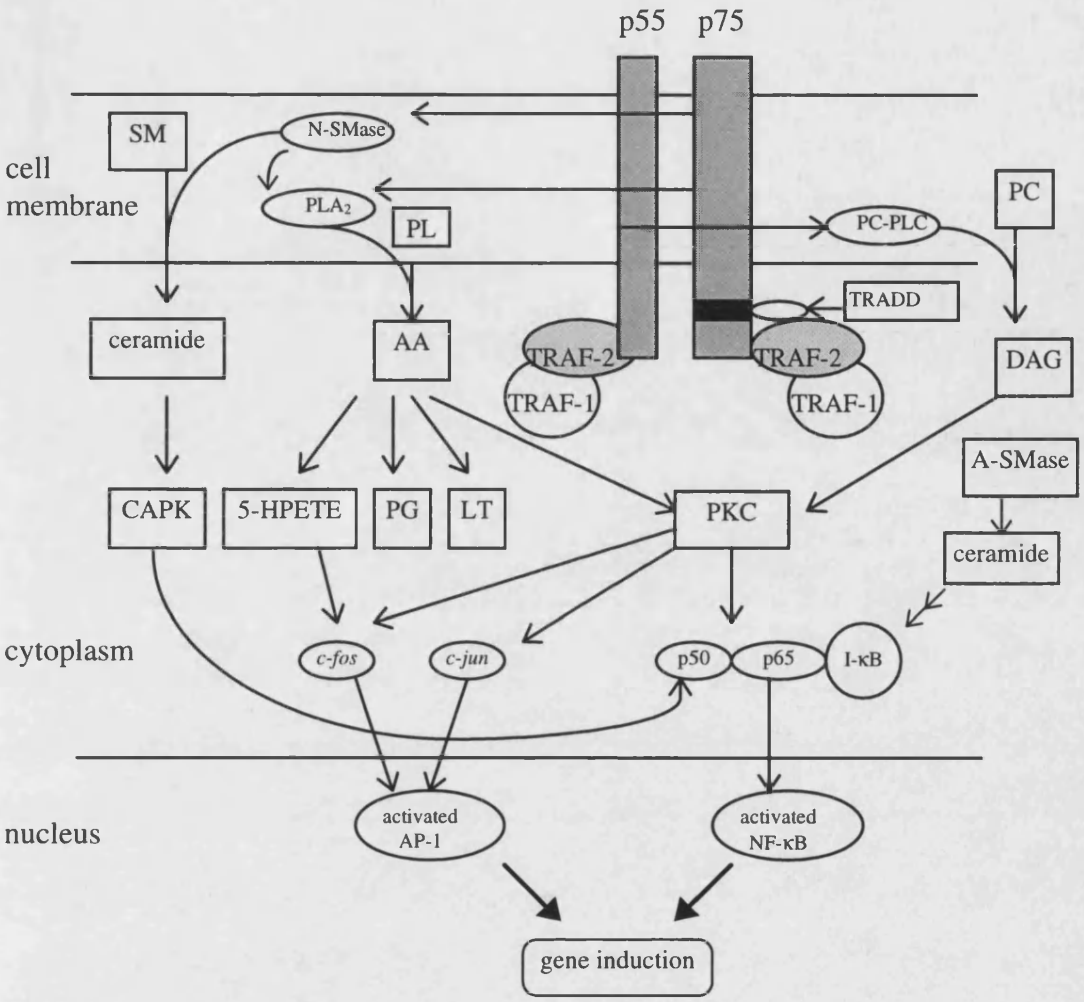


Figure 1.2: A schematic diagram to represent potential signalling pathways activated following activation of the p55 and p75 TNFα receptors. Both receptors are linked to PLA_2, PC-PLC and SMase ultimately resulting in gene induction by the pathways shown. The transcription factor NF-κB heterodimer is bound to an inhibitor protein, I-κB, which blocks the dimer's translocation to the nucleus. Phosphorylation and proteolysis of I-κB resulting in the activation of NF-κB involves multiple intermediates including ceramide, serine kinase, protein phosphatase and protein kinase. The transcription factor AP-1, which consists of homodimers (*jun* proteins) or heterodimers (*jun* and *fos* proteins) is also activated by TNFα.

AA, arachidonic acid; AP-1 and NF-κB are both transcription factor complexes, A-SMase, acidic sphingomyelinase; CAPK, ceramide activated protein kinase, DAG, diacylglycerol; I-κB, inhibitor of NF-κB; LT, leukotrienes; PC, phosphatidylcholine; PC-PLC phosphatidylcholine-specific phospholipase C; PG, prostaglandins; PL, phospholipid; PLA_2, phospholipase A_2; PKC, protein kinase C, p50+p65; proteins of NF-κB complex; N-SMase, neutral sphingomyelinase; SM, sphingomyelin; TRAF-1, -2, TNF receptor associated factor-1, -2; TRADD, TNF receptor associated death domain; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; black area of p75 receptor, death domain.

Soluble fragments of the TNFα receptors were initially isolated and purified as TNFα binding proteins (TNFα-BP) in the urine of patients with febrile illnesses or serum of patients with cancer (Seckinger *et al.* 1997; Gatanaga *et al.* 1990). Sequence analysis of TNFα-BP showed that the two proteins were the extracellular domains of the p55 and p75 receptors (Gray *et al.* 1990; Lantz *et al.* 1990a; Seckinger *et al.* 1990; Schall *et al.* 1990). Proteolytic cleavage of the membrane-bound receptors to produce soluble receptors (p55-sR and p75-sR) reduces both the level of cellular expression and the circulating levels of the active TNFα protein by the binding of the soluble receptors to TNFα, hence receptor shedding decreases the biological response by two mechanisms. In addition to this inhibitory effect, it is thought that the dissociation of bound TNFα from the soluble receptors may be capable of maintaining circulating TNFα at a biologically active level (Aderka *et al.* 1992; Bemelmans *et al.* 1993a; Van Zee *et al.* 1992).

1.1.5 Biological activity and disease pathogenesis

As described earlier during the cloning and purification of TNFα two diverse functions for the protein were discovered, these being the necrosis of tumours and regulating lipid metabolism during infection (hence the name cachectin). The production of the recombinant protein has lead to extensive studies of the activity of TNFα in *in vitro* and *in vivo* systems, the results from which have established a role for TNFα in the physiological and pathological state. It is believed that TNFα is important in a variety of normal physiological processes including embryogenesis (reviewed in Wride & Sanders, 1995), hematopoiesis (Jacobsen *et al.* 1992) and inflammation. However it is thought that the distinction between the physiological and pathological states may be due to the local TNFα concentration (Kunkel *et al.* 1989). At low levels TNFα acts to maintain/promote cellular and tissue homeostasis. By increasing the level, as in the case of a local tissue injury a controlled immune

response is orchestrated to repair the tissue, but if the concentration is further increased a pathological state is reached.

With the wide distribution of receptors TNFα can stimulate virtually any cell type acting in an autocrine and paracrine fashion. Following activation TNFα induces a range of biological activities including the alteration of growth, differentiation and metabolism of a variety of cell types. These effects are cell dependent, for example proliferation is induced by TNFα in fibroblasts (Vilcek *et al.* 1986) but suppressed in endothelial and keratinocyte cell cultures (Pillai *et al.* 1989; Schweigerer *et al.* 1987). This broad range of actions is reflected in the diverse pathological conditions in which TNFα is implicated. One of the first documented pathological roles for TNFα was as a major mediator of the lethal metabolic effects of endotoxin-mediated (septic) shock (Beutler *et al.* 1985b). The administration of TNFα to rats induces shock syndrome symptoms, hypotension, tachypnea, metabolic acidosis, hyperglycemia followed by hypoglycemia, and multiple end-organ damage with death usually resulting from respiratory arrest . Latterly, the involvement of TNFα has been suggested in two extreme conditions of body weight: cachexia and obesity. TNFα can modulate the metabolic activities of adipocytes suppressing lipoprotein lipase activity, reducing adipose tissue lipid accumulation and leading to the wasting associated with cachexia, and the inhibition of insulin-stimulated glucose uptake observed in obesity-related insulin resistance (Hotamisligil *et al.* 1993).

TNFα has both peripheral and central effects. Although only low amounts of TNFα cross the blood-brain barrier (Beutler *et al.* 1985) cells within the central nervous system (CNS) are capable of producing the protein (Robbins *et al.* 1987). TNFα stimulation of the CNS can produce anorexia (Moldawer *et al.* 1988), changes in sleep patterns, pyrogenic responses (fever) (Dinarello *et al.* 1986), induction of the release of adrenocorticotropic hormone and prolactin from the pituitary (Milenkovic *et al.* 1989). Furthermore the overproduction of TNFα by cells within the CNS may be

detrimental to the integrity of the blood brain barrier as TNFα is cytotoxic to oligodendrocytes, the myelin producing cells, which may facilitate demyelination (Selmaj & Raine, 1988) in, for example, multiple sclerosis.

Table 1.3: A partial list of the responses mediated by TNFα which may be involved in inflammatory responses.

Cell type	Responses mediated by TNFα	Reference
Endothelial cell	Rearrangement of the cytoskeleton to increase vascular permeability	(Brett *et al.* 1989)
	Coagulation (via down modulation of thrombomodulin) to generate thrombin and fibrinogen to induce cell contraction	(Nawroth & Stern, 1986)
	Increased expression of adhesion molecules (E-, P-selectin, ICAM-1, VCAM-1)	(Mantovani *et al.* 1992)
	Induction of bioactive mediators (e.g. IL-1, PAF, PGI_2) and nitric oxide synthase	(Naworth *et al.* 1986; Camussi *et al.* 1987; Kawakami *et al.* 1986)
	Induction of chemokine expression (e.g.IL-8, MCP-1)	(Brown *et al.* 1994)
	Increased cell surface antigen expression	(Pober *et al.* 1986; Collins *et al.* 1986)
Leukocytes	Induction of bioactive mediators (e.g. IL-1, PAF, TNFα, leukotrienes, prostaglandins, chemokines)	(Shalaby *et al.* 1985; Klebanoff *et al.* 1986)
	Stimulates phagocytosis and cellular degranulation	(Shalaby *et al.* 1985)
	Increase MHC expression	(Perlmutter *et al.* 1986)
	Induced superoxide production, release of lysozyme and hydrogen peroxide	(Tsujimoto *et al.* 1986; Klebanoff *et al.* 1986)
Resident tissue cells	Induction of bioactive mediators (e.g. IL-1, PAF, RANTES, MCP-1, IL-8) and nitric oxide synthase	(Lukacs *et al.* 1995a; Le *et al.* 1984; Robson *et al.* 1995; Jordan *et al.* 1995; Jordan *et al.* 1996)
	Cellular proliferation	(Vilcek *et al.* 1986)
	Acute-phase protein synthesis	(Perlmutter *et al.* 1986)
	Bone and cartilage resorption	(Saklatvala, 1986; Bertolini *et al.* 1986)

Perhaps the most important role of TNFα is as a primary mediator of inflammation. It is widely hypothesised that an inflammatory response is initiated, maintained and resolved via the interplay of the cytokine network. TNFα, thought to be an integral part of this network, is capable of inducing many of the events which are associated with inflammation as described in table 1.3. In addition to the indirect recruitment of leukocytes via increased vascular permeability, enhanced adhesion molecule expression and the induced release of chemoattractants and inflammatory mediators, TNFα also influences tissue remodelling. Tissue remodelling may potentiate the inflammatory condition by stimulating bone and cartilage reabsorption (Saklatvala, 1986; Bertolini *et al.* 1986) or help resolve the damage incurred during the inflammatory response by acting as a growth factor (Vilcek *et al.* 1986) and inducing the production of other cytokines which stimulate matrix production (Hajjar *et al.* 1987). Raised TNFα levels in body fluids have implicated the involvement of the protein in the pathogenesis of many inflammatory disorders including rheumatoid arthritis (Saxne *et al.* 1988), congestive heart failure (Levine *et al.* 1990), allograft rejection (Maury & Teppo, 1987), bowel necrosis (Tracey *et al.* 1986; Sun & Hsueh, 1988), multiple sclerosis (Selmaj.K *et al.* 1991) and bacterial meningitis (Leist *et al.* 1988).

1.1.6 Distribution and clearance of TNFα

TNFα release into the circulation following the administration of LPS has been extensively studied. Within minutes of endotoxin delivery there is a burst of serum TNFα that peaks in 1-2 hours and is rapidly cleared from the serum becoming undetectable by 4-6 hours, exhibiting a kinetic profile which is similar in most species (table 1.4). In these studies the rapid decline in TNFα bioactivity was not attributed to the presence of soluble TNFα-BP (Brittain *et al.* 1985; Remick *et al.* 1989), however increases in TNFα levels have been associated with increases in TNFα-BP (Spinas *et al.* 1990; Lantz *et al.* 1990b; Bemelmans *et al.* 1993a; Bemelmans *et al.* 1993b). Repeat administration of LPS two hours following the first injection failed to induce a

second serum TNFα peak and a state of tolerance was induced (Ulich *et al.* 1990). In accordance with earlier *in vitro* studies (Beutler *et al.* 1986a), *in vivo* studies demonstrated that dexamethasone was only effective at reducing serum TNFα levels if administered concomitant or prior to LPS (Remick *et al.* 1989). In studies monitoring TNFα production at ascites, where there was a ten fold increase in TNFα levels compared with plasma concentrations, dexamethasone if administered at the site of inflammation reduced both the local production and systemic (serum) appearance of TNFα protein. Both protein and mRNA levels at the ascites were reduced by dexamethasone, however administration 20 minutes following LPS did not reduce TNFα levels in the ascitic fluid or in the serum (Remick *et al.* 1989). The regulation of the decline of serum TNFα levels is believed to involve corticosteroids as serum levels in LPS challenged adrenalectomised animals remain at elevated levels. In this situation TNFα concentration is though to persist because the corticosteroid feedback loop, which normal reduces TNFα biosynthesis, is incomplete (Bertini *et al.* 1988).

Table 1.4: A comparison of kinetics of TNFα following *in vivo* LPS challenge. CFA: complete Freunds adjuvant.

Species	**Time of peak serum levels**	**Priming agent**	**Resolved to basal levels**	**Route of LPS administration**	**Reference**
rabbits	2 hours	none	4-6 hours	intravenous	(Beutler *et al.* 1985)
mice	2 hours	BCG	4 hours ?	intravenous	(Carswell *et al.* 1975)
	1 hour	none	4 hours	intraperitoneal	(Brittain *et al.* 1985)
	1 hour	CFA	4 hours	intraperitoneal	(Remick *et al.* 1989)
rats	1-2 hours	none	4 hours	intravenous	(Ulich *et al.* 1990)
	1-2 hours	*C. parvum*	4 hours ?	intravenous	(Ulich *et al.* 1990)

Studies injecting radio-labelled TNFα in mice provided evidence that the serum half-life of the protein is short, approximately 6 minutes in mice, and it is

rapidly (within 10 minutes of TNFα injection) concentrated in liver, skin, gastrointestinal tract, kidney and lung when administered via a peripheral vein. With the exception of lung and skin this accumulation was shown to be via TNFα specific binding sites (Beutler *et al.* 1985). Although a large amount of the administered TNFα was detected in the kidney only a small amount was observed in the urine as degraded products. The clearance of TNFα is thought to be via the kidney and liver. Experiments using nephrectomised mice (Bemelmans *et al.* 1993b) showed that following the administration of LPS the serum levels of TNFα peaked at 2 hours but the decline was slower than that observed in sham treated animals, with immunologically detectable levels still present 6 hours following challenge. It was suggested that this increase was accompanied by an increase in TNFα-BP as the bioactivity of the serum was similar to the serum levels of the sham treated animals and chromatography studies identified an immunological protein of 95kDa (possibly TNFα bound p75-sR). Similarly, following the administration of TNFα the biological activity, but not the immunological detectability, was rapidly removed from the circulation of nephrectomised animals. It would appear from studies following unilateral nephrectomy in mice that one functioning kidney is sufficient for the maintenance of normal TNFα clearance. The study by Bemelmans *et al.* 1993b, in agreement with others (Lantz *et al.* 1990a), implicated the involvement of the kidney in the clearance of TNFα and TNFα-sR from the circulation. Studies by Bemelmans *et al.* 1993a stress the importance of considering the methods used to measure TNFα levels when interpreting data. Although immunological detectable levels of TNFα (e.g. measured by ELISA) following LPS administration remained high the levels of biologically active TNFα rapidly returned to the level observed in control animals. This is due to immunological assays not distinguishing between the active and inactive (TNFα bound to the soluble receptor or fragmented TNFα) forms of TNFα, unlike the TNFα bioassays (e.g. WEHI 164 clone 13 cytolytic assay) which only measure biologically active TNFα.

1.2 Airway inflammation and asthma

Under normal physiological conditions an inflammatory reaction is an appropriate response by the body to rid the host of invading pathogens, but when the control on this system is in some way disturbed the inappropriate release of mediators from cells can lead to the destruction of healthy tissue, and the onset of a disease state.

Asthma, an inflammatory disease of the airways, displays reversible obstruction of the bronchi and bronchioles causing varying extents of dyspnea resulting from the much reduced airflow in the lungs. Asthmatics are often divided into one of 3 types; 1) extrinsic asthma, where the agent initiating the asthmatic attack originates outside the body, with initially a classical Type I hypersensitivity reaction occurring (atopic). 2) occupational asthma which is mainly atopic and 3) intrinsic asthma not being allergenic in nature (Corrigan & Kay, 1992). An asthmatic attack comprises of 2 main components: bronchial hyperreactivity, with the airways exhibiting increased sensitivity to a variety of non-allergenic stimuli and a localised inflammatory reaction occurring within the lung.

The allergic component of asthma was noted in the early 1900s and an extensive study by Huber & Koessler (Huber & Koessler, 1922) revealed increased eosinophil levels in the blood, sputum and lung tissue of asthmatic patients. The eosinophil has been shown to have a prominent role in asthma as: (1) asthmatic patients exhibit an increased number of eosinophils in the bronchial secretion and lung tissue and an increase in activated or hypodense eosinophils in the peripheral blood (which is often correlated with disease severity) (Bousquet *et al.* 1990; Fukuda *et al.* 1985), (2) increased eosinophil-derived toxic mediators including major basic protein (Frigas *et al.* 1981) and eosinophil cationic protein (Bousquet *et al.* 1990) are present in asthmatic sputum. Apart from lung eosinophilia histological studies show that the asthmatic patients' airway walls exhibit abnormal features; a degraded epithelium, a thickened basement membrane, increased intraluminal secretions and smooth muscle

hypertrophy and hyperplasia (Huber & Koessler, 1922; Laitinen *et al.* 1985; Jeffery, 1993; Dunnill, 1960; Barnes *et al.* 1988).

1.2.1 Allergic asthma

The allergic asthmatic attack consists of an early and late phase response with the first early phase response occurring within minutes of contact with the antigen, the agent to which the individual is sensitised. On initial exposure to the antigen an immune response occurs with the inappropriate synthesis of IgE antibodies. The total IgE level is much lower than the levels of other antibodies (in human serum 50-300ng/ml compared with 10mg/ml of IgG) however, the response is amplified via receptor activation (Sutton & Gould, 1993). The Fc region of IgE molecule binds to a high affinity receptor (FcεRI) present on the surface of mast cells, basophils, eosinophils and activated monocytes (atopic individuals) (Soussi Gounni *et al.* 1994; Maurer *et al.* 1994) and to a low affinity receptor (FcεRII) on a variety of cells, including B lymphocytes, T lymphocytes, eosinophils, macrophages and platelets. The bound IgE molecules act as receptors for the antigen. Cross linkage between the antigen and the cell bound IgE - FcεRI, principally involving mast cells and basophils, triggers cellular degranulation and mediator release from cellular granules by modifying the cell membrane permeability and activating a series of intracellular signalling pathways. Both mast cells, resident within the lung, and basophils contain a vast array of preformed mediators as well as the capacity to synthesise and secrete other mediators including cytokines, chemokines and biologically active lipids (Galli, 1993; Wodnar-Filipowicz *et al.* 1989; Plaut *et al.* 1989).

The released mediators exhibit an array of bronchoconstricting and inflammatory activities. Prior to the recognition of the involvement of cytokines eicosanoids were thought to be one of the major mediators of inflammation. The arachidonic acid metabolites leukotrienes (LT) C_4, LTD_4, LTE_4 (then called the slow reacting substance of anaphylaxis) were suggested, in the 1940's, to be released from

sensitised tissue following antigen challenge (Kellaways & Trethewie, 1940). Elevated eicosanoid levels have been detected in the bronchoalveolar lavage (BAL) of atopic asthmatics (Lam *et al.* 1988; Liu *et al.* 1990), especially LTE_4 (a metabolite of LTC_4 and LTD_4), prostaglandin (PG) D_2 and thromboxane (Tx) A_2 all of which induce bronchoconstriction. Unlike LTD_4 and LTE_4, which induce bronchconstriction within 5 minutes, the effect of LTC_4 is delayed occurring after 10-20 minutes (Drazen, 1988). Apart from inducing acute bronchoconstriction eicosanoids also exhibit inflammatory activities, for example PGD_2 increases vascular permeability and LTB_4 is a neutrophil and eosinophil chemoattractant (Ford-Hutchinson *et al.* 1980) both of which in turn enhance leukocyte accumulation. In the development of anti-asthmatic drugs much attention has also focused on two other mediators released from mast cells which demonstrate bronchoconstricting and inflammatory activities and are elevated in BAL from asthmatics, these being histamine (Casale *et al.* 1987) and platelet activating factor (PAF) (Averill *et al.* 1991; Wardlaw *et al.* 1986). Although H_1 receptor antagonists reduce anaphylaxis they have a limited role in the management of asthma, currently various approaches are under investigation and in clinical trials ranging from selective phosphodiesterase isoenzyme inhibitors to 5-lipoxygenase inhibitors, specific PAF and leukotriene antagonists to more specific muscarinic receptor antagonists. At present several of these new therapies relieve bronchospasm but their ability to prevent the underlying inflammatory damage to the lung is limited.

1.3 Evidence for the role of cytokines in asthma

The presence of cytokines in allergic airway inflammation has been demonstrated in the bronchial fluid and lung sections from asthmatic individuals and animal models of the disease. Changes in cytokine levels have been investigated at mRNA and protein levels using various techniques including polymerase chain reaction (PCR) and *in situ* hybridisation. Studies of bronchial fluid and biopsy specimens from asthmatic patients have shown elevated levels of several cytokines including IL-1, IL-6, (Borish *et al.* 1992; Mattoli *et al.* 1991; Marini *et al.* 1992) and TNFα (Gosset *et al.* 1991; Krishnaswamy *et al.* 1993; Cembrzynska-Nowak *et al.*

1993). In addition, recently three chemoattractant cytokines have also been identified in the BAL of antigen challenged asthmatics, lymphocyte chemoattractant factor (IL-16), macrophage inflammatory protein (MIP)-1α and IL-8 (Cruikshank *et al.* 1997; Yousefi *et al.* 1995).

Although the involvement of TNFα in asthma is acknowledged, the majority of studies have focused on the interrelationship between T lymphocyte derived cytokines, especially IL-5, and eosinophils. The activation of T lymphocytes is thought to be important in the late phase asthmatic response together with the recruitment of leukocytes into the lungs. Mature T lymphocytes are (mainly) divided into $CD4^+$ and $CD8^+$ populations based on surface expression markers. $CD4^+$ T lymphocytes are activated by processed antigen presented by antigen presenting cells (APC), in particular dendritic cells in the airway mucosa (Holt, 1993), in association with major histocompatibility complex (MHC) class II molecules. Less efficient APC active in the lung include macrophage/monocytes, epithelial cells, B lymphocytes and fibroblasts. The importance of $CD4^+$ T lymphocytes in antigen-induced pulmonary eosinophilia was demonstrated in antigen-challenged $CD4^+$ depleted mice where eosinophil accumulation was completely abolished (Gavett *et al.* 1994). $CD4^+$ lymphocytes are further divided into at least two sub-types, Th1 and Th2. This classification is based on the profile of cytokines released following activation (Mosmann *et al.* 1986), Th1 cells produce IL-2, TNFβ, and interferon (IFN) γ and Th2 cells produce IL-4, IL-5, and IL-10. Both subsets produce IL-3, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Mosmann *et al.* 1986, Minty *et al.* 1993).

Recently, a Th2-like cytokine profile in allergy and asthma has been indicated, implicating a role for Th2 lymphocytes (Robinson *et al.* 1992; Robinson *et al.* 1993). Both the BAL and the supernatant from peripheral blood T lymphocytes collected from allergic asthmatics have been shown to contain higher levels of IL-4 and IL-5 than samples from normal individuals (Walker *et al.* 1992; Walker *et al.* 1991). Furthermore, *in situ* hybridisation techniques have demonstrated increased IL-3,

GM-CSF, IL-4 and IL-5 mRNA in BAL cells from atopic asthmatics (Robinson *et al.* 1993). Asthmatic lung biopsies revealed that the IL-5 mRNA was localised to the inflammatory cells in the bronchial mucosa (Hamid *et al.* 1991). The Th1 derived cytokines, IL-2 and IFNγ, were unchanged or only marginally increased in asthmatic subjects at the mRNA and protein levels when compared with normal subjects (Robinson *et al.* 1992; Robinson *et al.* 1993; Walker *et al.* 1992). In addition, the expression of IL-3 and GM-CSF, hematopoietic growth factors which are linked to the activation and enhanced survival of eosinophils (Rothenberg *et al.* 1988; Owen *et al.* 1987), is increased in asthmatic subjects (Robinson *et al.* 1992; Kato *et al.* 1992; Broide & Firestein, 1991). Figure 1.3 shows a diagrammatic representation of the potential central role of Th2 lymphocytes in the propagation of the inflammatory response following antigen stimulation. Furthermore the importance of Th2 lymphocytes was indicated by the inhibition of antigen-induced BAL eosinophilia together with IL-4 and IL-5 production following the administration of IL-12 (Gavett *et al.* 1995), a cytokine which favours Th1 expression by suppressing the production of Th2 lymphocytes and Th2 cytokines (Manetti *et al.* 1993). The role of IL-4 and IL-13 is unclear but these cytokines are believed to be of importance in allergic reactions. IL-4 is a regulating factor in the commitment of naive T lymphocytes to the Th2 phenotype, which favours the initiation of an immediate antibody reaction (Anderson & Coyle, 1994). In addition to promoting the Th2 phenotype, IL-4 and IL-13 also promote isotype switching increasing B lymphocyte production of IgE and IgG4 (Snapper & Paul, 1987; Punnonen *et al.* 1993). Furthermore, IL-4 and IL-13 have demonstrated anti-inflammatory activities e.g. IL-4 and IL-13 inhibit monocyte /macrophage production of IL-1, TNFα and chemokines following LPS stimulation of the cells (Hart *et al.* 1991; Minty *et al.* 1993).

Once recruited to the lung tissue the eosinophils may then be primed by the eosinophil stimulating factors IL-3, GM-CSF and IL-5 released from the T lymphocytes. IL-5, a weak eosinophil chemoattractant (Wang *et al.* 1989), is of particular interest as unlike IL-3 and GM-CSF it promotes the selective differentiation

of myeloid cells into eosinophils (Campbell *et al.* 1988). IL-5 is also of importance as it prolongs eosinophil survival (Tai *et al.* 1991) and following IL-5 priming of the cells eosinophil release of cytotoxic products is enhanced (Coëffier *et al.* 1991). Hence, modulation of IL-5 bioactivity is a potential mechanism by which to reduce asthmatic

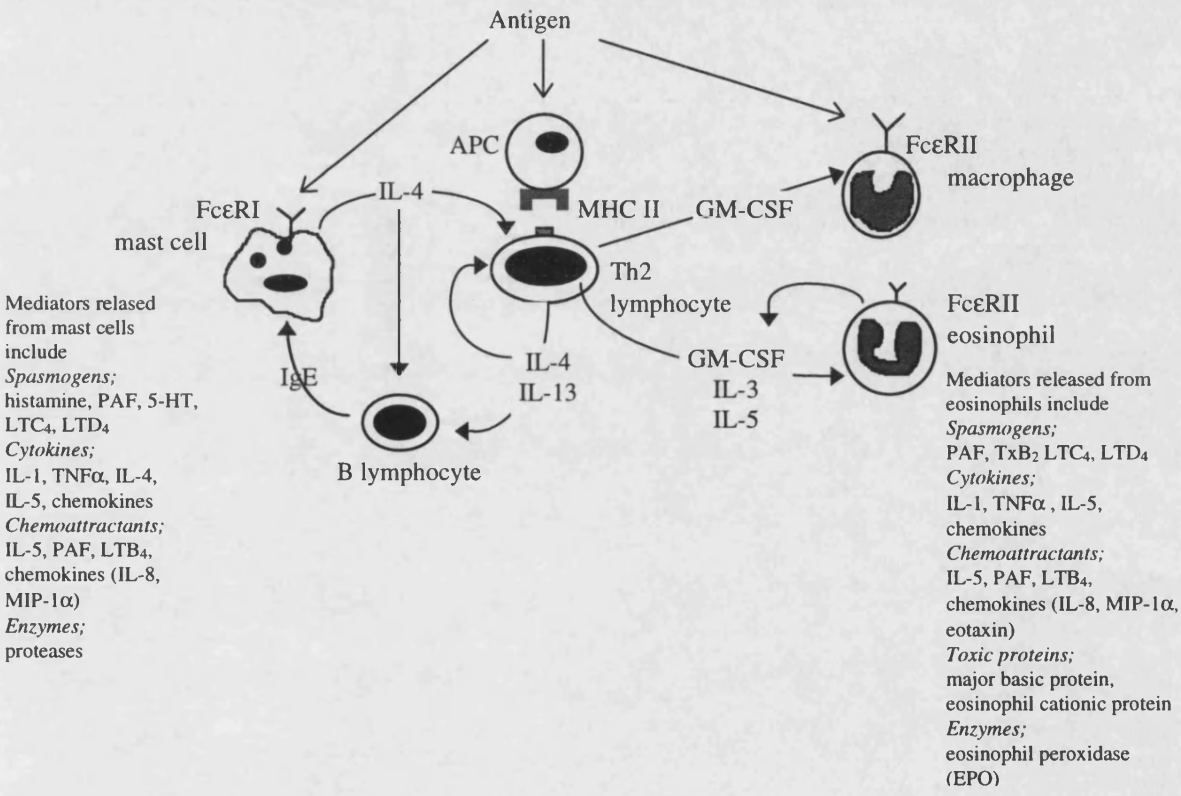


Figure 1.3: Diagram indicating the potential involvement of Th2 lymphocytes in the propagation of the allergic inflammatory response. Processed antigen is presented, by APC, in association with MHC class II molecule to Th2 lymphocytes. Released Th2-derived cytokines increase IgE production which promotes antigen-induced mast cell degranulation, and increase eosinophil activation and survival.

eosinophilia. Several studies have provided evidence that the administration of anti-IL-5 antibodies in asthmatic models reduces antigen induced lung eosinophil infiltration and bronchial hyperreactivity (Nagai *et al.* 1993; Van Oosterhout *et al.* 1993a; Gulbenkian *et al.* 1992; Chand *et al.* 1992; Mauser *et al.* 1993). Similarly, recombinant human IL-5 induces both lung eosinophilia and mild bronchial hyperreactivity (Fattah *et al.* 1990; Van Oosterhout *et al.* 1993b). Ohnishi (Ohnishi *et al.* 1993b; Ohnishi *et al.* 1993a) showed that following antigen challenge, the BAL from allergic subjects contained an activity which prolonged eosinophil survival in culture which was reduced by co-incubating the eosinophils with antibodies to IL-5 and this activity was correlated with eosinophil recruitment, degranulation and lung injury. It is also possible that as the local IL-5 concentration rises within the lung, significant levels of IL-5 could enter the circulation and induce a transient blood eosinophilia which could account for the high tissue eosinophil numbers. In support of this theory Collins *et al.* (Collins *et al.* 1995) have shown that after administration of IL-5 there is an increase in blood eosinophil numbers but a decrease in bone marrow levels.

In addition to neutralising antibodies, receptor antagonists have also become a useful tool in establishing the role of specific cytokines in animal models. The administration of an IL-1 receptor antagonist (IL-1ra) reduced antigen-induced lung eosinophil accumulation and bronchial hyperreactivity (Watson *et al.* 1993; Selig & Tocker, 1992). IL-1ra is a naturally occurring member of the IL-1 family which competes with IL-1 for the IL-1 receptor (reveiwed by Lennard, 1995). Interestingly, following aerosol administration of IL-1ra to antigen challenged guinea-pigs, TNFα bioactivity in the lung lavage was also reduced (Watson *et al.* 1993). This suggests that there is an interrelationship between these two pro-inflammatory cytokines and that the inhibition of cytokine activity may provide a possible therapeutic target in the treatment of asthma.

1.3.1 Leukocyte accumulation

In order for cells to migrate from the post capillary venules to the inflammatory site a series of events is required; rolling, activation and firm adhesion, diapedesis, and migration. For initial binding between the leukocyte and the vascular endothelium the leukocyte velocity must be reduced allowing the cells to roll along the endothelium. The phenomenon of cellular rolling is mediated by a family of adhesion molecules, called selectins (Lasky, 1992), which form loose tethering bonds between leukocytes and endothelial cells. The family comprises of transmembrane glycoproteins characterised by an NH_2-terminal lectin-like domain, an epidermal growth factor-like region and a number of complement regulatory protein repeat sequences. To date, three selectins have been identified E- selectin (endothelial), P-selectin (platelet) and L-selectin (leukocyte), the cellular expression and ligands for these proteins are summarised in table 1.5. The formation of loose interactions reduces the leukocyte flow velocity allowing the leukocytes to adhere strongly to the endothelium.

Firm adhesion is mediated via the interaction between two families of adhesion proteins, integrins and their immunoglobulin supergene family ligands (Albelda & Buck, 1990; Hynes, 1987). The integrins are a large family of transmembrane heterodimeric glycoproteins consisting of two non covalently linked α and β subunits. There are at least twelve different α subunits and eight different β subunits which associate to form at least fourteen different integrins. The $\beta 2$ integrins share a common β chain (CD18) and their expression is restricted to leukocytes, hence they are also known as leukocyte integrins. Three α subunits are associated with CD18, namely CD11a, CD11b and CD11c forming the integrins LFA-1, Mac-1 and p150,95 respectively. Each α subunit glycoprotein is similar in structure, having a NH_2-terminal signalling region, a relatively short cytoplasmic region and three putative divalent cation binding sites in the extracellular domain indicating the requirement of Ca^{2+} and Mg^{2+} for integrin function (Springer, 1990).

Table 1.5: A summary of the selectin adhesion molecules required for the rolling phenomena displayed in leukocyte-endothelium adhesion.

Selectin	Expression	Expression Time	Regulation	Ligand	Ligand expression	Reference
L-	leukocytes	constitutively expressed	receptor shedding following cellular activation	carbohydrate, e.g. sialyated Lewis a and x	endothelial cells	(Kishimoto *et al.* 1989)
P-	induced on endothelial cells, platelets	rapid, transient expression, translocated to surface ~10 minutes, preformed and stored in endothelial Weibel-Palade bodies, platelet α granules)	induced by histamine, thrombin. Reinternalised (30-60 minutes)	carbohydrate, e.g. sialyated Lewis a and x	leukocytes platelets	(Hattori *et al.* 1989)
E-	induced on endothelial cells	1-24 hours Peak 3-8 hours	transcriptionally regulated, *de novo* synthesis induced by TNF, IL-1, and LPS	carbohydrate, e.g. sialyated Lewis a and x	leukocytes	(Montgomery *et al.* 1991) (Bevilacqua *et al.* 1985) (Cotran *et al.* 1986)

The β1 (CD29) integrins are mainly receptors for extracellular matrix proteins including fibronectin and collagen. The principle β1 integrin involved in leukocyte adhesion is very late antigen (VLA)-4 (α4/β1, CD49d/CD29) which mediates cell-cell interactions in addition to fibronectin binding. VLA-4 is of particular interest in diseases which exhibit eosinophilia, such as asthma, as it is expressed constitutively on all leukocytes with the exception of neutrophils (Elices *et al.* 1990).

It appears that integrins are indirectly associated with the cytoskeletal actin filaments via a linkage involving talin, vinculin and other cytoskeletal associated molecules (Burridge *et al.* 1988). Integrin-induced changes in the cytoskeleton may be involved in the conformational changes in the extracellular domains of the integrin resulting in firm adhesion and a high avidity state ligand (Pardi *et al.* 1992). Cytoskeletal activation requires the β subunit and is possibly regulated via a tyrosine phosphorylation site in the cytoplasmic domain of β1 and β3 subunits (Tamkun *et al.* 1986). It has been postulated that phosphorylation results in the formation of a core of actin polymerisation and a polarised leading leukocyte edge which is in strong connection with the endothelial cell (Pardi *et al.* 1992). Integrin activation would therefore promote firm adherence to the endothelium by bond formation and by inducing leukocyte shape changes resulting in increased contact surface area of the cell.

The counter-ligands for the integrins are members of the immunoglobulin supergene family (Springer, 1990). Several members of this family are expressed by endothelial cells and are involved in leukocyte adhesion, these include intercellular adhesion molecule (ICAM)-1, -2 and vascular cell adhesion molecule (VCAM) -1. The interactions between integrins and their ligands are summarised in table 1.6. As VLA-4 binds to VCAM-1 this provides an alternative pathway for firm leukocyte adhesion which does not utilise CD11/CD18. The presence of eosinophils and mononuclear leukocytes, but not neutrophils, in inflamed tissue from patients

Table 1.6: A summary of integrin-immunoglogulin superfamily adhesion molecules required for firm leukocyte-endothelium adhesion.

Ligand	Ligand expression	Expression Time	Regulation	Integrin	Expression	reference
ICAM-1	endothelial cells, leukocytes and epithelial cells	12-48 hours peak 8-24 hours	transcriptionally regulated, induced by TNF, IL-1, LPS	LFA-1 Mac-1	constitutively on leukocytes (activatable)	(Dustin *et al.* 1986)
ICAM-2	monocytes, endothelial cells	constitutive		LFA-1		(Staunton *et al.* 1989)
VCAM-1	endothelial cells, monocytes	6 hours-1week peak 24 hours	induced by TNF, IL-1, LPS selectivley induced by IL-4	VLA-4	constitutively on leukocytes except for neutrophils (activatable)	(Osborn *et al.* 1989) (Elices *et al.* 1990) (Munoz *et al.* 1991)

Alternative names for integrin molecules:

LFA-1	CD11a/CD18	α_L/β_2
Mac-1	CD11b/CD18	α_M/β_2
VLA-4	CD49d	α_1/β_1

suffering from leukocyte adhesion deficiency syndrome (LAD) type I (Anderson *et al.* 1985), caused by a defected β2 subunit (Springer *et al.* 1984), reiterates the importance of this alternative pathway.

Once leukocytes have firmly adhered to the endothelium and diapedesed into the extracellular space by a process involving the cyclic modulation of integrin receptor avidity, they move to the inflammatory foci by chemotaxis. The leukocyte movement is directed by chemoattractants many of which are not leukocyte subtype selective, these include PAF, LTB_4 and C5a. Recently much attention has focused on the low molecular weight, leukocyte selective chemotactic cytokines, chemokines. These chemoattractants can be divided into distinct supergene families, C-X-C, C-C, and the recently identified C and C-X-X-X-C families, designated by the position of the first cysteine amino acid residue (Westwick *et al.* 1991; Bazan *et al.* 1997). The C-X-C chemokines are primarily chemotactic for neutrophils and are typified by IL-8, whereas the C-C family are principally chemotactic for mononuclear cells and eosinophils. Monocyte chemoattractant protein (MCP)-1, -2, -3, macrophage inflammatory protein (MIP)-1α/β and RANTES are all members of the C-C chemokine family. It has been proposed that chemokines use a hapatotactic mechanism by binding to glycosaminoglycan molecules present on endothelial cell surfaces (Rot, 1992). *In vitro* studies have suggested that the binding of chemokines to glycosaminoglycan molecules influences their effect on target cells. Webb *et al.* (Webb *et al.* 1993) demonstrated that the binding of IL-8 to heparin/heparan sulphate enhanced neutrophil responses and Tanaka (Tanaka *et al.* 1993) provided evidence that the C-C chemokine MIP-1β when bound to proteoglycan induces T lymphocyte adhesion. In addition to the chemotactic properties chemokines also directly effect leukocyte adhesion, IL-8 is capable of enhancing neutrophil adherence to endothelial cells by the upregulation of integrins (Carveth *et al.* 1989) and inducing the shedding of L-selectin (Detmers *et al.* 1990).

Once recruited to the airways, the leukocytes may be activated by antigen (via FcεRI/II) or agonist stimulation to induce the production of an array of inflammatory mediators. These substances can amplify the inflammatory response by promoting further leukocyte infiltration. Neutrophils, monocytes/macrophages (both phagocytic cells), and eosinophils are capable of generating numerous toxic substances, principally to remove foreign bodies but if inappropriately released these are often toxic to the surrounding tissue (Weiss, 1989; Gleich *et al.* 1988) and can destroy the architecture of the lung.

1.4 Animal model of airway inflammation

The OA sensitised guinea-pig demonstrates the characteristic features of asthma: bronchial hyperreactivity to a non-allergenic agent and lung inflammation with a pronounced lung eosinophilia. The guinea-pig is a classical model of allergic airway disorders which has been used by various workers since the early 1900's (Samter, 1949; Popa *et al.* 1973; Lefort & Vargaftig, 1978; Sanjar *et al.* 1990a). During this time several guinea-pig models of asthma have been developed focusing on both the early and late responses (Hutson *et al.* 1988; Iijima *et al.* 1987), and on airway hyperreactivity (Ishida *et al.* 1989) and lung eosinophilia (Dunn *et al.* 1987) induced by antigen or environmental / occupational stimuli. However, controversy about the realistic nature of the model arose as the primary antibody in guinea-pigs is IgG_1 whereas in man it is IgE. Andersson produced evidence that guinea-pig IgE levels could be increased by sensitising the animals using low antigen concentration in combination with aluminium hydroxide via intraperitoneal injection (Andersson, 1980a; Andersson & Bergstrand, 1981; Andersson & Brattsand, 1982; Andersson, 1982). He and others later demonstrated that this effect could be further enhanced by repeat administration of sensitisation solution over a period of weeks and by the administration of cyclophosphamide prior to the initial sensitisation (Andersson, 1980b; Andersson, 1981; Graziano *et al.* 1981). Ishida (Ishida *et al.* 1989) produced high IgE titers by incorporating *Bordetella pertussis* vaccine into the sensitisation solutions.

Various protocols have been used to produce antigen-induced airway inflammation, but important differences exist between individual models, in particular the routes of allergen sensitisation and challenge i.e. depot injection (as used by Andersson) or aerosol inhalation (Boichot *et al.* 1991; Ishida *et al.* 1989). Whereas in humans, inhalation of airborne allergens is a natural route for both sensitisation and challenge, delivery of aerosol antigen to guinea-pigs presents a problem as they are obligate nose breathers. It is predicted that 80% of the aerosol inhaled by conscious free breathing guinea-pigs is deposited in the nose with less than 12% being demonstrable in the lungs (Varley *et al.* 1991). Despite the methodological variations the antigen (notably OA) sensitised guinea-pig has been used extensively to assess the efficacy and mechanisms of action of potential anti-asthmatic drugs (Sanjar *et al.* 1990a; Havill *et al.* 1990; Sanjar *et al.* 1990b; Schellenberg *et al.* 1991; Manzini *et al.* 1993). In addition to the antigen sensitised guinea-pig, recently several other species have been used as models of antigen-induced airway inflammation including mouse (Lukacs *et al.* 1995c), rat (Sertl *et al.* 1988), rabbit (Marsh *et al.* 1985), sheep (Abraham *et al.* 1994), dogs (Chung *et al.* 1985) and primates (Wegner *et al.* 1990).

1.5 Aims of this project

The aim of this project was to assess the activity of TNFα *in vivo* and to investigate the role of TNFα in airway inflammation. Throughout this project guinea-pig models were used hence, in order to eliminate species selectivity, recombinant guinea-pig TNFα (gpTNFα) was produced. gpTNFα cDNA was cloned into *E. coli* and gpTNFα expressed and purified. gpTNFα was administered to non-sensitised (naive) animals and lung leukocyte accumulation characterised. Changes in bronchial hyperreactivity, airway leukocyte accumulation, the production of chemokine-like activity in the lung and airway TNFα levels in antigen-sensitised animals were investigated. Finally the effects of administering inhibitory cytokines and a TNFα antibody to animals, which were either antigen-sensitised or treated with gpTNFα, were examined.

2. Materials & Methods

2.1 Materials

All items were purchased from Sigma Chemical Company Ltd., Poole, Dorset UK. unless otherwise stated.

2-mercaptoethanol	BDH Laboratory Supplies Ltd., Lutterworth, UK
Aluminium hydroxide (F2200)	Reheis Ireland, Dublin, Ireland
anti DIG-AP Fab fragments	Boehringer Mannheim Uk Ltd., Lewes, UK
Antibodies:	
Monoclonal Hamster anti murine TNFα (IgG)	gift from Celltech, Slough, UK
Polyclonal Goat anti murine TNFα (IgG)	gift from Celltech, Slough, UK
Polyclonal Rabbit anti murine TNFα (sera)	gift from S. Kunkel Ann Arbor, USA
Polyclonal rabbit anti human factor X (IgG)	gift from DAKO Ltd., High Wycombe, UK
Aprotinin	Calbiochem Novabiochem Ltd., Nottingham, UK
Bordetella pertussis vaccine	Wellcome Foundation, Bechenham, UK
Bordetella bronchisepetica vaccine	Solvay -Duphar Veterinary, Southampton, UK
Calcium chloride	Fisons Pharmaceuticals, Loughborough, UK
Carbon dioxide	BOC Ltd., Guildford, UK
Cellophane membrane backing	Bio-Rad Laboratories, Hemel Hempstead, UK
Chromatography filter paper	Bio-Rad Laboratories, Hemel Hempstead, UK
CSPD	Boehringer Mannheim Uk Ltd., Lewes, UK
DIG-11-UTP	Boehringer Mannheim Uk Ltd., Lewes, UK
Digitonin	BDH Laboratory Supplies Ltd., Lutterworth, UK
dTTP	Boehringer Mannheim Uk Ltd., Lewes, UK
Dunkin-Hartley Guinea-pigs	University of Bath, UK
Electrophoresis molecular weight	Novex Experimental Technology, San Diego,

markers	USA (distributed by R&D systems)
Ethanol	Fisons Pharmaceuticals, Loughborough, UK
Factor Xa (bovine)	New England Biolabs Ltd., Hitchin, UK
Foetal calf serum	Gibco Life Technologies Ltd., Paisley, UK
Fura-2-acetoxymethyl ester	Calbiochem Novabiochem Ltd., Nottingham, UK
Gallamine	May and Baker Ltd., Dagenham, UK
Glacial acetic acid	Fisons Pharmaceuticals, Loughborough, UK
Glycerol	Fisons Pharmaceuticals, Loughborough, UK
Hams F12	Gibco Life Technologies Ltd., Paisley, UK
HBSS without calcium, magnesium and phenol red	Gibco Life Technologies Ltd., Paisley, UK
HBSS without phenol red	Gibco Life Technologies Ltd., Paisley, UK
Horse serum	Gibco Life Technologies Ltd., Paisley, UK
Hydrochloric acid	Fisons Pharmaceuticals, Loughborough, UK
Iso-pentane	BDH Laboratory Supplies Ltd., Lutterworth, UK
JH-4 clone 1	ECACC, Wiltshire, UK
Leishman's stain	R. A. Lamb Laboratory Supplies, London, UK
Magnesium chloride	Fisons Pharmaceuticals, Loughborough, UK
Marvel non-fat milk powder	Premier Brands UK Ltd., Stafford, UK.
Methanol	Fisons Pharmaceuticals, Loughborough, UK
OCT	Miles Inc. Diagnostic Division, Elkhart, USA
Penicillin	Gibco Life Technologies Ltd., Paisley, UK
Percoll	Pharmacia Biotech Ltd., St. Albans, UK
Polymyxin B columns	Pierce & Warriner, Chester, UK
Platelet activating factor	Bachem UK Ltd., Saffron Walden, UK
pMAL™-2 protein fusion and purification system	New England Biolabs Ltd., Hitchin, UK
PolyScreen PVDF transfer membrane	DuPont NEN Research Products, Boston, Massachusetts, USA
Protogel	National Diagnostics, Atlanta, USA

Recombinant human IL-1α/β	gift from Glaxo, Stevenage, UK
Recombinant human IL-8	gift from Glaxo, Stevenage, UK
Recombinant human IFNγ	Boehringer Mannheim Uk Ltd., Lewes, UK
Recombinant human MIP-1α	Preprotech, London, UK
Recombinant human MIP-1β	Preprotech, London, UK
Recombinant human RANTES	gift from Glaxo, Stevenage, UK
Recombinant human TNFα	gift from Bayer UK, Slough, UK
Recombinant human TNFα standards	NIBSC, Potters Bar, UK
RNAozl B	Tel-Test, Friendswood, Texas, USA
RPMI 1640	Gibco Life Technologies Ltd., Paisley, UK
Saline	Steripak, Runcorn, UK
Sodium chloride	Fisons Pharmaceuticals, Loughborough, UK
Sodium hydroxide	Fisons Pharmaceuticals, Loughborough, UK
Sodium pentobarbitone	Rhone Merieux, Dublin, Ireland
Sodium phenobarbitone	BDH Laboratory Supplies Ltd., Lutterworth, UK
Sterile distilled water	Steripak, Runcorn, UK
Sterile sodium bicarbonate (7.5%)	Gibco Life Technologies Ltd., Paisley, UK
Streptomycin	Gibco Life Technologies Ltd., Paisley, UK
WEHI 164 clone 13	NIBSC, Potters Bar, UK

2.2 Expression of gpTNFα

The expression and purification system used to produce recombinant gpTNFα from cDNA is summarised in figure 2.0 and detailed in sections 2.2.1-2.3.4. The cDNA was cloned and sequenced by Dr T. Yoshimura, NCI-FCRDC, Frederick, MD, USA, as described in section 2.2.1. The cDNA, provided inserted in pBluescript SK(-), was amplified by polymerase chain reaction (PCR) and used to express gpTNFα (sections 2.2.2-2.3.4).

2.2.1 cDNA for gpTNFα

gpTNFα cDNA was cloned from guinea-pig spleen cells essentially as described for guinea-pig IL-8 (Yoshimura & Johnson, 1993). Briefly, poly(A) RNA was prepared from guinea-pig splenocytes stimulated with concanavalin A (5μg/ml, 4 hours). A modification of the Gubler and Hoffman method (Gubler & Hoffman, 1983) was used to synthesis cDNA. This was used to prepare a library in a λ ZAP II vector (Short *et al.* 1988), and phages were probed with a 33 base oligo ^{32}P labelled probe based on a human TNFα cDNA using a sequence which was conserved between human and murine TNFα. Positive phagemids were rescued with helper phage and subcloned into pBluescript SK(-). cDNA was sequenced by the dideoxynucleotide triphosphate chain termination method (Sanger *et al.* 1977).

2.2.2 Transfection of gpTNFα cDNA into XL1-Blue

A 1 in 10 sub-culture of XL-1 Blue cells, from an overnight (37°C) culture, was grown for 2 hours in 40ml LB Broth (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, pH 7.0),with tetracycline (10μg/ml) at 37°C. The cells were harvested by centrifugation (3000g, 15min, 4°C), washed in 0.5 volume Milli Q H_2O and resuspended in a minimum volume of ice cold 10% glycerol (approx. 250μl). A 40μl aliquot of cells was placed in a cold 0.5ml tube and kept on ice. The gpTNFα

Figure 2.0: (please see next page).

gpTNFα cDNA

1. gpTNFα cDNA encoding the mature protein was amplified by PCR using flanking primers.

Bluescript KS(-) with gpTNFα cDNA inserted.

amplified gpTNFα cDNA.

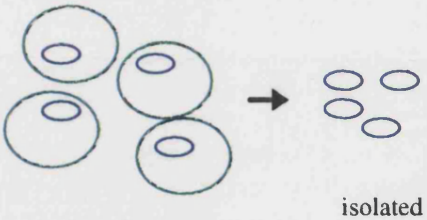
2. A T_4 polymerase and *Hind* III were used to enzymatically cleave cDNA in order to create cDNA with one blunt and one cohesive end.

during PCR the cDNA produced has non-uniform ends.

cDNA with one blunt end and a cohesive end created by *Hind*III.

pMAL-p2 plasmid

1. pMAL-p2 plasmid DNA was isolated from *E. coli.*



isolated pMAL-p2 plasmid

2. Plasmid DNA is double digested with *Xmn*1 and *Hind*III to create an insertion site which has a blunt end and a cohesive end corresponding to the cohesive end of the gpTNFα cDNA.

plasmid DNA with a blunt and cohesive (*Hind*III) ended insertion site.

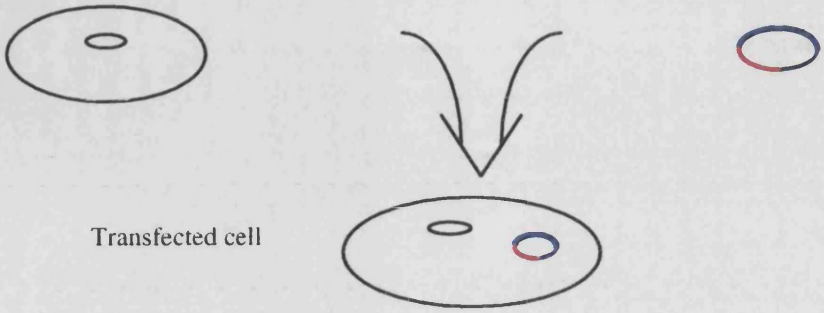
3. **Ligation of gpTNFα cDNA and pMAL-p2 plasmid.**

⇒ VECTOR

4. pMAL-p2 - gpTNF vector was cloned into the host TB1cells

E.coli HOST ***TB1***

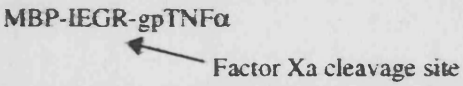
VECTOR



5. Transfected cells were induced by IPTG to express the fusion protein (MBP-gpTNFα).

6. MBP-gpTNFα was isolated from *E. coli* lysate by passing over an amylose column.

7. The fusion protein was cleaved with factor Xa.



8. Cleaved gpTNFα was purified by a series of affinity chromatography columns.

1) hydroxyapatite column 2) amylose column 3) polymixin B column

Figure 2.0: A simplified diagram to show the expression and purification of gpTNFα.
(MBP; maltose binding protein, the methodology is detailed in section 2.1-2.2)

supplied cDNA inserted in pBluescript SK(-) was washed twice in cold 70% ethanol, pelleted by microfugation, and vacuum dried before resuspending in 50µl Milli Q H_2O. cDNA template (2-5µl) was added to the 40 µl aliquot of XL-1 Blue cells and stored on ice. The remaining template was frozen at -20°C for future use. Cell-cDNA mixture (42-45µl) was transfered to a sterile electrophoresis cuvette. Electroporation was performed at 2.5kV, 200 Ohms, 25µFD with a electroporation time constant of approx. 5 ms. Immediately after electrophoresis 1ml of conditioned culture media (LB Broth + 1mM Mg^{2+}) was added, the cuvette was mixed and contents were transfered to a conical culture flask containing 2ml LB Broth. The cells were agitated at 37°C for 1 hour.

Following incubation, 100µl of XL-1 Blue cell culture was plated out on an agar LB Broth plate (LB Broth, 1.5% agarose) containing ampicillin (100µg/ml) and tetracycline (12.5µg/ml) which had been over dried to remove surface moisture. Inverted culture plates were incubated at 37°C overnight. Since XL1-Blue cells are tetracycline resistant and ampicillin sensitive, whereas the cDNA template pBluescript SK(-) are tetracycline sensitive and ampicillin resistant, only cultures which contain the template will grow in media containing tetracycline and ampicillin.

2.2.3 Storing bacterial cultures at -70°C

A stable frozen stock of cDNA was generated by freezing the XL1-Blue colonies which contained the Bluescript / cDNA template, to ensure a continued supply of non-contaminated template which is readily accessible. Bacterial cultures were prepared for storage by adding 0.75ml LB Broth to the agar surface and suspending the cultures in the media by gentle scraping. The cells were removed, placed in a 2ml screw capped sterile tube and an equal volume of LB Broth containing 30% glycerol added. The tube was vortexed to ensure that the glycerol was completely dispersed and cultures were stored at -70°C.

2.2.4 Preparation of oligo nucleotide primers

A putative start site for mature gpTNFα was determined from the base sequence of gpTNFα cDNA, provided by Dr T. Yoshimura, and the published start sequences of human, murine and guinea-pig TNFα. Oligonucleotide primers were designed to amplify the region of cDNA encoding the putative mature protein. Primer sequences are shown in figure 2.1.

Forward primer 5' T C A G C T T C T C A A A A C G A T A A T
Reverse primer 3' G T G A G T C A G G A A G G C C A A T T C G A A C C C

Figure 2.1: The primers used for the amplification of gpTNFα cDNA. The reverse primer has a *Hin*d III cleavage site incorporated at the 5' end (underlined).

The primers (Biochemistry, Bath University) were supplied attached to resin columns and displaced using concentrated ammonia solution (88%). Ammonia (0.2ml) was applied to the column every 20 min with a 1ml syringe and the eluted primer collected in a 5ml syringe. The eluted primer was transferred to a screw capped 1.5ml tube and sealed with polythene film. This was then placed in a glass bottle containing 0.5ml ammonia solution, the bottle was capped, double sealed and incubated overnight at 55°C. Samples were cooled to room temperature, excess moisture removed by vacuum drying the samples for 1.5-2 hours. The dried primers were precipitated by the addition of 200µl Milli Q H_2O, 20µl 3M sodium acetate and 400µl cold ethanol (-20°C) followed by microcentrifugation (12,000g, room temperature, 10 min). The above precipitation was repeated then the single stranded DNA was washed in 1ml 70% cold ethanol and repelleted by microcentrifugation. The

ethanol was removed and the pellet vacuum dried for 5-10 min before solubilising in 300µl Milli Q H_2O.

Oligonucleotide concentration was ascertained by optical density (OD) at 260nm, assuming an OD_{260} 1.0 = 37µg/ml for single stranded RNA/DNA. Since the average molecular weight of a nucleotide is 350 the molarity of each primer was calculated.

2.2.5 Plasmid vector generation

A plasmid vector is a small circular molecule of double-stranded DNA used to transport the cDNA of interest into a host cell, which upon replication produces large quantities of cDNA. The plasmid, pMAL-p2 (New England Biolabs Ltd., Hitchin, UK), was used since it encodes a maltose binding protein (MBP) which is coexpressed with the gpTNFα enabling simple purification of the MBP-gpTNFα fusion protein using a MBP affinity column.

As the gpTNFα primers were designed to only amplify the region of gpTNFα cDNA sequence required, it was not necessary to excise the cDNA template from the pBluescript SK(-) prior to amplification by PCR. The concentration of the cDNA template previously prepared and stored at -20°C was determined by OD at 260nm, assuming OD_{260} 1.0 = 50µg/ml double stranded DNA.

2.2.5.1 Amplification of gpTNFα cDNA

The cDNA template was amplified using PCR, to minimise contamination the reagents were added to the PCR vials in the order stated in table 2.1. Nujol mineral oil (75µl) was layered on the top of the reaction mixture to prevent evaporation during the heating cycles. cDNA was amplified in a Crocodile II thermal cycler (Appligene, Pleasanton, California) using an initial 2 min at 95°C followed by 30 cycles of

denaturation (95°C, 60 s), annealing (55°C, 60 s) and extension (72°C, 60 s) followed by a final extension at 72°C for 5 min. The expected PCR product, a low molecular weight fragment of 540 base pairs, was visualised by running on a 1.5% agarose gel containing 0.5µg/ml ethidium bromide (2.2.5.2).

Table 2.1: PCR-amplification protocol detailing the order in which the reagents were added to minimise contamination.

Order to add reagents	Final concentration
Milli Q H_2O	
MgCl	2.5mM
NH_4 Buffer	1x
dATP/dCTP/dTTP/dGTP	200µM
PRIMERS Forward	0.5µM
Reverse	0.5µM
Taq	25U/ml
TEMPLATE	10ng/ml - 10µg/ml
Total volume	100µl

2.2.5.2 Tris-Borate EDTA (TBE) agarose gels

Briefly, agarose (1.5% w/v) was solubilised in 30ml TBE buffer (45mM Tris-borate, 1mM EDTA) by heating in a microwave oven for 1-2 min. The solution was allowed to cool to room temperature and Milli Q H_2O added to compensate for liquid lost by evaporation during heating. Ethidium bromide was added to a final concentration of 0.5µg/ml. The cooled solution was poured into a gel plate (15cm x 6cm) containing an 8 lane comb (0.5cm/lane) and allowed to set. The gel was immersed in a running tank containing TBE buffer.

An aliquot of PCR sample (10µl) was mixed with 2µl 5x sample buffer1 (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll-type 400), and the samples loaded into the gel wells. *Hin*d III molecular weight standards (2µl) were run in an outer lane to ensure the product was of the correct size. The gel was electrophoresed at 200V for 15-20 min. Following visualisation using a UV light source the product from the optimal PCR reaction (i.e. maximum product obtained using the minimum template concentration) was immediately excised from the agarose gel and 'cleaned' (see 2.2.5.3).

2.2.5.3 'Cleaning' DNA recovered from agarose gels or in solution

The DNA was 'cleaned' using a Geneclean kit (BIO 101 Inc., La Jolla, CA) according to manufacturer's instructions. Briefly, the slice of agarose gel containing the cDNA was weighed and incubated with 4.5 volumes sodium iodide (6M) and 0.5 volume TBE modifier at 50°C for 10 min. Once the gel had melted, glassmilk (5µl) was added, the solution mixed and incubated on ice for 10 min. The DNA was pelleted by microcentrifuging for 30 s, the supernatant removed, and the pellet washed three times in 0.5ml NEW Wash. On the last wash the supernatant was removed, the pellet was then re-spun and any remaining supernatant removed to obtain a dry pellet. Milli Q H_2O or low salt buffer (5µl) was added to the dry pellet and incubated at 50°C for 10 min. During this step the DNA disassociates from the glass particles into the low salt solution. The glass particles were pelleted by microcentrifugation and the supernatant, containing DNA, was transfered to a sterile 500µl tube. The volume of DNA was restored to 10µl with Milli Q H_2O and a 1µl sample was electrophoresed on a 1.5% agarose gel to ensure that the DNA has dissociated from the glassmilk. Remaining DNA was stored at -20°C.

To 'clean' DNA which is in solution, 3 volumes sodium iodine (6M) and 5µl glassmilk were added to the DNA, the solution mixed and incubated on ice for 10 min. The DNA was then pelleted and washed in NEW Wash as described above.

2.2.5.4 Preparing gpTNFα cDNA for insertion into pMAL-p2 plasmid

When using PCR to amplify a length of cDNA a phenomenon of 'overshooting' often occurs whereby a series of adenosine residues are added to the 3' end of the template. This overshoot is removed by performing T4 polymerase digestion. T4 polymerase has a potent 3′→5′ exonuclease activity which removes protruding 3′ termini from double stranded DNA to create a blunt ended fragment.

The 'cleaned' cDNA was added to the T4 polymerase digestion buffer (50mM Tris hydrochloride pH 8.0, 5mM $MgCl_2$, 5mM DTT, 100µg/ml BSA, 10mM dNTPs, final volume 49µl). The reaction was started by the addition of 1µl T4 polymerase (3U/µl) and the mixture incubated at 11°C (optimum temperature for T4 polymerase enzymatic activity) for 20 min. The reaction was stopped by denaturing the enzyme at 75°C for 10 min. A 5µl sample was run on a 1.5% agarose gel to ensure that the DNA was not fragmented during digestion. The DNA in solution, was 'cleaned' using geneclean kit detailed in 2.2.5.3, and finally resuspended in 10µl Milli Q H_2O.

In order to create a cohesive end which will orientate the cDNA when it is inserted into the pMAL-p2 plasmid the cDNA must be treated with *Hin*d III. A *Hin*d III cleavage site had been incorporated into the primer sequence (figure 2.2).

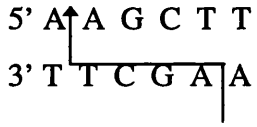


Figure 2.2:. *Hin*d III cleavage site. The enzyme recognises the 6 bp and cleaves along the black line.

The T4 polymerase treated cDNA was incubated at 37°C for 2-3 hours with 10U *Hin*d III in 10mM Tris.Cl, 10mM $MgCl_2$, 50mM NaCl, 1mM DTT (final volume

20µl). *Hind* III was inactivated by incubating at 75°C for 20 min. A sample of reaction product (2µl) was run on a 1.5% agarose gel to ensure the cDNA had not been fragmented. DNA was 'cleaned' using the Geneclean kit (2.2.5.3) and resuspended in 10µl Milli Q H_2O. The amplified gpTNFα cDNA was now suitable for cloning into the plasmid, and was stored at -20°C until required.

2.2.6 Preparation of pMAL-p2 plasmid

2.2.6.1 Isolation of pMAL-p2 plasmid DNA

Plasmid DNA was isolated using a Qiagen kit (Qiagen, Hilden, Germany). A 500ml secondary culture of *E.coli* containing the correct plasmid (pMAL-p2) was grown overnight at 37°C in LB Broth containing ampicillin (50µg/ml). The cells were pelleted by centrifugation (4000g, 15 min, 4°C) and resuspended in 10ml ice cold buffer P1 (50mM Tris hydrochloride, 10mM EDTA pH 8.0, 100µg/ml RNase A). The suspension was transferred to a sterile ultracentrifuge tube containing 10ml prewarmed (37°C) buffer P2 (200mM NaOH, 1% SDS) and gently mixed by inverting the tube 5 times, producing a viscous lysate. After 4 min 10ml chilled buffer P3 (3.0M potassium acetate pH 5.5) were added and the mixture gently mixed by inverting. The mixture was incubated on ice for 30 min prior to ultracentrifugation (25,000g, 90 min, 4°C). The clear, yellow, supernatant was carefully removed, promptly transferred to a 50ml tube and placed on ice.

A Qiagen Tip 500 column was washed with 10ml QBT buffer (750mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7.0) by gravity induced flow. The collected supernatant was added to the column and washed through with 2 x 30ml QC buffer (1.0M NaCl, 50mM MOPS, 15% ethanol, pH 7.0). DNA was eluted with 15ml QF buffer (1.25M NaCl, 50mM Tris hydrochloride, 15% ethanol, pH 8.5) and the eluate collected in a sterile glass ultracentrifuge tube.

Isopropanol (10.5ml) was added to the eluate and ultracentrifuged (25,000g, 60 min, 4°C) to precipitate the DNA. Supernatant was carefully removed from the pelleted DNA, which resembled a fine spray on the glass, the pellet was washed with 15ml 70% cold ethanol and ultracentrifuged (25,000g, 45 min, 4°C) to repellet. The supernatant was discarded and the DNA pellet was air dried for 10 min before being redissolved in 200-500µl TE buffer (10mM Tris hydrochloride, 1mM EDTA pH 8.0).

DNA was quantified by measurement of OD at 260nm and 280nm and the 260:280 ratio was calculated to assess the amount of background contamination in the preparation. The ratio obtained was >1.8 indicating low contamination with proteins. The DNA content was determined assumming OD_{260} 1 = 50µg/ml double stranded DNA.

In order to produce a MBP fusion protein, the gpTNFα cDNA must be incorporated into the circular plasmid structure in the same translational reading frame as the plasmid's *malE* gene. To create an insertion point for gpTNFα cDNA a section of the plasmid's *malE* gene was enzymatically removed using *Xmn*I to create the blunt end and *Hin*d III to create a sticky end which will adhere to the sticky (*Hin*d III cleaved) end of the gpTNFα cDNA. *Xmn*I and *Hin*d III were chosen as both enzymes have unique cleavage sites in the *malE* gene (figure 2.3) preventing fragmentation of either the plasmid or gpTNFα cDNA.

2.2.6.2 Double digestion of pMAL-p2 with XmnI and Hind III

pMal-p2 plasmid DNA (10µg) was incubated with *Xmn*I 12U, 2µg BSA, in 10mM Tris.Cl, 10mM $MgCl_2$, 50mM NaCl, 1mM DTT (final volume 20µl), at 37°C (the optimum temperature for *Xmn*1 enzymatic activity) for 2-3 hours. The enzyme was heat inactivated by incubating the mixture at 75°C for 20 min. To assure *Xmn*1 digestion had occurred digested pMal-p2 DNA (2µl) and an equal amount of DNA

from control non-digested pMal-p2 DNA was run and visualised on a 1.5% agarose gel. The digested plasmid had fewer base pairs, hence ran further on the gel than the non-digested fragment. The remaining digested fragment was 'cleaned' (2.2.5.3) and resuspended in 10µl Milli Q H_2O. *Hind* III digestion was performed by incubating 10µl digested plasmid with 10U *Hind* III in 10mM Tris.Cl, 10mM $MgCl_2$, 50mM NaCl, 1mM DTT (final volume 20µl), at 37°C for 2-3 hours. The enzyme was heat inactivated (75°C, 20 min) prior to running 2µl samples of, control non-digested, single digested, and double digested DNA on a 1.5% agarose gel which was visualised to ensure that each sample contained only a single band and that the double digested product had fewer base pairs than the non-digested control. The double digested product was 'cleaned' (2.2.5.3), resuspended in Milli Q H_2O to a final volume of 10µl and stored at -20°C.

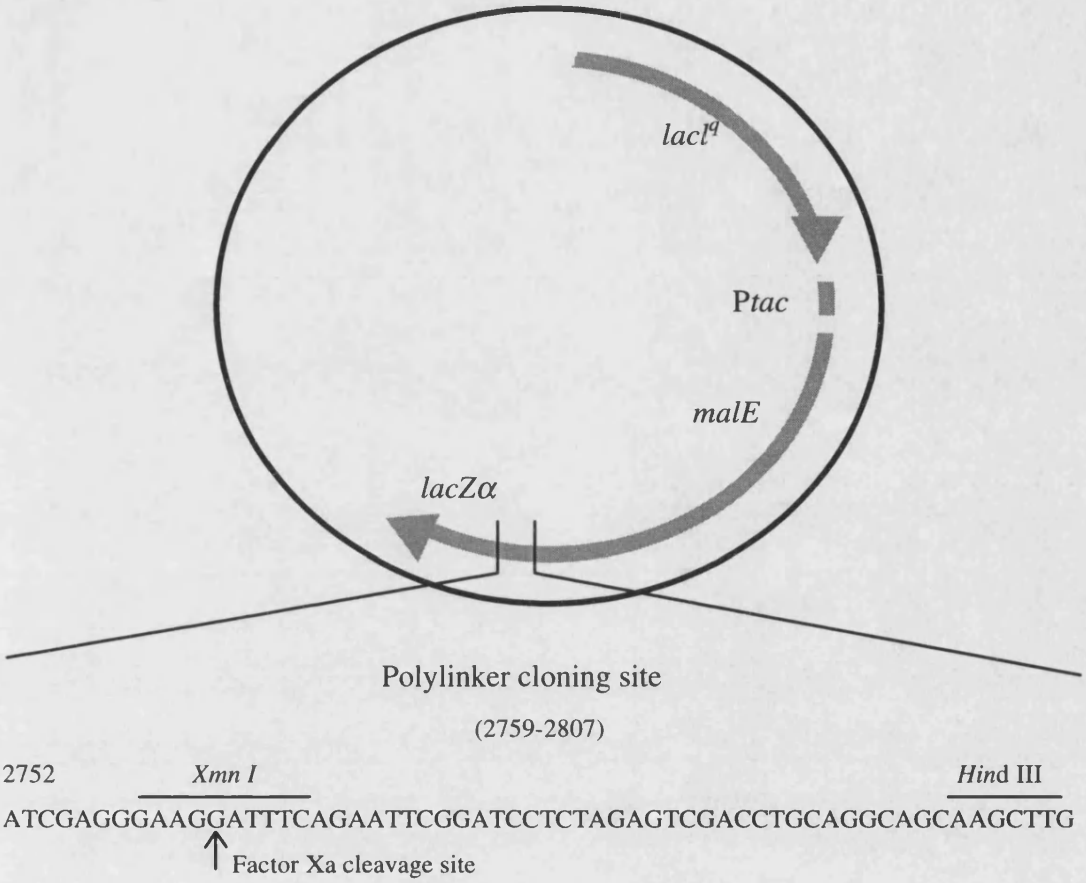


Figure 2.3: A map of the pMAL-p2 plasmid. The plasmid contains the inducible Ptac promoter, positioned to transcribe a *malE-lacZα* gene fusion. The *lacI*^{q} gene encodes the Lac repressor, which turns off transcription from P*tac* until IPTG is added. The polylinker provides restriction endonuclease sites to insert the gene of interest, fusing it to the *malE* gene. The unique *Xmn I* and *Hin*d III cleavage sites are located in the polylinker. Nucleotide numbering starts at the beginning of the *lacI*^{q} fragment. (Adapted from pMAL-p2 map, New England Biolabs, Hitchin, Hertfordshire).

2.2.7 Cloning of gpTNFα cDNA into pMAL-p2 plasmid

Ligation of the plasmid and cDNA was performed using T4 DNA ligase which catalyses the formation of phosphodiester bonds between adjacent 3'-hydroxyl and 5'-phosphate termini in DNA. Briefly, double digested plasmid (4µl) was added to the reaction mixture containing 1200U T4 DNA ligase, 5µl gpTNFα cDNA in 50mM Tris.Cl (pH 7.5), 10mM $MgCl_2$, 0.75µg BSA, 10mM dithiothreitol, 1mM ATP (final volume 30µl). The reaction mixture was left at room temperature overnight to ligate (T4 DNA ligase optimal ligation temperature is 16°C) and the reaction stopped by heat inactivation (75°C, 20 min). The ligation mixture was 'cleaned' (2.2.5.3) and resuspended in a final volume of 10µl in Milli Q H_2O. The ligated plasmid-gpTNFα cDNA construct, the vector, was stored at -20°C.

2.2.8 Preparing the host cell

To prepare the host cell, competent *E. coli* (TB1) cells were cultured from frozen stocks (-70°C). LB Broth (20ml) was innoculated with TB1 cells and cultured overnight at 37°C. A 1 in 20 sub-culture was grown in 10ml prewarmed LB Broth for a further 2-3 h, at 37°C, until the cell density was approximately $2x10^8$ cell/ml, OD 600nm of 0.2.

2.2.9 Cloning of vector into host cell

Electroporation of the vector (pMAL-p2-gpTNFα) and host cell (TB1) was performed as previously described (2.2.2). Briefly, 40µl TB1 cells was mixed with either 2-5µl vector or 2-5µl Milli Q H_2O and subjected to electroporation. Cells were incubated in 1ml conditioned culture media (1 h, 37°C, shaking). Control and vector containing-cells (100µl/plate) were plated on agar plates containing 100µg/ml ampicillin and agar plates containing 100µg/ml ampicillin, 80µg/ml Xgal, 0.16mM

IPTG. Plates were incubated, inverted, at 37°C overnight before assessing the colour of the colonies on the plates visually.

The presence of white colonies on the plate containing Xgal and IPTG indicated the disruption of the *malE-lacZα* gene which results in the inactivation of the β-galactosidase α fragment, hence the cells were unable to metabolise Xgal into the blue product bromochloroindole, table 2.2.

Table 2.2: The expected growth of TB1 cells on agar plates containing ampicillin.

TB1 Cells :	**agar plate**	**agar + Xgal + IPTG plate**
control	No colonies	No colonies
vector insertion	White colonies	Blue + White colonies

A master plate (agar + 100μg/ml ampicillin) and an agar plate containing 100μg/ml ampicillin, 80μg/ml Xgal, 0.16mM IPTG were prepared for sub-culturing the vector inserted colonies. Identical guideline grids with numbered squares were placed under each plate and the start position marked on the plates. Using sterile toothpicks colonies from the overnight culture plate without Xgal + IPTG were randomly picked by touching the surface of a single colony with the toothpick and then pricking identical squares on the master and Xgal/IPTG plate (figure 2.4), this was repeated until all the squares had been innoculated with a single colony. Both plates were incubated, inverted, 37°C overnight.

Five white colonies and 1 blue colony on the plate containing Xgal + IPTG were selected, randomly, and the corresponding colonies on the master plate i.e. colonies that have not seen IPTG were picked and streaked onto separate agar plates (containing 100μg/ml ampicillin) and cultured overnight. The blue colony was used as a control since it contained cells which had not incorporated the vector. The white colonies contained a disrupted *malE-lacZα* gene, but the vector may still not have

been inserted correctly. Due to the strength of the P*tac* promoter, transformants taken from a plate containing IPTG can contain mutant plasmids that have either 1) lost part or all of the fusion gene or 2) no longer express it at high levels, thus colonies selected for sub-culturing should have had no contact with IPTG.

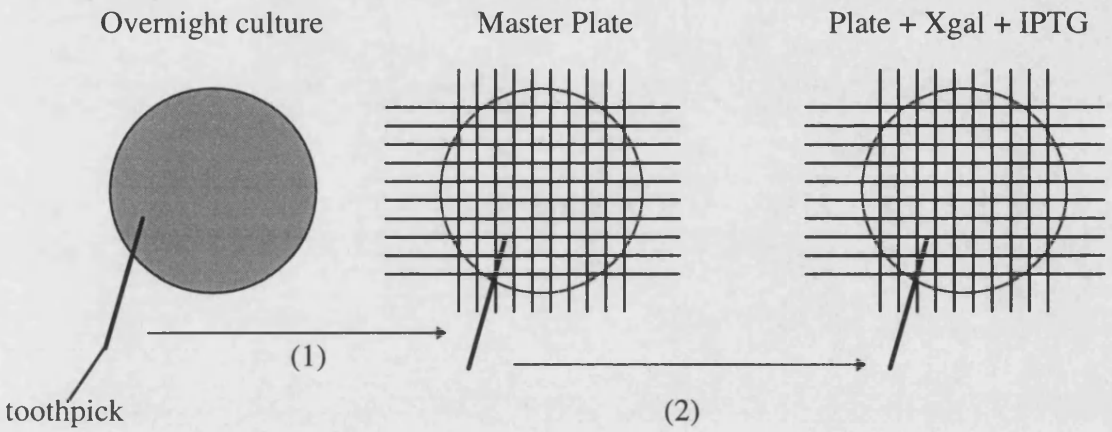


Figure 2.4: Selection of individual colonies to identify those containing the vector. A toothpick was used to select a colony from the overnight culture plate and seed onto the master (1) and a corresponding Xgal/IPTG plate (2).

Aliquots (20ml) of LB Broth containing ampicillin (100µg/ml) were inoculated with single colonies from each plate and cultured overnight in a shaking water bath at 37°C. A 1.5ml aliquot from each flask was sub-cultured into 20ml pre-warmed LB Broth containing ampicillin (100µg/ml) and incubated at 37°C for 2-3 h, until the cell density was approx. 2 x 10^8 cells/ml.

2.2.10 Screening for the presence of vector insertion

A 1ml aliquot was removed from each culture prior to induction of the fusion protein, samples were microcentrifuged, the pellets were resuspended in 50µl SDS-

sample buffer (see section 2.5.1) and retained on ice until required (these were the uninduced samples). IPTG (0.3mM) was added to each of the cultures incubated for a further 2 hours to induce fusion protein expression. Following induction, a 0.5ml sample of each culture was removed, microcentrifuged and the pellets resuspended in 100µl SDS-sample buffer (these were the induced samples). The uninduced and induced samples (15µl) were run on two 10% SDS polyacrylamide gels which were used for protein staining and western blotting respectively (see section 2.5). The relative molecular mass of gpTNFα is ~17kDa, the MBP-β-galactosidase fusion protein has a mass of ~ 51kDa and the gpTNFα-MBP fusion protein is ~60kDa. Hence, the culture which demonstrated the most predominant protein band shift, corresponding with the incorporation of the gpTNFα gene, following IPTG induction was chosen for the production of gpTNFα. This culture was referred to as C/MBP-gpTNFα.

A pilot experiment was carried out to assess which cellular fraction contained the MBP-gpTNFα fusion protein. An aliquot (0.8ml) of an overnight culture of C/MBP-gpTNFα was use to innoculate 80ml rich media (1% bacto-tryptone, 0.5% bacto-yeast extract, 0.5% NaCl, 0.2% glucose, 100µg/ml ampillicin). The culture was grown at 37°C for 2 hours prior to removing a 1ml sample of the uninduced culture, this was microcentrifuged and the cell pellet resuspended in 50µl SDS-sample buffer. The sample was vortexed and stored at -20°C. Following the addition of 0.3mM IPTG the remaining culture was incubated for a further 2 hours when a 0.5ml sample was removed. This induced sample was microcentrifuged, the cell pellet resuspended in 100µl SDS-sample buffer and stored at -20°C.

The cellular fraction of the culture was collected by centrifugation (4000g, 10 min, 4°C) and the pellet resuspended in 10ml column buffer (20mM Tris pH 7.4, 200mM NaCl, 1mM EDTA). The suspension was frozen (-20°C) for at least 18 hours. Once thawed, the cells were lysed by sonication (10 cycles of 30 s sonication, 30 s

rest) in an ice-water bath. The lysed cells were pelleted (9000g, 20 min, 4°C), the supernatant decanted and placed on ice, this was called the 'soluble crude extract fraction'. The pelleted cellular debris was resuspended in 10ml column buffer, this was the 'insoluble crude extract fraction'. Samples of the soluble (10µl) and insoluble (10µl) crude extract fractions were each added to 10µl SDS-sample buffer, and stored at -20°C.

To ensure that during expression the fusion protein had not lost the ability to bind to the amylose resin a small volume was tested. Amylose resin (200µl) from the amylose affinity column (see 2.3.1) was washed twice in 1.5ml column buffer and resuspended in 200µl. 50µl of amylose resin slurry was mixed with 50µl crude extract and incubated on ice for 15 min. The sample supernatant was discarded after microcentrifugation and the pellet washed with column buffer and resuspended in 50µl SDS-sample buffer. This was called the 'protein bound to amylose' fraction. Pilot experiment SDS samples (table 2.3) were boiled for 5-10 min, microcentrifuged for 1 min, run on a 15% polyacrylamide gel and stained with Coomassie blue.

Table 2.3: Volume of each pilot experiment/SDS sample loaded onto an SDS gel.

Sample	Volume sample/gel lane
Uninduced cells	20µl
Induced cells	20µl
Soluble crude extract	10µl
Insoluble crude extract	10µl
Protein bound to amylose	20µl

2.2.11 Production of gpTNFα-MBP fusion protein

The pilot experiment demonstrated that the fusion protein was expressed as a soluble protein in the crude extract fraction hence, the pilot experiment was scaled up. Rich media (1l) was inoculated with 10ml of an overnight culture of C/MBP-gpTNFα and grown at 37°C for 2 hours shaking. The culture was induced with IPTG (0.3mM) and incubated for a further 2 hours. The culture was centrifuged (4000g, 20 min, 4°C), and the pellet resuspended in 50ml cold lysis buffer (20mM Tris pH 7.4, 200mM NaCl, 1mM EDTA 40U/ml aprotonin, 1mM phenyl methyl-sulfonyl fluoride, 1ng/ml leupeptin, 4°C). The cells were frozen at -20°C for at least 18 hours.

Protein degradation during harvesting and cell lysis was minimised by performing all steps at 4°C and in the presence of protease inhibitors. The suspension was thawed, placed in an ice-water bath and sonicated in short pulses (10 cycles of 30 s sonification, 30 s rest). The insoluble material was pelleted by centrifugation (9000g, 20 min, 4°C), the supernatant decanted off and retained on ice. The supernatant, which was the soluble crude extract containing the fusion protein, was diluted 1 in 5 with column buffer and applied to the amylose resin column.

2.3 Purification of gpTNFα

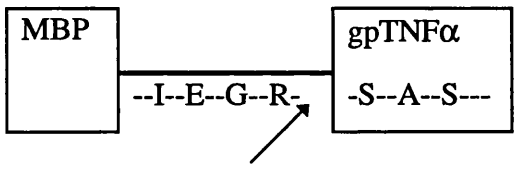
2.3.1 Affinity chromatography over amylose

Amylose resin (New England Biolabs Ltd., Hitchin, UK) was poured in a 2.5 x 10cm glass chromatography column and allowed to settle. All subsequent steps were performed at 4°C. The column was washed with 200ml column buffer (20mM Tris.Cl, 200mM NaCl, 1mM EDTA) and the flow rate adjusted to 1 ml/min. The diluted soluble crude extract was applied and the column washed overnight with a further 300ml column buffer.

The fusion protein was eluted with 60ml column buffer containing 10mM maltose and collected in 3ml fractions. Fractions containing protein, assessed by measuring the OD at 280nm, were pooled (fractions 1-8) and the sample was stored at -20°C. The amylose column was regenerated by washing with 25ml distilled water, 75ml 0.1% SDS, 75ml distilled water, 75ml column buffer, 50ml column buffer 0.05% NaN_3, and stored at 4°C. The column was reused up to 5 times.

2.3.2 Cleavage with factor Xa

Cleavage of the MBP from gpTNFα was performed by factor Xa cleavage using the specific amino acid recognition sequence, IEGR, incorporated into the vector design (see figure 2.5). As thrombin cleaves at the arginine-serine (R-S) bond and there are no other R-S bonds in the amino acid sequence of gpTNFα, cleavage of the fusion protein with thrombin was also attempted.



factor Xa and thrombin
cleavage site

Figure 2.5: The factor Xa and thrombin cleavage site, in the MBP-gpTNFα fusion protein. Factor Xa recognises the amino acid sequence IEGR and cleaves at the next bond (R-X).

To determine that factor Xa and thrombin were able to cleave the fusion protein an inital pilot experiment was carried out. Fusion protein (20µl at a concentration of ~1mg/ml) was incubated at room temperature with factor Xa (1µl, 200µg/ml) or thrombin (1µl, 2mg/ml) for 2, 4, 8, 18 or 24 hours. To stop the reaction SDS-sample buffer was added and the samples were boiled for 5-10 min. Samples

(20µl) were run on 15% SDS polyacrylamide gels, for protein staining and western blotting. A sample of uncleaved fusion protein boiled in SDS-sample buffer was run as a negative control and human TNFα (10µg/ml) as a positive control.

The pilot study indicated that optimum conditions for cleavage were 18 hours at room temperature with factor Xa (10µg/ml). These conditions were used in subsequent preparations to cleavage the MBP-gpTNFα fusion protein.

2.3.3 Removal of MBP

MBP was removed from the cleaved fusion protein mixture by passing over a hydroxyapatite column followed by an amylose column. Binding to hydroxyapatite caused maltose to be displaced from the MBP. The high affinity of the maltose-free MBP for amylose enabled it to be separated from gpTNFα by passage over an amylose column.

2.3.3.1 Hydroxyapatite column

Hydroxyapatite (1g) was allowed to swell in 30ml column buffer, the fines were removed by washing the resin in column buffer 3 times. The resin was poured into a 1 x 10cm polyproylene column (Bio-rad Laboratories, Hemel-Hampstead, UK) and allowed to settle at room temperature.

The cleaved fusion protein mixture was loaded onto the column and the column washed with 80ml column buffer, under gravity flow, to remove the maltose. Proteins were eluted from the column by 0.5M sodium phosphate buffer (1.61% $NaH_2PO_4 \cdot H_2O$ 10.26% $Na_2HPO_4 \cdot 7H_2O$, pH 7.2) and collected in 2ml fractions. Protein containing fractions, assessed by OD at 280nm, were pooled.

2.3.3.2 Amylose column

A 15ml amylose column was prepared as described previously (section 2.3.1). The proteins eluted from the hydroxyapatite column were run over the amylose column and washed with 75ml column buffer. Eluate was collected in 5ml fractions and protein content assessed by OD 280nm. Samples were removed for SDS-PAGE and proteins were detected by silver staining and western blotting (section 2.5). Western blotting was performed using antibodies against gpTNFα and factor Xa.

2.3.4 Removal of endotoxin

A polymixin B column (detoxi-gel, Pierce Chemical Company, Chester, UK) was used to remove endotoxin contamination from the purified protein. Under sterile conditions the column was regenerated by washing with 5ml 1% sodium deoxycholate and rinsed with 3ml H_2O. The column was equilibrated with sterile PBS and the protein sample applied. Eluate was collected in 1ml fractions and TNFα bioactivity of the samples assessed (2.4).

The purified recombinant gpTNFα was concentrated prior to storage at -20°C using centriplus-10 concentrators (Amicon Ltd., Stonehouse, UK, molecular weight cut-off 10,000 Da) and the buffer was replaced with PBS 0.1% BSA.

2.4 TNFα cytotoxicity bioassay and immunological activity

2.4.1 TNF bioassay

TNF bioactivity was assessed using a mouse fibrosarcoma cell line, WEHI 164 clone 13 (supplied by Dr A. Meager, NIBSC, Potters Bar, UK) cytotoxicity assay (Espevik & Nissen-Mayer, 1986). WEHI 164 cells were rountinely cultured in RPMI 1640 medium supplemented with 10% FCS, 10U/ml penicillin and 10μg/ml streptomycin. Cells were passaged twice weekly, when confluent, and split 1:20.

Aliquots (180μl/well) of WEHI cell suspension (2 x10^5/ml) in RPMI 1640, 10% FCS, 10U/ml penicillin, 10μg/ml streptomycin were dispensed into a 96 well microtitre plate (NUNC, Gibco Life Technologies Ltd., Paisley, UK) and incubated (37°C in a humidified, 5% CO_2/air environment) for 4 hours. Samples (including gpTNFα [2.3], BAL supernatant [2.6.4], lung homogenates [2.4.2]) or human TNFα (1pg/ml-10ng/ml) standards (20μl), diluted in the appropriate buffer, were added to the wells in triplicate and the cells incubated for a further 24 hours. The supernatant was carefully removed and the adhered cells fixed with 50μl/well 95% methanol for 30 s. The methanol was removed and the cells were stained with crystal violet stain (100μl/well) for 15 min, then the plate was extensively washed with distilled water. To solubilize the remaining cell associated dye 33%v/v glacial acetic acid (200μl/well) was added and the optical density of the wells read at 540nm in a microtitre plate reader (MR5000, Dynatech Laboratories, Chantilly, Virginia).

2.4.2 Whole lung tissue sample preparation

Frozen (-70°C) peices of lung tissue were ground in liquid nitrogen using a mortar and pestle over dry ice. The lung powder was transferred to a glass homogeniser and frozen tissue (200mg/ml) homogenised in PBS containing 0.05%

Triton X-100, 1% FCS and 100U/ml aprotinin on ice. Homogenates were transferred to sterile tubes, microcentrifuged and the supernatant was stored at -20°C.

2.4.3 Neutralisation of TNFα cytolytic activity

In neutralisation experiments TNFα containing samples were incubated with rabbit anti murine TNFα antiserum or rabbit anti murine TNFα IgG (2.4.4) for 30 min at room temperature prior to testing in a WEHI bioassay.

2.4.4 Preparation of rabbit anti murine TNFα IgG fraction

Rabbit anti murine TNFα antiserum was a gift from Dr S .L. Kunkel, University of Mitchigan, Ann Arbor. An IgG fraction was produced using a 1ml protein A affinity column. The column was washed with 10ml PBS followed by 10ml 1M Tris buffer pH 8.0 under gravity flow. A 1ml sample of antiserum was added to the column prior to washing with 10ml 100mM Tris buffer pH 8.0 and 10ml 10mM Tris buffer pH 8.0. The IgG fraction was eluted from the column using 100mM glycine (pH 3.0) and 1.5ml fractions were collected in tubes containing 50µl 1M Tris buffer (pH 8.0). The protein content of the fractions was determined by OD at 280nm. Protein containing fractions were pooled and concentrated using Centriplus-10 concentrators (Amicon Ltd., Stonehouse, UK, molecular weight cut-off 10,000 Da) to approx 1mg/ml (1 OD is approx. 0.8mg/ml for IgG fractions).

The column was reused once after residual antibodies were stripped off the column by washing sequentially with 2M urea, 1M lithium chloride and 100mM glycine pH 2.5. The column was equilibrated by washing with PBS before applying 1.5ml whole rabbit serum to obtain a control IgG fraction.

2.4.5 ELISA for gpTNFα

In order to detect gpTNFα in biological samples, a murine TNFα ELISA (enzyme linked immunosorbent assay) known to cross react with rat TNFα was investigated. This ELISA was developed in co-operation with by Dr W. A. Buurman, University of Limburg, Maastricht, The Netherlands.

A 96 well plate (Immunoplate Maxisorb, NUNC, Gibco Life Technologies Ltd., Paisley, UK) was coated with monoclonal hamster anti murine TNFα (IgG) diluted 1 in 200 in PBS (100µl/well) and incubated at 4°C overnight. The wells were emptied, 150µl 1%BSA (Sigma A-7906) in PBS was added to block non-specific binding sites, and the plate incubated at room temperature, covered, for 1 hour. The plate was then washed 5 times in wash buffer (Milli Q H_2O 0.1% Tween 20).

The standard murine TNFα samples (0.03-30ng/ml) and gpTNFα samples (0.1-100ng/ml) were diluted in murine serum 20 min prior to addition to the plate. Samples (100µl/well) were added in duplicate to the plate, the plate was covered, and incubated for 1 hour at room temperature. The plate was then washed 5 times in wash buffer. The secondary antibody, rabbit anti murine TNFα, was diluted 1 in 10 in PBS containing 0.1%BSA and 100µl/well added to the wells. The plate was incubated for a further 1 hour at room temperature prior to washing 5 times in wash buffer.

Goat anti rabbit IgG peroxidase conjugate (100µl/well) was diluted 1 in 1000 in PBS 0.1%BSA and added to the plate which was covered and incubated for 1 hour at room temperature. The plate was washed for a further 5 times in wash buffer and 100µl/well 3,3',5,5'-tetramethylbenzidine (TMB) detection substrate was added. The reaction was incubated in the dark for 10-15 min before stopping with 100µl/well 3M H_2SO_4. The OD at 450nm was measured on a microtitre plate reader (MR5000 Dynatech Laboratories, Chantilly, Virginia.). The murine TNFα standards were used to as a positive control and murine serum as the vehicle control.

2.5 SDS-PAGE and western blotting

To avoid protein contamination all SDS-PAGE equipment was thoroughly cleaned with alcohol prior to use.

2.5.1 Sample preparation for PAGE gels

2.5.1.1 Protein containing solutions

Protein containing solutions for SDS-PAGE or western blotting were prepared by boiling with an equal volume of SDS-sample buffer (2% SDS, 20% glycerol, 160mM Tris pH 6.8, 9% 2-mercaptoethanol, 0.2% bromophenol blue) for 5-10 min. The samples were then cooled prior to use or frozen at -20°C.

2.5.1.2 Whole lung tissue

Lung tissue was ground as described in section 2.4.2 and homogenised in PBS containing 0.05% Triton X-100, 0.1% BSA, 1mM EDTA, 2μg/ml leupeptin, 1μg/ml pepstatin A, 100μg/ml PMSF and 2μg/ml aprotinin on ice. An aliquot (200μl) of homogenate supernatants was retained to determine the protein content (2.5.1.3) and the remainder boiled with SDS-sample buffer as described above (2.5.1.1). Lung homogenate supernatants were run on 15% PAGE gels, loading equal amounts of protein for each sample.

2.5.1.3 Protein assay

The protein content of samples was calculated using a Bio-Rad Bradford-based protein assay. BSA protein standards (1-40μg/ml) or supernatant samples were added in triplicate to a 96 well microtitre plate (200μl/well). Dye reagent concentrate (50μl) was added to each well, the samples mixed thoroughly and incubated at room temperature for 30-50 min. OD at 595nm was measured on a microtitre plate reader

(MR5000, Dynatech Laboratories, Chantilly, Virginia). Protein content of the samples was calculated following the generation of a BSA (standard) protein calibration curve.

2.5.2 SDS gel preparation

SDS-PAGE was carried out essentially as described by Laemmli (Laemmli, 1970) using Bio-Rad mini gel system (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) with 1.5mm thick gels. The resolving gel of the appropriate acrylamide percentage was prepared as descibed in table 2.4 and poured to approximately 0.5cm below the point where the bottom of the combs would reach. The poured gel was overlaid with water, to reduce contact with air, and left to polymerise. Once set, all the liquid was removed from the top of the resolving gel. This was overlaid with stacking gel and the plastic comb carefully inserted. When the stacking gel had set, the comb was removed and the running chamber assembled. Electrophoresis was carried out in running buffer containing 0.25M Trizma base, 1.92M glycine, 0.1%SDS.

Samples (10-20µl) were added to individual lanes and molecular mass protein standards (4-250kDa) were included on each gel (3µl pre-stained for western blotting). When available, a control sample which was known to cross react with the primary antibody used in western blotting was included. Electrophoresis was carried out at a constant voltage of 100V through the stacking gel (~ 20 min) and 150V through the resolving gel (1-1.5 h). The gels were removed and a selected corner was marked for orientation/identification. The gels were either stained to indicate the position of the proteins, with Coomassie blue (2.5.3.1) or silver stain (2.5.3.2), or used for western blot transfer (2.5.5).

Table 2.4: Protocol for 10% and 15% SDS polyacrylamide gels.

Components (in order of addition)	**10% polyacrylamide gel** (optimal resolution ~30-60kDa proteins)		**15% polyacrylamide gel** (optimal resolution ~5-30kDa proteins)	
	Running gel	Stacking gel	Running gel	Stacking gel
30% Acrylamide Stock	5.0ml	1.67ml	7.5ml	1.67ml
Tris Buffer 1.0M pH 8.8	5.6ml	-	5.6ml	-
Tris Buffer 1.0M pH 6.8	-	1.25ml	-	1.25ml
Water	4.35ml	6.0ml	1.85ml	6.0ml
10% SDS	150µl	100µl	150µl	100µl
10% AMPS	100µl	50µl	100µl	50µl
TEMED 6.6M	10µl	10µl	10µl	10µl
Total volume required for 2 mini gels	15.21ml	9.08ml	15.21ml	9.08ml

2.5.3 Detection of proteins in SDS-PAGE gels

2.5.3.1 Coomassie blue staining

The gel was submerged in Coomassie blue stain (table 2.5) and shaken for 30-45 min allowing the proteins to be both fixed and stained simultaneously. The gel was then placed in destain to remove background colouration for 3-4 hours.

Table 2.5: Protocol for comassie blue stain and destain.

Components	**Gel stain**	**Gel destain**
Coomaissie Brillant Blue R-250	0.25%	-
Methanol	40%	40%
Acetic acid	7%	7%
Water	53%	53%

2.5.3.2 Silver staining

All containers used were washed extensively prior to use and reserved exclusively for silver staining to prevent background artefacts. Solutions were made using sterile, double distilled water in sterile vessels.

Silver staining was performed essentially as described by Johnstone and Thorpe (Johnstone and Thorpe, 1987). Proteins in the gel were fixed by agitating the gel in 50% methanol for 1-1.5 hours. The methanol was replaced with the staining solution (0.0756% w/v NaOH, 0.21M NH_4OH, 0.8% w/v silver nitrate) and the gel agitated for 15 min. The gel was washed 3 x 10 min in water, then developed in 0.005% w/v citric acid, 0.019% w/v formaldehyde. The stain was developed until protein bands were visible on the gel (approx. 10 min). The reaction was stopped by rinsing in water and the gel stored in 45% methanol / 5% acetic acid. For longer storage gels were dried using a Bio-Rad gel dryer (Bio-Rad Laboratories, Hemel Hempstead, UK) in accordance to manufacturers instructions. The gel was placed on dampened filter paper, covered with a piece of soaked cellophane membrane and air bubbles carefully removed. The gel was dried for 70 min at 80°C under vacuum.

2.5.4 Electrophoretic transfer of proteins to nitrocellulose membrane

Proteins were transfered using a Tris-glycine system and mini Trans-blot cells (Bio-Rad Laboratories, Hemel Hempstead, UK). The transfer sponge pads, 2 pieces of filter paper/gel (cut slightly larger than the gel size) and the gel were soaked for 5 min in transfer buffer (0.25M Trizma base, 1.92M glycine). The PVDF nitrocellulouse membrane (NEN Research Products, Boston, MA, USA), cut to match the size of the gel, was activated by immersing in methanol (3 s), distilled water (1 min) and transfer buffer (1-2 min). A "western sandwich", figure 2.6, was then assembled under transfer buffer. A transfer pad was placed on the transfer cell, then a piece of filter paper followed by the gel. A glass pasteur pipette was used to roll out air bubbles. The membrane was placed on the gel, air bubbles again carefully removed, and the filter

and remaining transfer pad positioned. The transfer cell was closed, placed in the Trans-blot and then in the transfer tank with the gel nearest to the cathode.. The tank which contained an ice pack and magnetic stirrer was filled with transfer buffer. The blot was transfered at a constant current of 250mA for 1 hour whilst being stirred.

Figure 2.6: The "*Western sandwich*".

Negative terminal (cathode)				*Proteins migrate* →			*Positive terminal (anode)*
Black side of transfer cell	*Sponge*	*Filter*	*Gel*	*Nitrocellulose membrane*	*Filter*	*Sponge*	*Red side of transfer cell*

Following transfer the membrane was ready for immunoblotting. Alternatively, the membrane was stored overnight in PBS, 0.1% NaN_3, 4°C or carefully placed between filter paper, wrapped in tin foil and stored at -20°C. The gel was then stained with Coomassie blue to ensure the protein transfer was complete.

Efficiency of protein transfer was checked by staining with Ponceau S stain (0.2% Ponceau S, 3% trichloroacetic acid, 3% sulfosalicylic acid) for 5-10 min. Ponceau S staining was non-permanent and was removed during immunoblotting wash procedures.

2.5.5 Blocking and antibody probing of membranes

All stages were carried out on an orbital shaker at room temperature with 1 membrane/container. Each membrane was covered with blotting buffer (170mM NaCl, 10mM Tris, pH 7.4) containing 5% non-fat milk protein for 1.5-2 hours to reduce non-specific protein binding. This was removed and the membrane washed

twice in blotting buffer (10 min/wash) and once in wash buffer (0.05% Tween 20, blotting buffer).

The primary antibody, diluted as detailed in table 2.6 in wash buffer, 0.02% NaN_3 was incubated with the membrane for 2 hours. After probing the antibody was stored at 4°C and reused up to 5 times. The membrane was washed 3 times in wash buffer (10 min/wash) prior to incubating with the secondary antibody alkaline phosphate conjugate for 1-$1^1/_4$ hours. After discarding the secondary antibody the membrane was washed once in wash buffer for 10 min and twice in a high salt wash buffer (500mM NaCl, 170mM Tris, 0.05% Tween 20) for 15 min/wash.

Protein-antibody complexes were detected using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The membrane was surmerged in detection solution of NBT (330µg/ml), BCIP (166µg/ml) dissolved in Tris/magnesium buffer (100mM Tris, 100mM NaCl, 50mM $MgCl_2$, pH 9.5). Bands were allowed to develop and the reaction was stopped by covering the membrane in cold blotting buffer (4°C). The membrane was then rinsed twice in blotting buffer, air dried and sealed in polythene bags for storage.

Table 2.6: The primary and secondary antibodies used for protein detection in western blot analysis.

Protein to be detected.	**Primary antibody**	**Dilution**	**Secondary antibody**	**Dilution**
TNFα	goat anti murine TNFα IgG	1 in 1000	anti goat IgG alkaline phosphate conjugate	1 in 2000
TNFα	rabbit anti murine TNFα sera	1 in 1000	anti rabbit IgG alkaline phosphate conjugate	1 in 2000
Factor Xa	rabbit anti human Factor Xa	1 in 500	anti rabbit IgG alkaline phosphate conjugate	1 in 3000

2.6 Northern blot analysis of mRNA

To minimise degradation of RNA, by RNases, all solutions were prepared using RNase and pyrogen free Milli Q H_2O or distilled water was treated with 0.1% diethylpyrocarbonate (DEPC), overnight at 37°C, followed by autoclaving. Sterile equipment was used and gloves were worn throughout. Whenever possible the samples were kept on ice.

2.6.1 Extraction of total RNA from samples

Frozen (-70°C) pieces of lung tissue were ground (2.4.2) and homogenised in RNAozl B (5ml/g lung tissue) whereas cell pellets were mixed, using a plastic pasteur pipette, with 1ml RNAzol B, samples were kept on ice or stored at -70°C until required. Each sample was vortexed with 100µl chloroform for 15 s, placed on ice for 5 min, and centrifuged at 12500g, 4°C for 15 min. The aqueous (upper) layer of the sample was transfered into a sterile tube containing 0.5ml ice cold propan-2-ol, taking care not to transfer or disturb the aqueous/organic interface which contained proteins including RNases. The tube was inverted several times to mix and placed on ice for 15 min before pelleting the RNA (12500g, 15 min, 4°C). The supernatant was removed and the pellet washed with 1ml 75% ethanol (at -20°C), the pellet was vortexed breifly to resuspend and then repelleted by centrifugation at 7500g, 4°C for 8 min.

Supernatant was removed and the pellet was air dried for 30-45 min. The dry pellet was resuspended in 22µl water, vortexed and heated for 10-15 min at 60°C. The samples were then kept on ice. To quantify the amount of total RNA present the OD at 260nm of a small sample was measured in 0.1% NaOH (equation 2.1).

OD 260 x dilution factor x 40 x volume sample (ml) = µg total RNA.

Equation 2.1: Measurement of RNA concentration.

2.6.2 Agarose/formaldehyde gel electrophoresis

A 1% agarose gel was prepared by dissolving agarose (3g) in 230ml water/gel, by microwaving for 2-3 min. Once dissolved 15ml of 20 x MOPS pH 7.0 (0.4M morpholinopropansulfonic acid [MOPS], 0.02M EDTA, 0.2M sodium acetate pH 5.2) and 54ml formaldehyde solution (38%) were added. The gel was allowed to cool before pouring into a gel holder with the combs already positioned. The gel was placed in a tank containing cold running buffer (1x MOPS) in an ice bath.

RNA samples were prepared in RNA loading buffer (2.34 x MOPS, 303µg/ml ethidium bromide, 60% formamide, 8.1% formaldehyde solution), 5-10µg RNA per 30µl buffer per well. The samples were vortexed, heated at 80°C for 20 min, and quenched on ice. 2µl/well bromophenol blue solution (0.25% bromophenol blue, 30% glycerol) was added to the RNA samples and microcentrifuged to mix. RNA samples were loaded into the wells and the power pack connected with the samples migrating towards the anode. Electrophoresis was carried out at 100V for 1.5-2 hours until the bromophenol blue had migrated 3-5cm from the wells.

The completed gel was visualised under UV light (Hoefer mighty bright UVTM 25), and photographed. The 18S and 28S ribosomal RNA bands were visible and could be used to demonstrate equal loading of the lanes.

2.6.3 Transferring RNA from the gel to nitrocellulose membrane

The RNA was transferred from the gel onto a nylon membrane by capillary action. The gel was cut to the desired sized and 1 peice of nylon membrane (Boehringer Mannheim, Lewes, Sussex, UK) and 3 pieces of 3mm filter paper cut to the same size. A length of filter paper was cut and placed in the blotting tank with each end in a reservoir of 20 x SSC transfer buffer (3M NaCl, 0.3M sodium citrate,

pH 7.0), and the central platform area above this level so that the filter paper acts as a wick. The platform region was dampened with 20 x SSC and air bubbles were rolled out using a glass pipette. The gel was inverted on the platform such that the lower with the surface previously touching the gel tray was now uppermost. The gel was covered with nylon membrane and 3 pieces of filter paper taking care to exclude air bubbles. A stack (~8cm) of absorbant paper (paper hand towels) cut to match the gel size were placed on top of the filter paper and weighted down with a glass plate and 500g weight, at least 20 hours was allowed for transfer to be completed.

Following transfer, the appropriate corner of the membrane was marked for orientation/identification purposes, the membrane was baked for 20 min at 120°C. The 18S and 28S ribosomal RNA bands were visualised by exposure to UV light and their position marked on the edge of the membrane. The membrane was sealed in a polythene bag and stored ready for probing.

2.6.4 Digoxigenin (DIG)-labelled probes for northern analysis

β-actin and eotaxin mRNA were detected using DIG-labelled oligonucleotide probe cocktails (3 x 30mers) purchased from R&D Systems, Abingdon, Oxon, UK. The β-actin probe was designed to hybridise with human β-actin mRNA but it was found to detect guinea-pig mRNA (E. Campbell, University of Bath). The eotaxin probe was designed to in accordance to guinea-pig eotaxin nucleotide sequence (Jose *et al.* 1994).

Guinea-pig IL-8 and gpTNFα mRNA were detected using DIG-labelled double stranded cDNA probes. PCR was utilised to incorporate the DIG label into the cDNA. By using a 1:4 digoxigenin-11-dUTP (DIG-dUTP):dTTP ratio during amplification of the cDNA the DIG-dUTP randomly replaced dTTP, hence labelling the cDNA. PCR was performed as described in section 2.2.5.1 using the reagents in table 2.7. Primers were designed based on the cDNA sequences (table 2.8). Guinea-pig IL-8 cDNA was

supplied by Dr T. Yoshimura supplied in pBluescript SK(-) (Yoshimura & Johnson, 1993), the primer was based on the sequence registered with Genbank, accession no. L04986.

Table 2.7: PCR protocol for the synthesis of DIG-labelled cDNA probes.

Order to add reagents	**Final concentration**
Milli Q H_2O	
$MgCl_2$	2.5mM
NH_4 Buffer	1x
dATP/dCTP/dGTP	200µM
dTTP	150µM
DIG-dUTP	50µM
PRIMERS Forward	0.5µM
Reverse	0.5µM
Taq	25U/ml
cDNA template	100ng/ml
Total volume	100µl

DNA was amplified in a GeneAmp 2400 thermal cycler (Perkin-Elmer, Warrington, UK) using an inital 2 min at 95°C followed by 30 cycles of denaturation (95°C for 60s), annealing (55°C for 60 s) and extension (72°C for 60 s) and a final extension at 72°C for 5 min. Following amplification a sample of each reaction mixture was run on a 1.5% agarose gel containing ethidium bromide, to ensure a single product was obtained. DIG incorporation was verified by immunoblotting. Briefly, 1 in 10 dilutions of the sample were dotted onto a nylon membrane and DIG was detected as described in section 2.6.6.

Table 2.8: Primers used for synthesis of DIG-labelled cDNA probes.

Primers	Forward	Reverse
guinea-pig IL-8	5'ATGGTCGTGACAAAGTT GGTC	5'CCCAAGCTTCCAATTCC TGAAGTAGA
guinea-pig TNFα	5'TCAGCTTCTCAAAACGA TAAT	5'CCCAAGCTTAACCGGAA GGACTGAGTG

2.6.5 Hybridisation of northern blots with DIG labelled probes

Membrane was prehybridised with 10-20ml of hybridisation solution 1 (5 x SSC, 0.1% sarcosyl, 0.02% SDS, 1% blocking buffer), for oligonucleotide probes, or solution 2 (5 x SSC, 0.1% sarcosyl, 7% SDS, 2% blocking buffer, 50mM sodium phosphate pH 7.0, 50% formamide) for cDNA probes. The hybridisation solution was added to the membrane in the polythene bag and the bag resealed removing all air bubbles. The membrane was incubated at 42°C (oligonucleotide probes) or 50°C (cDNA probes) for 1 hour.

The top corner of the polythene bag was cut and the hybridization solution drained out. The oligonucleotide and cDNA probes, diluted in hybridization solution to a final concentration of 10ng/ml was added to the membrane (2.5ml/50cm^2) and the polythene bag was resealed removing air bubbles. At this stage it was important not to allow the membrane to dry out. The membrane was hybridised overnight at the prehybridisation temperature.

2.6.6 Immunodetection of DIG-labelled probes for northern analysis

The membrane was removed from the polythene bag, placed in a suitable wash tank, and washed at the prehybridisation temperature twice in stringency wash buffer 1

(2 x SSC, 0.1% SDS) for 5 min and twice in stringency wash buffer 2 (0.1 x SSC, 0.1% SDS) for 5 min.

All washes and incubations for the following stages, unless otherwise stated, were performed at room temperature with shaking. The membrane was washed for 5 min in wash buffer, 0.3% Tween 20 in maleic acid buffer (0.1M maleic acid, 0.15M NaCl), before incubating for 30 min in blocking buffer (1% blocking reagent in maleic acid buffer). The blocking buffer was removed and the membrane incubated with anti-DIG Fab fragments conjugated to alkaline phosphatase, diluted 1 in 10,000 in blocking buffer, for 30 min. The antibody solution was discarded and the membrane washed 3 x 10 min in wash buffer. The wash buffer was poured off and the membrane equilibrated in detection buffer (0.1M Tris.Cl, 0.1M NaCl, 50mM $MgCl_2$, pH 9.5) for 2-5 min.

Chemiluminescent alkaline phosphate substrate, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD), was used for detection. CSPD (25mM) was diluted 1 in 100 in detection buffer. The membrane was removed from the detection buffer, excess buffer shaken off, placed on a polythene sheet and 1ml CSPD was evenly pipetted over the membrane. The membrane was covered with a top polythene sheet, the CSPD gently spread over the membrane surface and the membrane incubated for 5 min in the dark. The membrane was sealed in a new polythene sheet and incubated for 30 min at 37°C in the dark.

The membrane, in the sealed bag, was initially exposed to X-ray film for 1-1.5 hours. If necessary longer exposures of upto 12 hour were performed subsequently.

2.7 Induction of chemokine-like activity by TNFα

2.7.1 Guinea-pig peritoneal macrophage and eosinophil collection

Dunkin-Hartley guinea-pigs (500-650g, male and female) were each given a minimum of 4 injections of Horse Serum (0.5ml, i.p., 2 injections/week). A final "booster" injection was given the day before cell collection. The guinea-pigs were asphyxiated with CO_2, the lower abdominal fur was removed and the skin washed with methanol. An incision was made along the mid-ventral line and the peritoneal cavity lavaged with HBSS containing 0.1% BSA and 1mM EDTA (4x10ml). The lavage was centrifuged at 400g for 10 min and the supernatant discarded. Contaminating red blood cells in the pellet were lysed by hypotonic shock, distilled H_2O (250µl, 4°C) was mixed with the pellet for 10-15 s before resuspending the cells in 25ml HBSS containing 0.1% BSA (buffer A). The collected cells were washed twice in buffer A, centrifuging for 10 min at 400g. The cells were resuspended at $5x10^7$/ml in Percoll (1.070g/l) containing 1% BSA and carefully layered over a discontinuous isotonic Percoll gradient with densities 1.070, 1.080, 1.085, 1.090 and 1.100g/l (Gärtner, 1980), table 2.9. The gradient was centrifuged for 25 min at 1500g and the cell layers were removed sequentially from the top. The relevant leukocyte population (figure 2.7) was collected and washed twice in buffer A (400g, 10 min).

Table 2.9: Protocol for Percoll gradients.

Percoll density (g/l)	†Light Percoll (ml)	‡HBSS+ (ml)
1.100	1.60	0.41
1.090	2.74	1.26
1.085	1.25	0.75
1.080	1.14	0.86
1.070	0.91	1.09

†Light Percoll = 9 parts Percoll with 1part 10 x HBSS with calcium and magnesium, pH 7.2-7.4. ‡HBSS+ = 1 x HBSS with calcium and magnesium, pH 7.2-7.4.

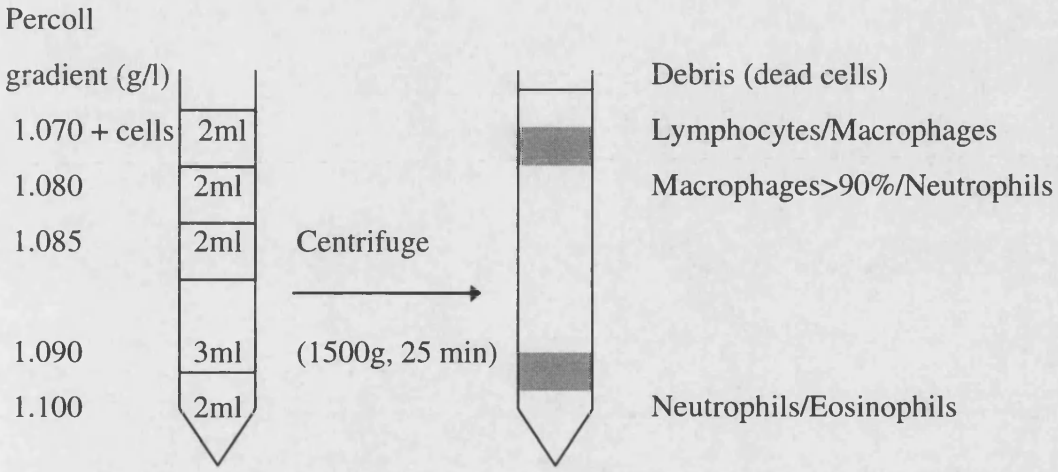


Figure 2.7: Layering of the Percoll Gradient.

2.7.2 Production of gpTNFα by stimulated macrophages

A crude preparation of guinea-pig TNFα was prepared from guinea-pig macrophages as described by Tamatani *et al.* 1989. Briefly, guinea-pig peritoneal macrophages ($1x10^7$/ml) were stimulated with 10μg/ml LPS for 1 hour (37°C in a humidified, 5% CO_2/air environment) in RPMI 1640, 10% FCS, 10U/ml penicillin, 10μg/ml streptomycin. The cells were washed twice in PBS and cultured for 12 hours in 5ml RPMI 1640, 10U/ml penicillin, 10μg/ml streptomycin. The cells were pelleted (400g, 10 min) and the supernatant aliquoted and stored at -20°C. This supernatant was shown to contain TNFα by WEHI bioassay.

2.7.3 Stimulation of guinea-pig spleen cells, peritoneal macrophages and JH-4 cells with gpTNFα and recombinant human cytokines

JH-4 clone 1 are a guinea-pig lung fibroblastic cell line, obtained from European Collection of Animal Cell Cultures (Wiltshire, UK), which were derived

from the lung of a young guinea-pig strain 13 and subsequently cloned. The cells were routinely cultured in Hams F12 medium supplemented with 10% FCS, 10U/ml penicillin and 10µg/ml streptomycin. Cells were passaged when confluent using 0.25% trypsin and seeded at a density of 3 x 10^4 - 4 x 10^4 cells/cm^2 (splitting 1:3 - 1:4). Cells were cultured until passage 39 (the cells were passage 34 when supplied).

Spleen cells were collected by removing the spleens from naive Dunkin-Hartley guinea-pigs (500-650g, male and female) and sieved through a fine wire mesh with saline. The resulting suspension was centrifuged (400g, 10 min), the red blood cells in the pellet were lysed with 150mM NH_4Cl (5ml) for 15 min at 4°C and made up to 25ml with RPMI 1640 medium, 10% FCS, 10U/ml penicillin, 10µg/ml streptomycin. The cells were pelleted by centrifugation at 400g, 20°C for 10 min then rewashed twice and resuspending in medium at 5x10^5cells/ml.

Cells were plated in 24 well plates (Gibco Life Technologies Ltd., Paisley, UK) at a density of 2x10^5cells/well (JH-4 cells were grown to confluence and the medium replenished prior to stimulation) in their respective medium containing 10% FCS, 10U/ml penicillin, 10µg/ml streptomycin. Cells were stimulated and then incubated at 37°C in a humidified, 5% CO_2/air environment for the duration of the experiment. Experiments were designed to incorporate cellular stimulation with macrophage derived gpTNFα, recombinant gpTNFα, recombinant human cytokines, TNFα antibody neutralisation and time course studies. The supernatants were collected, stored at -20°C prior to assessing the bioactivity (2.7.4) and in some experiments the adhered cells were scraped into 1ml RNAozl B and stored at -70°C for northern blot analysis (2.6).

2.7.4 Elevations in intracellular free calcium concentration $[Ca^{2+}]_i$ in guinea-pig peritoneal leukocytes

Guinea-pig peritoneal macrophages and eosinophils were prepared as described previously in section 2.7.1 and peritoneal neutrophil collection is described in section 2.7.4.1.

2.7.4.1 Guinea-pig peritoneal neutrophil collection

Dunkin-Hartley guinea-pigs (430-620g, male and female) were injected with 0.1% glycogen in saline (15-20ml/animal, i.p.). After 2-4 hours animals were sacrificed by inhalation of carbon dioxide and peritoneal lavage performed as described above (2.7.1). The red blood cells were lysed as described previously, the cells were repelleted by centrifugation (400g for 10 min) and the pellet resuspended in 5ml buffer A. Cell viability and percentage purity was determined by Trypan Blue exclusion and staining of cytospin preparations respectively (2.8.4).

2.7.4.2 Measurement of $[Ca^{2+}]_i$ in fura-2 loaded guinea-pig peritoneal leukocytes

Leukocytes were loaded with the fluorescent dye by incubating the cells ($1x10^7$/ml in buffer A) with fura-2-acetoxymethyl ester (2.5μM) at 37°C for 30 min. The loaded cells were washed twice and resuspended in HBSS containing 0.1% BSA, 1mM Mg^{2+} and 100μM Ca^{2+} at $2x10^6$cells/ml.

Measurements of $[Ca^{2+}]_i$ were obtained by using a dual excitation spectrophoto-fluorimeter (PTI Inc., NJ, USA, Deltascan) with excitation wavelengths of 340nm and 380nm and an emission wavelength of 510nm, the slit width was 5nm. The fluorescence signals from both excitation wavelengths were automatically recorded by the computer with the ratio of these being used to calculate $[Ca^{2+}]_i$ as described by Grynkiewicz and co-workers (Grynkiewicz *et al.* 1985) (see section

2.7.4.3). The apparatus was calibrated at least twice (at the start and end) of each experiment to reduce any time induced cellular variations. Fura-2 loaded leukocytes (1ml, $2x10^6$cells) and 1ml buffer A containing 1mM $MgCl_2$, 2mM $CaCl_2$ (to restore the extracellular calcium concentration to 1mM), were added to a quartz cuvette and placed in the fluorimeter. The cuvette was maintained at 37°C and a magnetic stirrer was used to ensure thorough mixing. Once the fluorescence signal stabilised the maximum $[Ca^{2+}]_i$ fluorescence (F_{max}) was measured by lysing the cells with 80µl digitonin (4mg/ml). After 20 s 40µl NaOH (2M) and 80µl EDTA (500mM) were added to the cells to establish a minimum background fluorescence value (F_{min}). Following calibration the cuvette was soaked in 95% ethanol for 5 min to remove any remaining digitonin.

To monitor the effect of agonists on $[Ca^{2+}]_i$, fura-2 loaded cells (1ml, $2x10^6$cells) and 1ml buffer A containing 2mM $CaCl_2$, 1mM $MgCl_2$ were placed in the cuvette and allowed to equilibrate in the fluorimeter for 4 min at 37°C. The basal fluorescence signal was measured prior to agonist addition (100µl) and any fluorescence changes during the next 120 s were recorded.

2.7.4.3 Calculating $[Ca^{2+}]_i$

The binding of calcium ions to fura-2 causes an increase in fluorescence intensity and a hypsochromic shift in absorption and excitation spectra, such that maximum absorption of free fura-2 anion is 380nm and the Ca^{2+}-fura-2 complexed state is 340nm. A ratio of absorption at 340/380nm was used to calculate $[Ca^{2+}]_i$ as this method minimised variability due to apparatus efficiency and the effective concentration of dye (Grynkiewicz *et al.* 1985).

From the calibration response the values for F_{max} and F_{min} were obtained from the 380nm measurements. F_{max} and F_{min} are equivalent to S_{f2} and S_{b2} the proportionality coefficients for free and bound Fura-2 at 380nm. Using the

340nm/380nm ratio measurements R_{max} and R_{min}, the maximum and minimum fluorescence ratios between the wavelengths, were evaluated. The apparent Kd for fura-2 binding calcium at 37°C is equal to 2.24×10^{-7}M, hence, by applying equation 2.2 the $[Ca^{2+}]_i$ at any one time is calculated. Once calibrated the computer automatically calculated $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$, following agonist stimulation, were measured as the difference between basal $[Ca^{2+}]_i$ and the maximum $[Ca^{2+}]_i$.

$$[Ca^{2+}]_i = \frac{Kd \times (R-R_{min}) \times (S_{f2})}{(R_{max}-R)\ (S_{b2})}$$

Equation 2.2: To determine the $[Ca^{2+}]_i$ of fura-2 loaded cells.

2.8 In vivo studies of airway inflammation in the guinea-pig

The effect of instilling cytokines and/or antibodies into the airways of both antigen sensitised and non-sensitised (naive) animals was examined and is summarised in figure 2.8.

2.8.1 Antigen sensitisation procedure and antigen challenge

Dunkin-Hartley guinea-pigs (200-400g, male and female) were sensitised over a period of 4 weeks to an antigen, ovalbumin (OA), via a series of intraperitoneal (i.p.) injections according to the method described in table 2.10. Aerosol challenge was performed by restraining the animal by the neck whilst the snout of each animal was placed in a nose-cone attached to a nebulizer. Animals were exposed to an aerosol of OA (0.1% in saline 10ml) using a compressed air (7l/min) nebuliser until dry. To prevent anaphylactic shock animals were injected with mepyramine (10mg/kg i.p.) 15 min prior to challenging. In some studies lung function tests (2.8.3) were performed 2-48 hours following aerosol challenge.

2.8.2 In vivo cytokine and murine anti TNFα antibody challenge

Naive (non-treated) or sensitised Dunkin-Hartley guinea-pigs (200-400g, male and female) were challenged with recombinant cytokines (TNFα, IL-13, IL-4) or rabbit anti-murine TNFα antibody. Inital studies compared 3 methods of administering the agents, as detailed below, but tracheal instillation (2.8.2.3) was used for the majority of the studies as this method allowed a known amount of agent to be delivered to the airways.

2.8.2.1 Aerosol challenge using a nose-cone

Naive guinea-pigs were restrained by the neck whilst the snout of each animal was placed in a nose-cone attached to a nebulizer. Animals were exposed to an aerosol

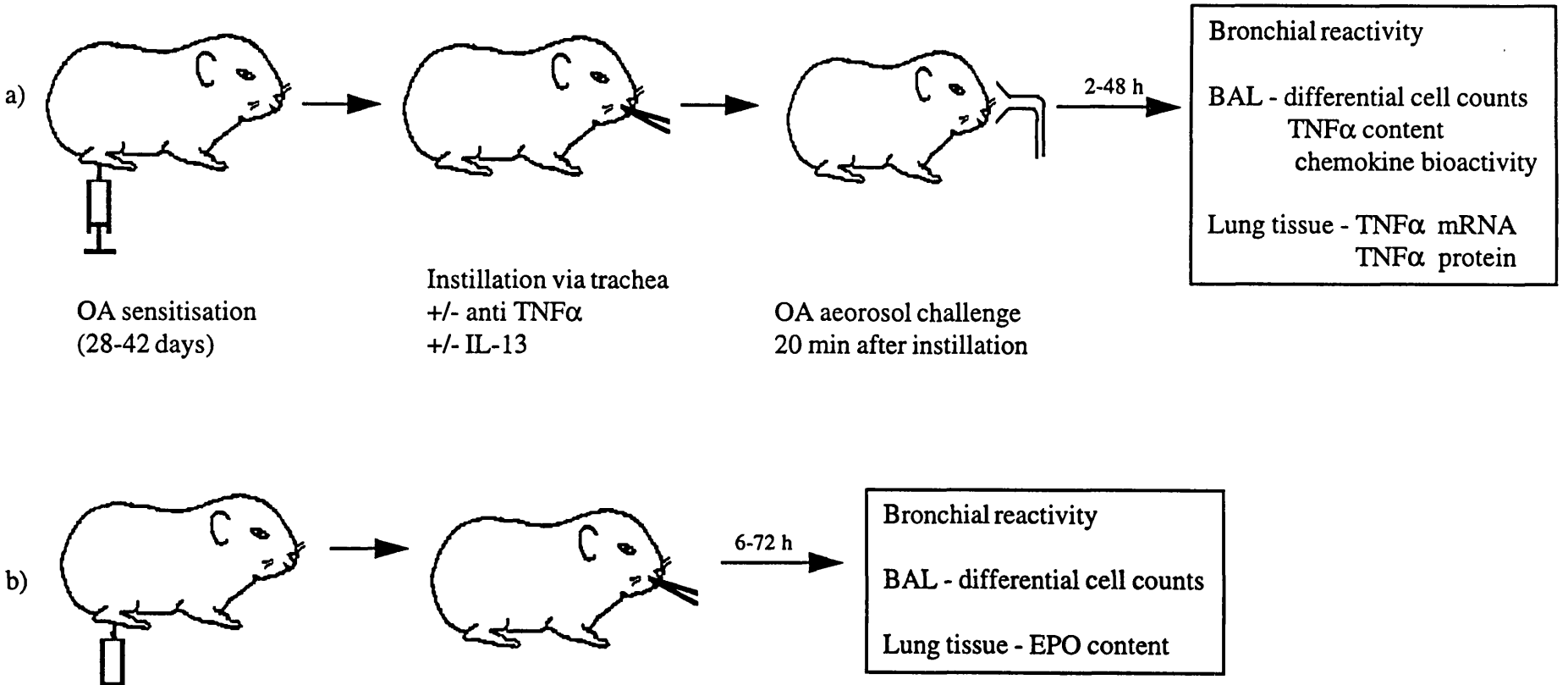
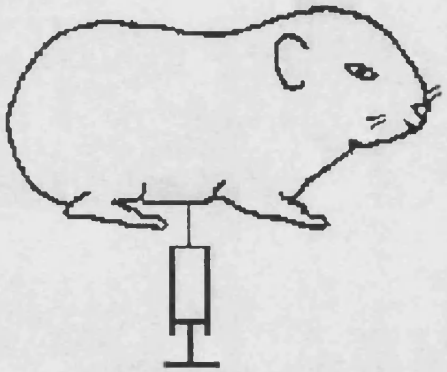


Figure 2.8: A flow diagram to show the sequence of the *in vivo* and *in vitro* methodologies used.
a) OA sensitised animals
b) naive animals instilled with gpTNFα

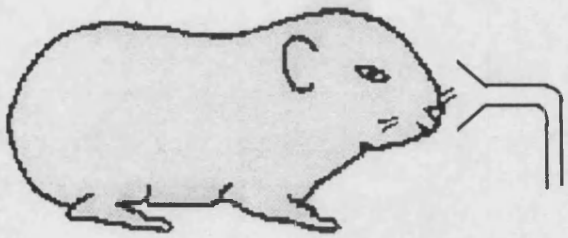
Table 2.10: Protocol for guinea-pig sensitisation methodology.

BV; *Bordertella bronchisepetica* vaccine.

All injection solutions were incubated for 1 hr at room temperature prior to use, to allow adjuvant (aluminium hydroxide) - antigen (OA) binding.



1ml intraperitoneal injection on days:



Day	0		14		14-28 days after final injection
sensitisation solution prepared in saline	$Al(OH)_3$ OA BV	2mg/ml 10µg/ml 50µl/ml	$Al(OH)_3$ OA BV	2mg/ml 10µg/ml 50µl/ml	Aerosol challenge 10ml OA (0.1%)

of 5ml agent using a compressed air (7 l/min) nebuliser until dry. Lung function tests were performed 2 or 6 hours following challenge (2.8.3).

2.8.2.2 Tracheal aerosol challenge

Naive guinea-pigs were anaesthetised, ventilated via the trachea cannula and prepared for lung function analysis, as described in 2.8.3. Bronchial reactivity in response to i.v. histamine (1-10μg/kg) was measured (2.8.3). The ADC nebulizer (PMS, Mumed, London, UK) was then attached between the trachea cannula and the flow transducer. Inspiration by the animal activated the ADC nebulizer to generate an aerosol for 50% of the inspiration time. The animal was exposed to the aerosol on alternate breaths for 60 min during which time approx. 1.2ml of the agent was dispensed. A resting period of 30 min was allowed prior to reassessing bronchial reactivity.

2.8.2.3 Tracheal instillation

Guinea-pigs (male and female, 200-550g) were anaesthetised (ketamine 40mg/kg, xylazine 5mg/kg, i.m.) and kept on warming blankets until the desired degree of anaesthesia was achieved. Animals were positioned prone and any food particles removed from the inside of the mouth using cotton buds. The mouth was opened and an adapted pediatric laryngoscope used to depress the tongue so that the glottis and vocal cords were visible. Agents were adminstered using an elongated-gel loading pipette tip which was passed between the vocal cords before expelling the agent. Animals were maintained on the warming blanket until fully recovered and lung function tests and/or bronchoalveolar lavage (BAL) was performed at set times following instillation.

To ensure the agents were entering the lungs, inital studies were performed by instilling 50μl Evans' blue dye (1mg/ml) into the airways. The animals were allowed

to recover before sacrificing, by an overdose of pentobarbitone, and removing the intact lungs to determine the distribution of the dye visually.

In neutralisation experiments gpTNFα was incubated with anti TNFα antibody at room temperature for 30 min prior to instillation. To examine the effect of co-instilling 2 cytokines, both were mixed immediately prior to instillation.

The instillation of sensitised animals, with rabbit anti murine TNFα antibody or IL-13, was performed 20 min prior to aerosol OA challenge. Mepyramine (10mg/kg i.p.) was administered 15 min before the animals were OA challenged.

2.8.3 Measurement of guinea-pig lung function in vivo

Dunkin-Hartley guinea-pigs (300-500g, male and female) were anaesthetised with sodium phenobarbitone (100mg/kg) and sodium pentobarbitone (30mg/kg) i.v. The animals were placed supine on a heated bed and their body temperature maintained at 37°C. Once in the state of surgical anaesthesia, a 3cm incision was made, the trachea exposed and cannulated. The trachea cannula was connected to a pneumotachograph (Fleish 0000) attached to a differential pressure transducer (MP45-14-871; Validyne, Northridge, CA) (See figure 2.9). The animal was ventilated (7ml/kg/breath) with a mixture of air and oxygen (1:1 v/v) at 60Hz using a small animal ventilator (Sandoz, Basel, Switzerland). The right carotid artery was cannulated with a heparinised saline filled cannula (1.02mm outside diameter, Portex Ltd., Kent, UK) connected to a pressure transducer (Washington PT400 Elcomatic Ltd.) to allow the measurement of blood pressure. The left jugular vein was cannulated, using a saline filled cannula with a volume of > 0.2ml, and attached to a saline containing infusion pump (B.Braun Melsungen AG). Transpulmonary pressure was assessed using a differential transducer (MP45-24-871; Validyne) connected to the trachea cannula and a blunt intrathoracic cannula inserted between ribs 6 and 8 (figure 2.9). Air introduced into the pleural cavity was removed via a 3-way tap

leaving approx.1ml residue for pulmonary measurements. Changes in the lung function (lung resistance and dynamic compliance) were calculated via a computerised respiratory analyser (PMS, Mumed, London, UK). The animal was paralysed with gallamine (10mg/kg i.v.) and allowed to stabilise for 15 min.

To assess the airway responsiveness, 0.2ml of a spasmogen (e.g. histamine, metacholine at concentrations between 1-56μg/kg) was administered at 10 min intervals via the jugular vein, each dose was infused into the vessel with saline (0.4ml). The lung resistance and dynamic compliance were measured for 1min prior to and 3 min following spasmogen administration.

2.8.4 Bronchoalveolar lavage (BAL) cell accumulation

Immediately after the assessment of lung function the animal was sacrificed with an overdose of anaesthetic (sodium pentobarbitone 200mg/kg i.p.). The lungs were lavaged with 2 x 10ml PBS containing 0.1% BSA and 1mM EDTA, the BAL was centrifuged at 400g for 10 min and the supernatant stored at -20°C. The lavage was repeated and combined with the cell pellet from the first lavage. Total BAL leukocyte numbers were assessed using a haemocytometer and Trypan blue exclusion. Aliquots of BAL were cytospun (500rpm for 5 min) onto poly-lysine coated slides using a Shandon II cytospin (Life Sciences, Ireland). Slides were either wrapped in foil and stored at -70°C for subsequent immunohistochemical analysis or stained with Leishman's (1.5g/l methanol) or Diff-Quik stain. Differential leukocyte percentages, based on counting 700-1000 stained cells/sample, were used to determine the BAL leukocyte population numbers.

2.8.5 Storage of lung tissue

Lungs which had been lavaged were dissected from the carcase, washed in saline and chopped into small pieces. The lung pieces were frozen in iso-pentane for

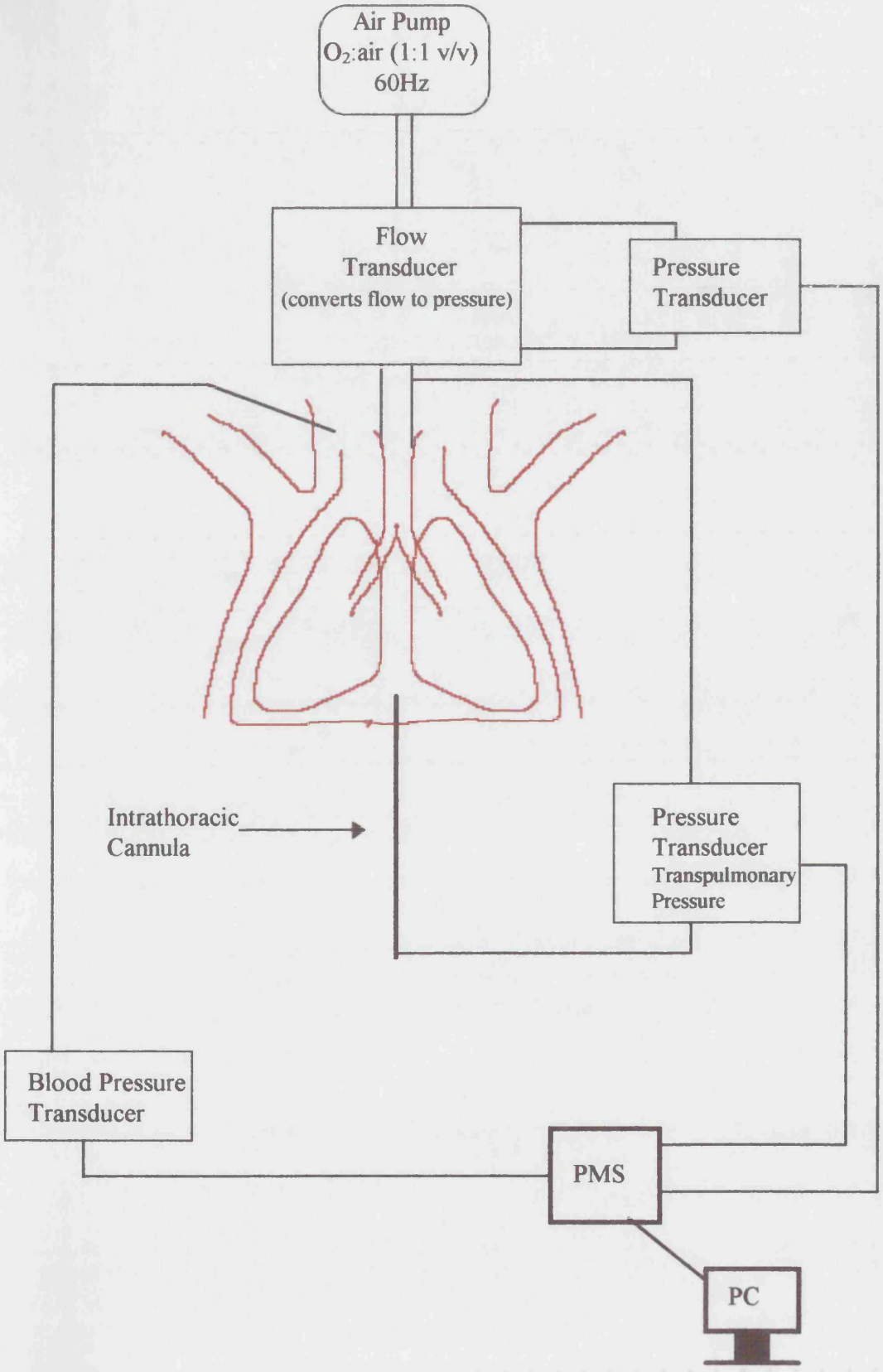


Figure 2.9: Diagram to show the basis lung function apparatus. (PMS; pulmonary monitoring system, red lines represent the guinea-pig)

15-30 s and samples stored in air-tight bags at -70°C. Fresh lung tissue, stored in 10% formaldehyde fixative solution following dissection, was cryostat sectioned and haeamatoxylin and eosin stained by Pathology, Royal United Hospital, Bath.

2.9 Measurement of TNFα and chemokine protein content and bioactivity in BAL and lung tissue

Biologically active TNFα in BAL and lung tissue was assessed using a TNFα cytotoxicity assay (2.4), and the presence of antigenic TNFα was determined by western blot analysis (2.5) for lung tissue and immunohistochemistry for BAL cells. The ability of BAL to induce mobilisation of intracellular calcium in leukocyte subtypes (2.7.4) was used as an indication of chemokine-like bioactivity. Changes in gpTNFα and chemokine mRNA levels in lung tissue were determined by northern blot analysis (2.6).

2.9.1 Immunohistochemical analysis of BAL cell TNFα content

BAL cell cytospins were thawed at room temperature, the cells fixed in 1:1 methanol:3% hydrogen peroxide for 30 min and the slides rinsed twice in TBS pH 7.6. Non specific binding was blocked by incubating the slides in blocking buffer (TBS pH 7.6, 5% rabbit serum) for 15 min at 37°C. The slides were drained, but not rinsed, and the primary antibody (goat anti murine TNFα Ig G) diluted 1 in 500 in blocking buffer was added to the cells. A control antibody, goat IgG, was included for each BAL cytospin. Slides were incubated at 4°C overnight in a humidified chamber.

The slides were washed three times in TBS pH 7.6 on an orbital shaker for 5 min at room temperature. Rabbit anti goat Ig G peroxidase conjugate, diluted 1 in 100 in blocking buffer, was added and incubated at room temperature for 30 min. The secondary antibody was removed by washing the slides three times in TBS pH 7.6 for 5 min at room temperature. The peroxidase conjugate was detected by

diaminobenzidine (DAB) peroxidase substrate using a tablet substrate kit. The DAB solution was applied to the slides for 5-10 min before counterstaining with Mayer's haematoxylin for 3-7 min and rinsing the slides in distilled water.

Slides were allowed to air dry before being examined under a microscope in a blind fashion. Positive staining was scored using a quantal scoring scale (table 2.11) for the number of cells stained in 5 fields of view.

Table 2.11: The quantal scale used for scoring the percentage of stained cells.

Scale	Percentage of cells stained positive
1	less than 25%
2	25-50%
3	50-75%
4	75-100%

2.10 Measurements of eosinophil peroxidase (EPO) activity in lung tissue

Frozen (-70°C) pieces of lung tissue were ground in liquid nitrogen (2.4.2) and homogenised in PBS 0.5% hexadecyltrimethylammonium bromide (HTAB) on ice, 2g lung tissue/8ml buffer. The homogenate was sonicated for 1 min prior to ultracentrifugation at 40,000g ,4°C for 5 min. The supernatant was decanted and repelleted at 48,000g, 4°C for 60 min. The pellet was resuspended in 3ml of 1mM Tris pH 7.4, centrifuged for 5 min at 12,000g, 4°C, the supernatant was aliquoted and stored at -20°C overnight. The aliqouts were thawed, heated for 2 hr at 60°C and stored on ice prior to use.

Horseradish peroxidase standards (1-100ng/ml) or test samples were added to a 96 well microtitre plate in triplicate (100µl/well). An equal volume of *o*-phenylenediamine (OPD) detection substrate was added. OPD solution was freshly

prepared by dissolving 10mg OPD in 25ml 0.05M phosphate citrate buffer pH 5.0 containing 10µl H_2O_2. The reaction was incubated in the dark for 30 min before stopping with 50µl/well of 3M H_2SO_4. The OD at 450nm was measured on a microtitre plate reader (MR5000 Dynatech Laboratories, Chantilly, Virginia.). A protein assay (2.5.1.3) was used to determine the amount of protein per sample and hence, the EPO activity (horseradish peroxidase equivalents) per mg protein was calculated.

2.11 Statistical analysis

The data is expressed as mean ± SEM. One-way analysis of variance (ANOVA) was performed on log transformed data (normalised) to determine significance between groups, unless otherwise stated. If significance was found Dunnett's test was used to assess differences between means. A value of $p \leq 0.05$ was considered significant. Statistical analysis was performed using Minitab for Windows (version 10).

3. Expression & purification of gpTNFα

3.1 Predicted protein sequence of gpTNFα

The sequence of gpTNFα cDNA, provided by Dr T. Yoshimura, and predicted amino acid sequence, are shown in figure 3.1. The gpTNFα cDNA is 77.8% and 74.7% homologous with human (Wang *et al.* 1985) and murine (Pennica *et al.* 1985) TNFα over the 1071 bp obtained. The amplified region encoded a 154 amino acid protein of 17 kDa which is approximately 80% identical with other mammalian sequences at the amino acid level, figure 3.2.

3.2 Expression of gpTNFα

cDNA was amplified using a forward primer corresponding with the NH_2 terminus of the secreted form of gpTNFα (Tamatani *et al.* 1989) to yield a DNA fragment of 540 bp (figure 3.3). This fragment was inserted into the pMAL-p2 plasmid to generate the vector which was subsequently cloned into TB1 cells. The cloning of the vector, using the electroporation conditions described, resulted in the disruption of the *malE-lacZα* gene in the colonies which had incorporated the vector (approximately 30% of the colonies). IPTG induction of cultures containing the disrupted gene promoted MBP-gpTNFα fusion protein expression in one of the five cultures randomly selected. SDS-PAGE and western blot analysis of the bacterial lysate indicated TNFα immunoreactivity at approximately 60 kDa, representing the fusion protein, figure 3.4. The fusion protein was isolated from the soluble crude bacterial extract by MBP-affinity chromatography using an amylose column. Factor Xa cleavage of the fusion protein resulted in the release of MBP (43 kDa) and gpTNFα (17 kDa), which was optimal following 8-24 hours incubation as shown in figure 3.5. Only limited cleavage was obtained by incubating the fusion protein with thrombin at room temperature for 24 hours.

```
-89       -80         -70         -60         -50         -40         -30
    GAAATAAGCCCAGACAATCCAGACAGGCAGGTTTGGTCCCTCTCACACACACCACCCCAGACTC
       -20         -10           1           10          20          30
    CAGAGGTCCCTCTGCAGAAGACGCC ATG AGC ACA GAA AGC ATG ATC CGG GAC GTG
                              Met Ser Thr Glu Ser Met Ile Arg Asp Val
                 40          50          60          70          80
    GAG CTC GCA GAG GAG CAG CTC CCC AAG AAG GCA GGG GGC CCC CAG GGC TCC
    Glu Leu Ala Glu Glu Gln Leu Pro Lys Lys Ala Gly Gly Pro Gln Gly Ser
              90          100         110         120         130
    AGG CGG TGC TGG TGC CTC AGC CTC TTC TCC TTC CTG CTG GTG GCA GGG GCC
    Arg Arg Cys Trp Cys Leu Ser Leu Phe Ser Phe Leu Leu Val Ala Gly Ala
             140         150         160         170         180
    ACC ACG CTC TTC TGC CTG CTG CAC TTT GGG GTG ATC GGC CCC CAG CGG GAA
    Thr Thr Leu Phe Cys Leu Leu His Phe Gly Val Ile Gly Pro Gln Arg Glu
            190         200         210         220         230
    GAG CAG TTC TCC AGT GGC CCC CCC TTC AGA CCC CTG GCC CAG ACG CTC ACA
    Glu Gln Phe Ser Ser Gly Pro Pro Phe Arg Pro Leu Ala Gln Thr Leu Thr
          240         250         260         270         280
    CTC AGA TCA GCT TCT CAA AAC GAT AAT GAC AAG CCG GTG GCT CAT GTT GTG
    Leu Arg^Ser Ala Ser Gln Asn Asp Asn Asp Lys Pro Val Ala His Val Val
         290         300         310         320         330
    GCA AAC CAG CAA GCA GAG GAG GAG CTG CAG TGG CTC AGC AAG CGT GCT AAC
    Ala Asn Gln Gln Ala Glu Glu Glu Leu Gln Trp Leu Ser Lys Arg Ala Asn
        340         350         360         370         380
    GCC CTC CTG GCC AAT GGC ATG GGC CTG AGC GAC AAC CAG CTG GTG GTG CCT
    Ala Leu Leu Ala Asn Gly Met Gly Leu Ser Asp Asn Gln Leu Val Val Pro
      390         400         410         420         430
    TCG GAT GGG CTG TAC CTC ATC TAC TCC CAG GTC CTC TTC AAG GGC CAA GGC
    Ser Asp Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly
     440         450         460         470         480
    TGC CCC TCC TAC CTG CTT CTC ACC CAT ACC GTC AGC CGC TTG GCC GTC TCC
    Cys Pro Ser Tyr Leu Leu Leu Thr His Thr Val Ser Arg Leu Ala Val Ser
    490         500         510         520         530         540
    TAC CCG GAA AAG GTC AAC CTT CTC TCT GCC ATC AAG AGT CCC TGC CAG AAG
    Tyr Pro Glu Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Lys
                550         560         570         580         590
    GAG ACC CCA GAA GGG GCT GAG CGC AAG CCC TGG TAT GAA CCC ATC TAC CTG
    Glu Thr Pro Glu Gly Ala Glu Arg Lys Pro Trp Tyr Glu Pro Ile Tyr Leu
              600         610         620         630         640
    GGA GGC GTC TTC CAG CTG CAG AAG GGT GAC CGG CTC AGC GCT GAG GTC AAC
    Gly Gly Val Phe Gln Leu Gln Lys Gly Asp Arg Leu Ser Ala Glu Val Asn
            650         660         670         680         690
    CTG CCT CAG TAC CCT GAC TTT GCC GAT TCC GGG CAG ATC TAC TTT GGG GTC
    Leu Pro Gln Tyr Pro Asp Phe Ala Asp Ser Gly Gln Ile Tyr Phe Gly Val
          700         710                                         750
    ATT GCC CTG TGA GAAGGACGGACGTCTCCATCCATCCCTTCTCCTTCCCCCACCAGTTCCTT
    Ile Ala Leu End
                                                       800
    GTGCCACTCAGTCCTTCCGGTTGGGTCCCCTCACCTTCTCCTGGTTCAGAAAGGGAATTAGGGGT
                                                850
    CAAGGCCAAAACCTGAGCTTAAAACTTGAAACAATACCATTGCTCAGGAACTCAGGGTGCAGGGA
                    900
950
    TGCCTGGTGTAGACCGTGGGGTGCTCACTAACCACGTCAAGAACTAACTGGGCTTCCAGAACTCA
                                                   982
    CTGGGGCTCACAGTTTGGATCCCTGAATTC
```

Figure 3.1: The nucleotide and deduced amino acid sequence of gpTNFα. The ^ symbol between bases 240-241 denotes the position of the cleavage site to release the mature protein. The sequence is available from GenBank under accession number U77036.

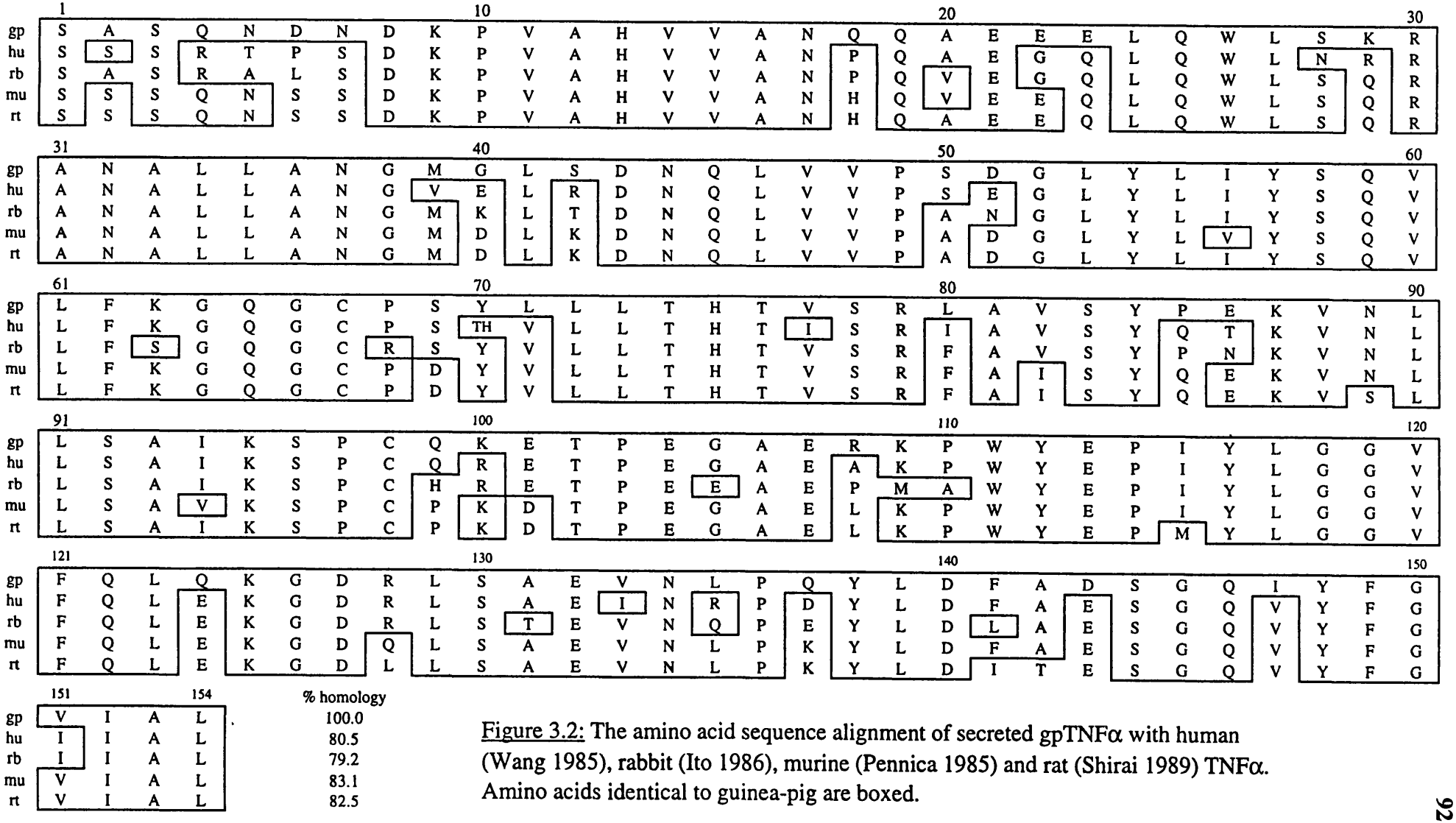


Figure 3.2: The amino acid sequence alignment of secreted gpTNFα with human (Wang 1985), rabbit (Ito 1986), murine (Pennica 1985) and rat (Shirai 1989) TNFα. Amino acids identical to guinea-pig are boxed.

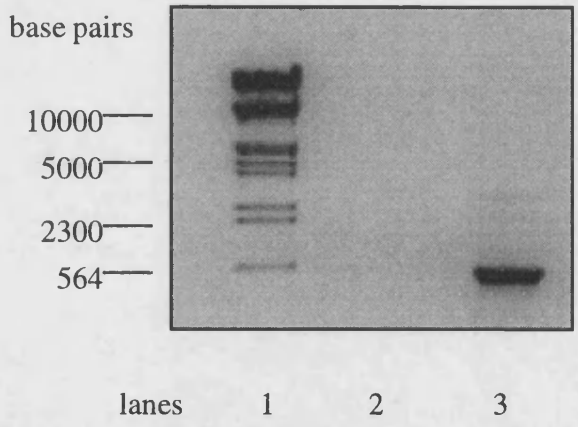


Figure 3.3: gpTNFα cDNA was amplified by PCR, using specific primers, to yield a single PCR product.
lane 1: λ base pair ladder, 2: control, no cDNA in PCR reaction 3: gpTNFα cDNA

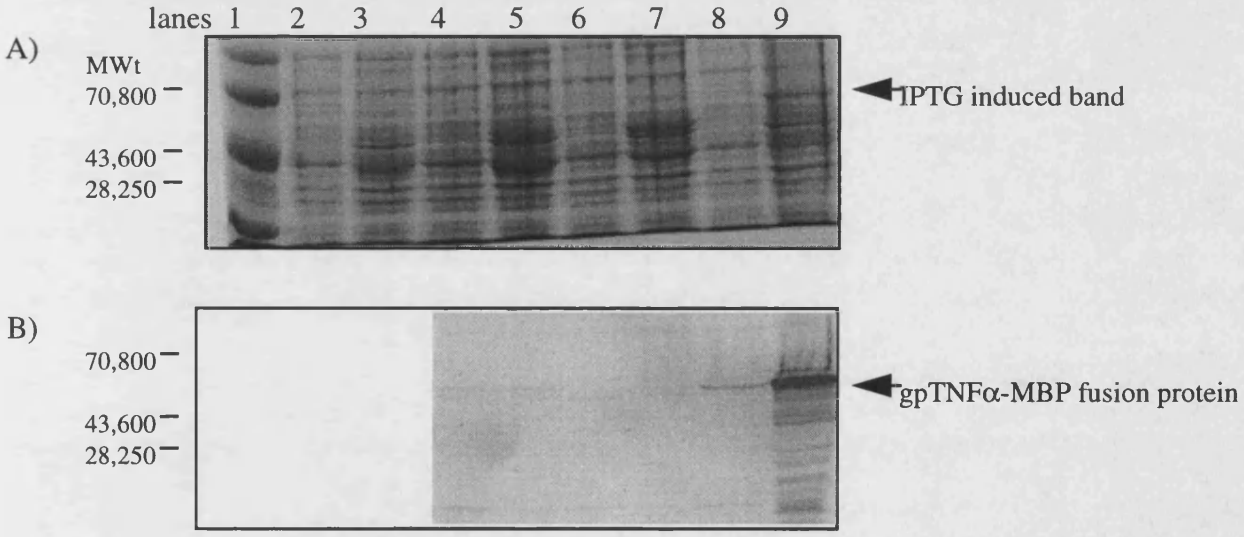


Figure 3.4: gpTNFα-MBP fusion protein expression following IPTG-induction of TB1 cells. Samples were run on a 10% SDS-PAGE gel and stained with coomassie blue (A) or transferred to nitrocellulose membrane and TNFα detected by western blotting (B). Molecule weight markers (MWt, kDa) were run in lane 1 and TB1 cell samples run in lanes 2-9, lane 2 and 3 :uninduced and induced cells without pMal-p2 insertion, lanes 4/5, 6/7 and 8/9 uninduced/induced cells, respectively, which exhibited inactive β-galactosidase activity (pMal-p2 inserted), but IPTG only induced significant fusion protein expression in one of the colonies (lanes 8 and 9) which was detected by anti murine TNFα antibody.

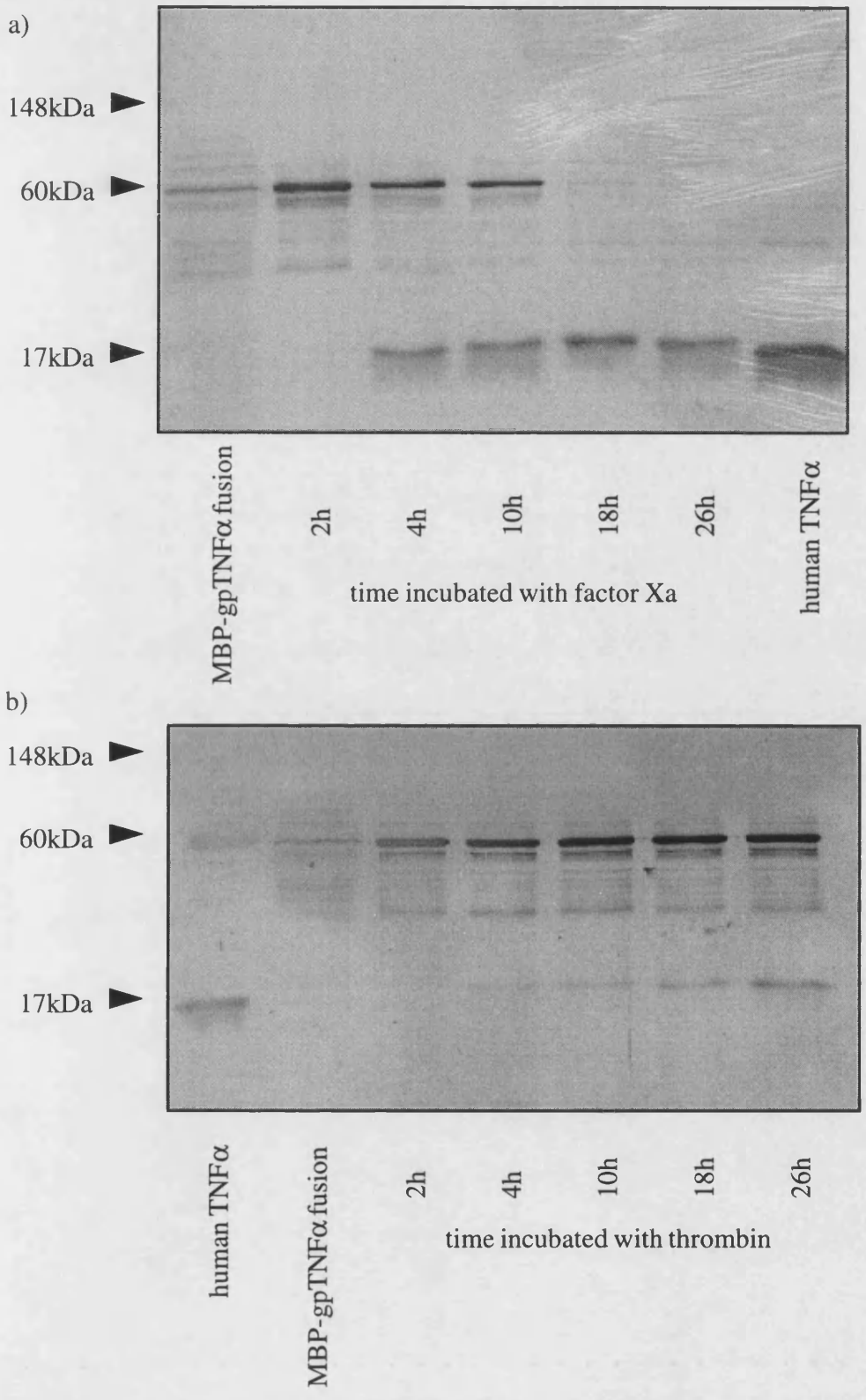


Figure 3.5: Western blot detection of gpTNFα by anti murine TNFα following cleavage of the MBP-gpTNFα fusion protein by Factor Xa (a) or thrombin (b).

3.3 Purification of gpTNFα

gpTNFα was purified by a series of affinity chromatography columns (amylose, hydroxyapatite, 2nd amylose and polymixin B) to yield a single band on a silver stained SDS-PAGE gel (figure 3.6) at 17 kDa. The purified protein was positively immunoblotted with goat and rabbit anti murine TNFα antibodies. The immunoblotting of gpTNFα with an anti factor Xa antibody was negative (figure 3.7) indicating that the factor Xa had been removed, although an affinity column had not been incorporated in the methodology to specifically remove factor Xa.

In initial experiments reverse phase HPLC, barium sulphate precipitation and heparin binding columns were used in attempts to isolate gpTNFα from the cleaved fusion protein mix, but these proved unsuccessful (not shown).

3.4 Cytolytic activity of gpTNFα

The WEHI 164 clone 13 cytotoxicity assay was used to determine the bioactivity of gpTNFα. Both the precleaved fusion protein and the purified gpTNFα exhibited cytolytic activity detailed in figure 3.8. The cytotoxic effect of 0.25ng/ml gpTNFα on WEHI 164 cells was dose dependently neutralised by increasing the concentration of rabbit anti murine TNFα sera (1 in 100 - 1 in 10000) pre-incubated with the protein, representative data is shown in figure 3.9. gpTNFα had an activity of 160IU/ng, compared with 40IU/ng of human TNFα standard.

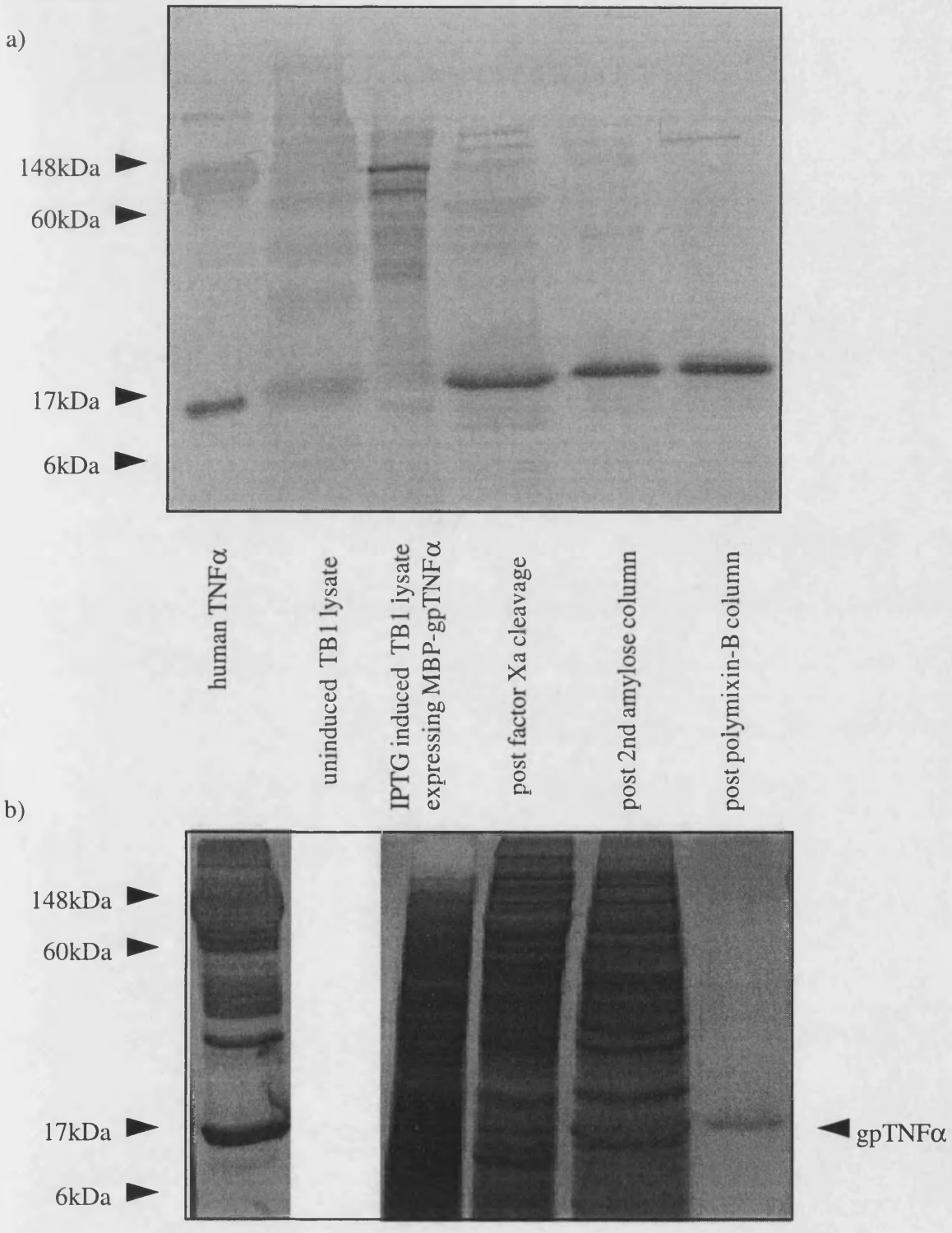


Figure 3.6: Western blot (a) and silver stained SDS gel (b) to show the presence of gpTNFα at various stages of purification. The MBP-gpTNFa fusion protein, expressed in TB1 *E. coli,* was cleaved with factor Xa and gpTNFα purified to a single band on a silver stained gel (b).

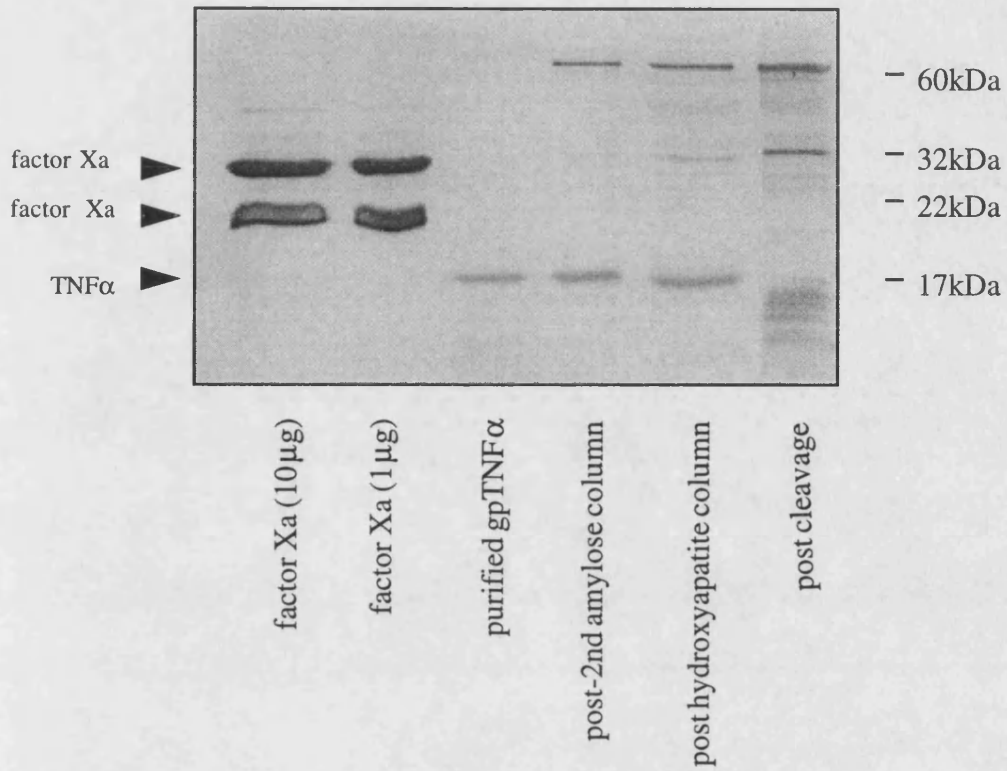


Figure 3.7: Western blot to show to the removal of factor Xa during the purification of gpTNFα. Factor Xa, which under denaturing conditions is detected as 30kDa and 20kDa proteins, was immunodetected using rabbit anti human factor Xa. The membrane was probed for a second time with goat anti murine TNFα to determine the presence of gpTNFα in the samples.

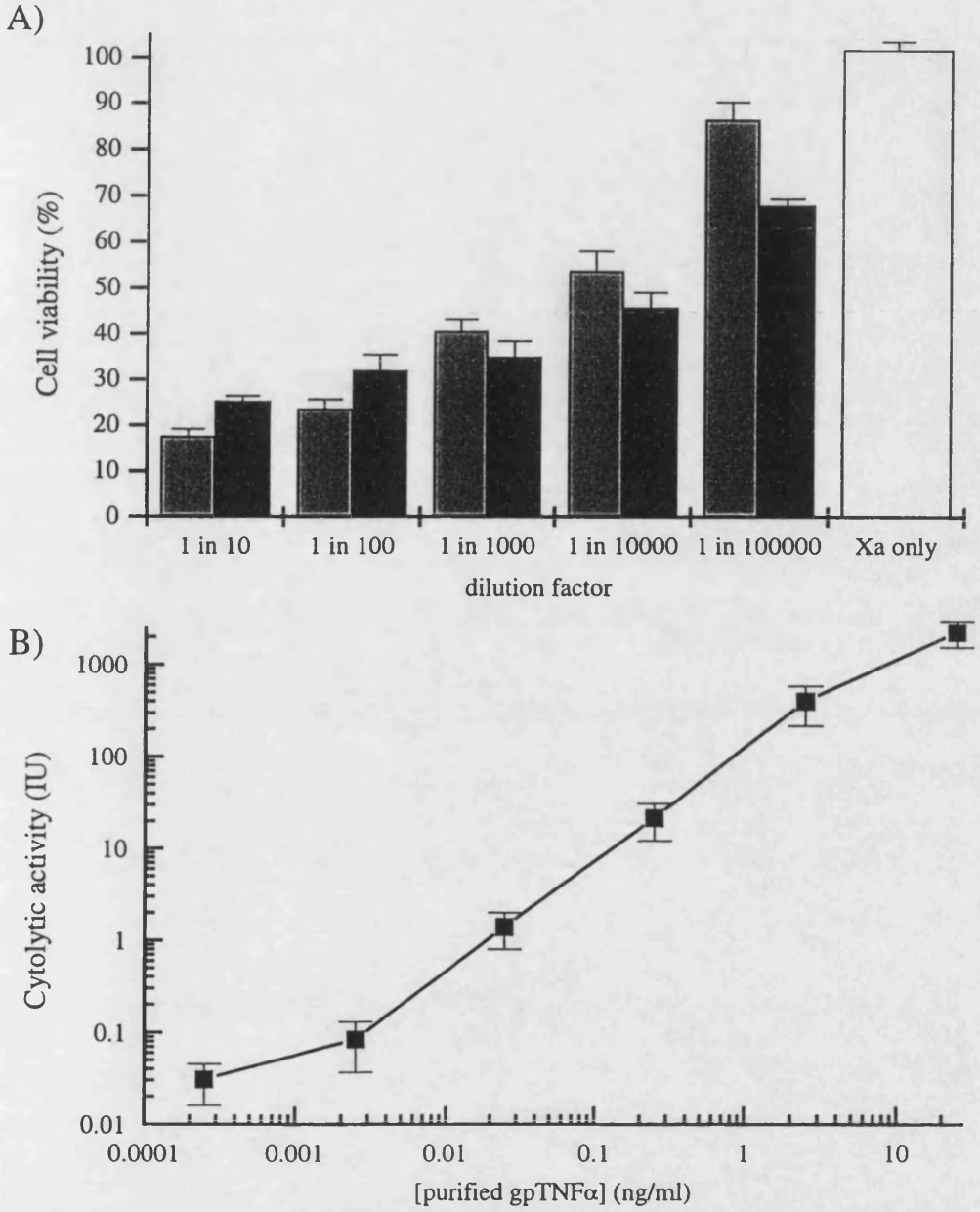


Figure 3.8: Bioactivity of gpTNFα was assessed various stages of purification by WEHI cytotoxicity assays.

A) The bioactivity of MBP-gpTNFα fusion protein before (▒▒) and after cleavage with factor Xa (■■). Factor Xa (200μg/ml) was incubated with the fusion protein for 18 h at room temperature. Cytotoxicity is expressed as cell viability with WEHI cells incubated with medium alone representing 100% viability, cells were incubated with factor Xa alone (□) as a control. Bars represent mean +/- sem from triplicate determinations.

B) Purified gpTNFα had a specific activity of 160IU/ng compared to recombinant human TNFα standards which had an activity of 40 IU/ng.
Points (—■—) represent mean +/- sem from triplicate determinations.

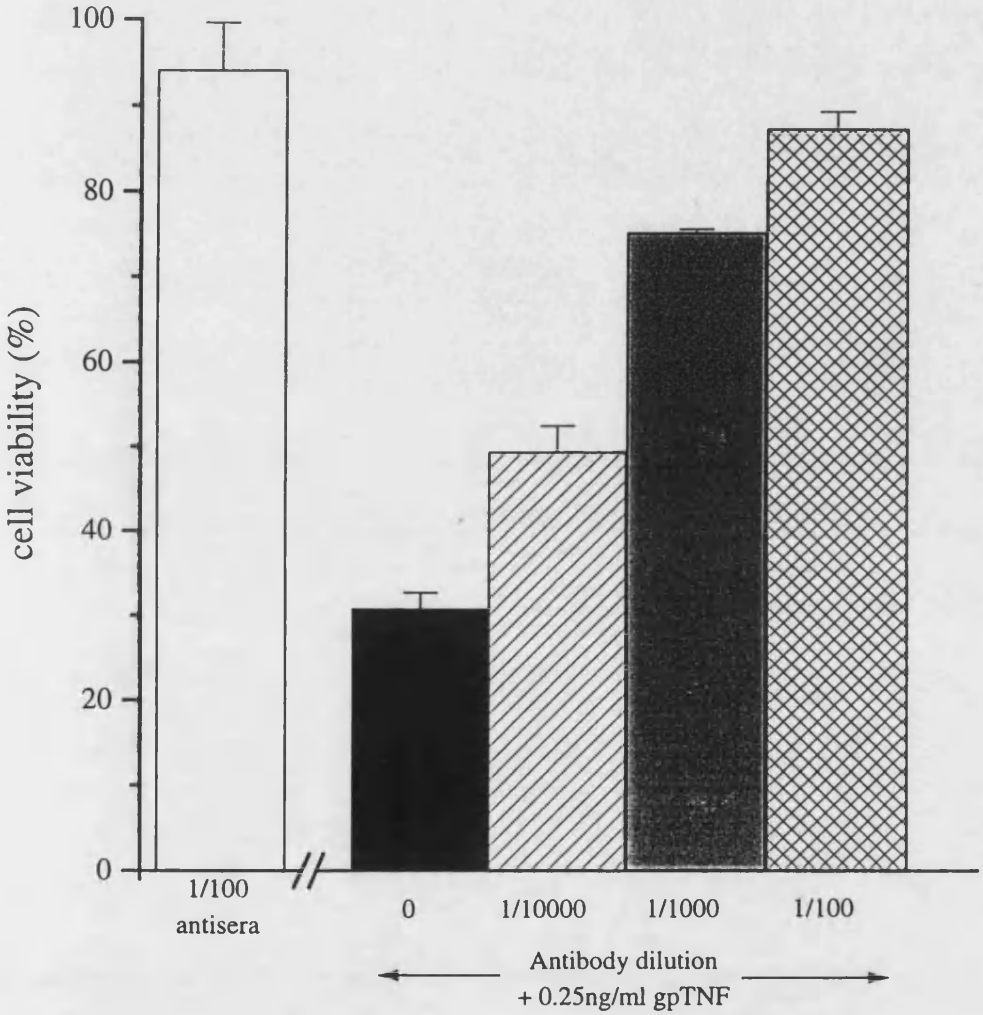


Figure 3.9: The effect on gpTNFα cytolytic activity by pre-incubating gpTNFα with anti TNFα antisera.
gpTNFα (■ , 25ng/ml) was pre-incubated with rabbit anti murine TNFα antisera 1 in 1000 (▨), 1 in 1000 (▩) or 1 in 10000 (▨) for 30 min at room temperature prior to addition to WEHI cultures. Cytotoxicity is expressed as cell viability with WEHI cells incubated with medium alone representing 100% viability, cells were incubated 1 in 100 rb α muTNFα alone (□) as a control.
Bars represent the mean +/- sem from triplicate determinations.

3.5 Detection of TNFα by ELISA

The murine TNFα ELISA detected murine TNFα over the range of 1-30ng/ml but did not detect gpTNFα (100ng/ml), data presented in figure 3.10. As the secondary antibody (polyclonal rabbit anti murine TNFα) is known to cross react with gpTNFα this suggests that the primary antibody (monoclonal hamster anti murine TNFα) did not cross react with the guinea-pig protein.

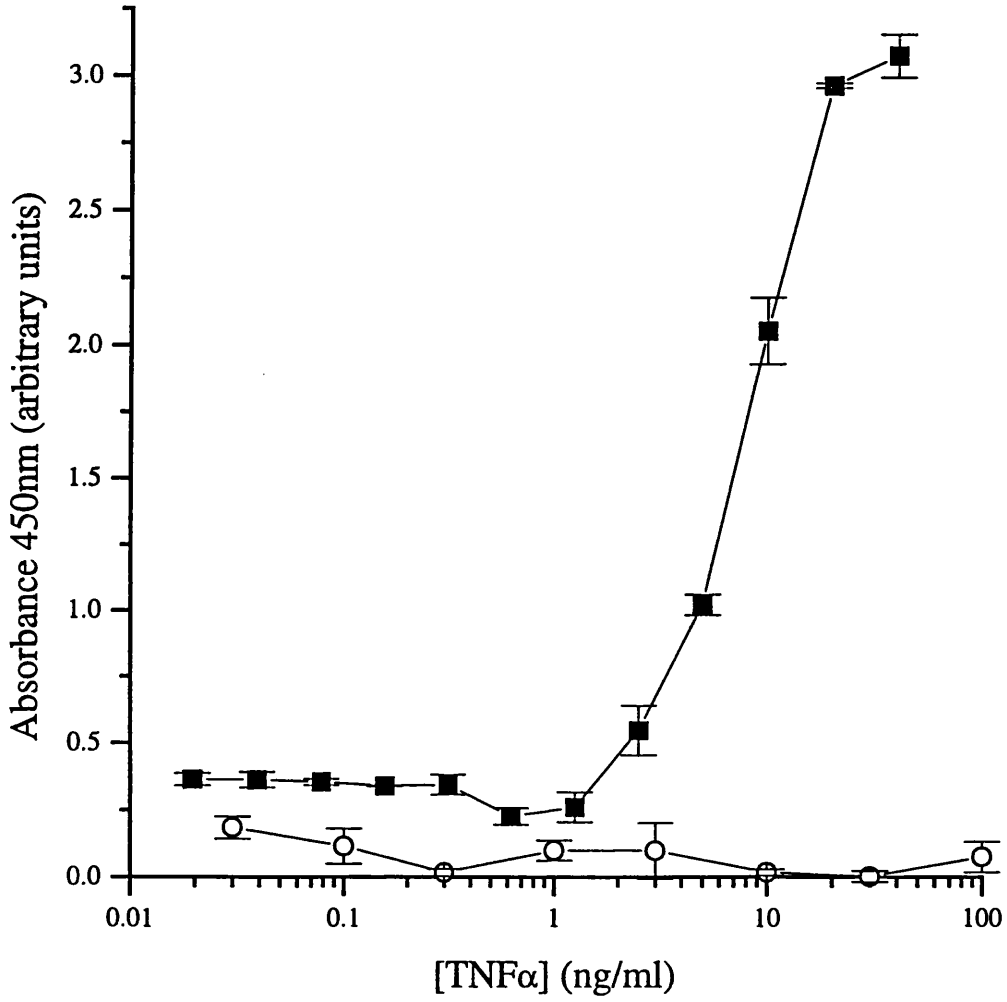


Figure 3.10: Measurement of gpTNFα using a murine TNFα ELISA.
gpTNFα (0.03-100ng/ml, —O—) and murine TNFα (0.01-30ng/ml, —■—) standards were compared in the asssay. Points represent mean absorbance values, with basal vehicle absorbance subtracted, of triplicates +/- sem.
Representative data for 2 experiments.

3.6 Summary of expression and purification of gpTNFα

- gpTNFα cDNA exhibited a 77.8% and 74.7% homology with murine and human TNFα cDNA respectively.
- Mature gpTNFα was expressed as a 154 amino acid, 17 kDa, protein which was 83.1%, 82.5%, 80.5 and 79.2% homologous with murine, rat, human and rabbit TNFα respectively.
- MBP affinity chromatography and purification using hydroxyapatite and polymixin B columns resulted in a single 17 kDa band on SDS-PAGE.
- Purified gpTNFα and the MBP-gpTNFα fusion protein was recognised by goat and rabbit anti murine TNFα antibodies.
- This recombinant gpTNFα has a cytolytic activity of 160IU/ng, compared with 40IU/ng recombinant huTNFα standards.
- gpTNFα cytolytic activity was neutralised by rabbit α murine TNFα antibodies.

4. Induction of chemokines by TNFα

4.1 The induction of chemokines by TNFα

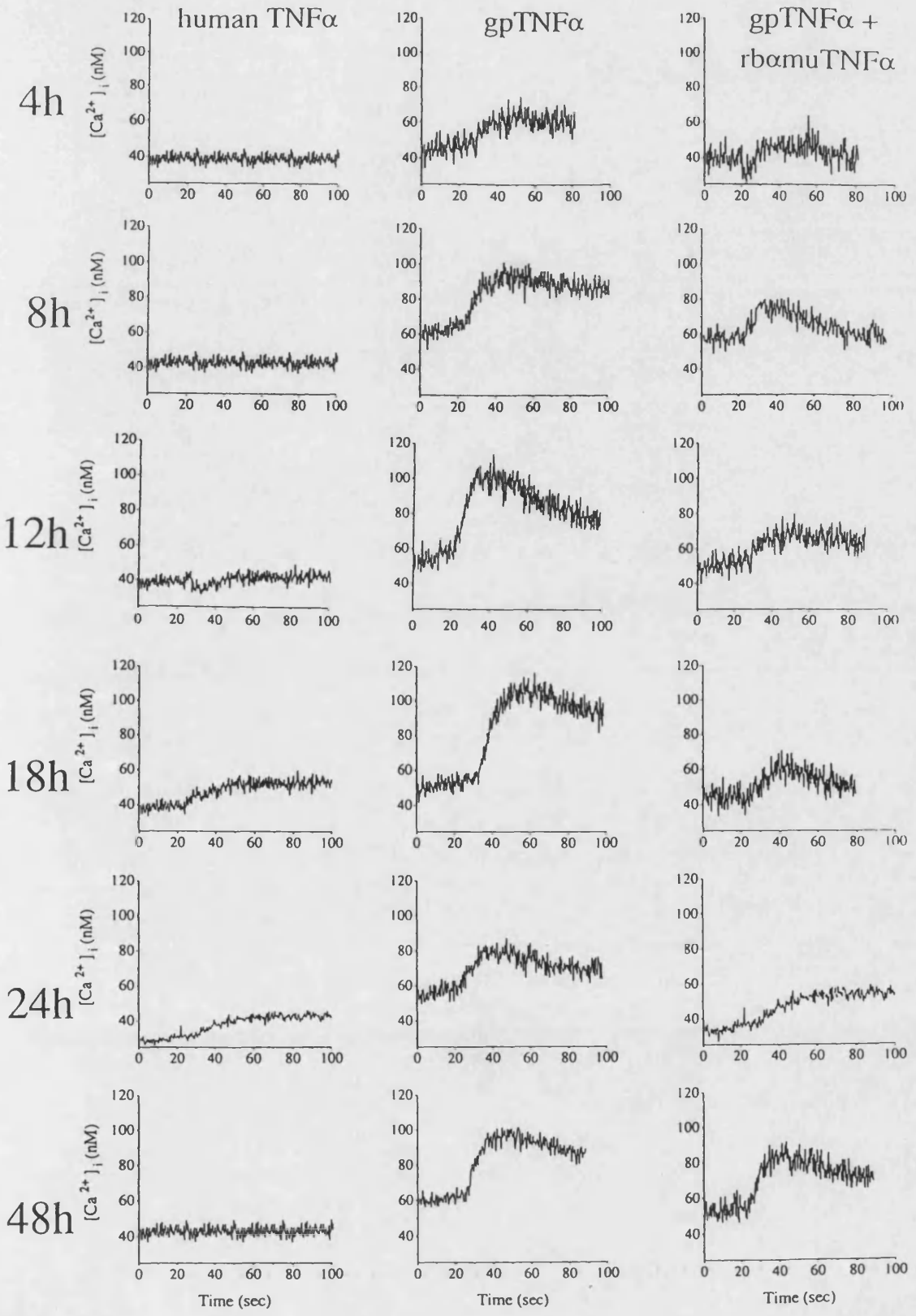
The ability of gpTNFα (recombinant and macrophage derived) and human TNFα to stimulate the release of chemokine-like bioactivity from JH-4 cells was assessed by measuring cell culture-supernatant induced calcium mobilisation in guinea-pig peritoneal leukocytes. JH-4 IL-8 mRNA production following gpTNFα (recombinant) stimulation was also examined.

Supernatants from the incubation of JH-4 cultures with recombinant gpTNFα for 4-48 hours induced an increase in $[Ca^{2+}]_i$ of peritoneal neutrophils. Maximum $[Ca^{2+}]_i$ elevations were observed in supernatants from 18 hours incubation with gpTNFα. The ability of gpTNFα to stimulate JH-4 cells to produce calcium mobilising activity was reduced to control levels by coincubating with rabbit anti murine TNFα sera, examples of the experimental traces are shown in figure 4.1. Culturing cells with rabbit anti murine TNFα sera alone (control) did induce chemokine-like activity in the supernatants but for each time matched control the $[Ca^{2+}]_i$ elevation was less than the rises induced by supernatants from incubating cells with gpTNFα alone. The supernatants from incubations of JH-4 with human TNFα only induced slight increases in $[Ca^{2+}]_i$ in peritoneal neutrophils following 18 and 24 hours incubation, shown in figure 4.1.

Guinea-pig IL-8 mRNA was detected in JH-4 cells incubated with gpTNFα for 6 and 12 hours by northern blot analysis (figure 4.2). This mRNA expression was reduced by co-incubating with rabbit anti murine TNFα sera. IL-8 mRNA was not detected following the incubation of JH-4 with human TNFα at 6 or 12 hours.

Figure 4.1 (please see next page):

Figure 4.1 (see facing page): Experimental traces to show the elevation of $[Ca^{2+}]_i$ in fura 2 loaded guinea-pig peritoneal neutrophils induced by JH-4 culture supernatants. Confluent JH-4 cells were stimulated with human TNFα 100ng/ml, gpTNFα 100ng/ml (gpTNFα) or gpTNFα 100ng/ml + rabbit anti murine TNFα sera 1 in 100 (gpTNFα + rbαmuTNFα) for 4 - 48 hours. Guinea-pig peritoneal neutrophils ($1x10^6$ cells/ml) were stimulated with 100µl supernatant at approximately 25 seconds. Experimental traces are representative of 2 experiments.


human TNFα
gpTNFα
gpTNFα + rbαmuTNFα
4h
8h
12h
18h
24h
48h
[Ca2+]i (nM)
Time (sec)

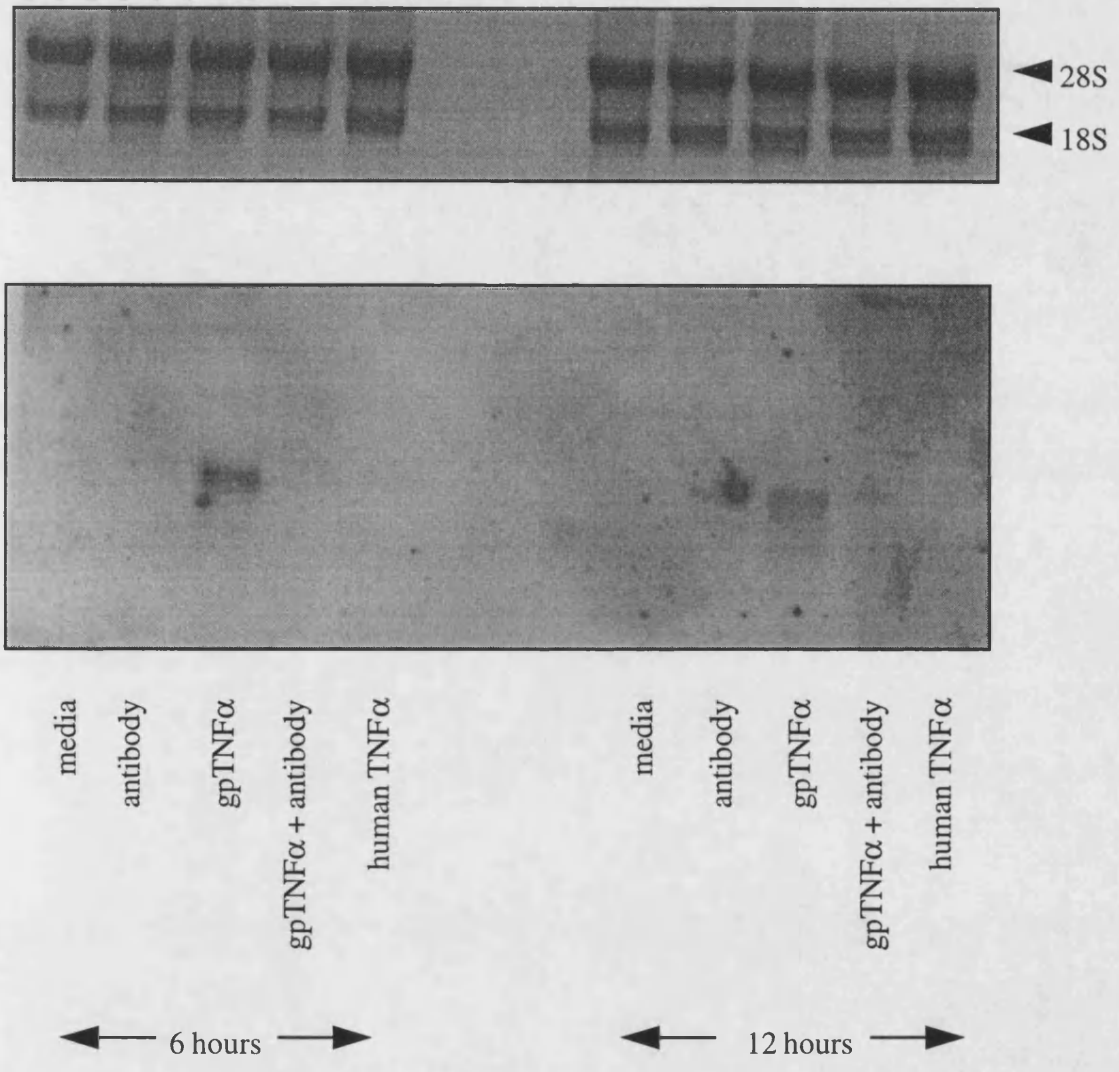


Figure 4.2: gpIL-8 mRNA expression in stimulated JH-4 cells, measured by Northern blot analysis. JH-4 cells were incubated with gpTNFα (100ng/ml), gpTNFα (100ng/ml) +rabbit anti murine TNFα, sera (1 in 100), human TNFα (100ng/ml) and antibody (1 in 100) or media alone as controls for 6 and 12 hours. The top panel shows equal loading of the gel as denoted by the 18S and 28S bands.

Experiments to determine the ability of human cytokines to induce chemokine release from guinea-pig cells were performed. Supernatants from cell cultures of guinea-pig spleen cells, peritoneal macrophages and JH-4 cell line, stimulated with human cytokines (TNFα, IL-1α and IFNγ either alone or in combinations) for 24 hours were assayed for the ability to induce elevation of $[Ca^{2+}]_i$ in guinea-pig peritoneal leukocytes. With the exception of the supernatant from JH-4 cells cultured with human TNFα, which elicited small amounts of IL-8-like (neutrophil stimulating) activity, none of the human cytokines, when added alone or in combination, were able to induce chemokine activity in the culture supernatants. All supernatants were assayed on peritoneal neutrophils, macrophages and eosinophils. For example, fura-2 loaded peritoneal neutrophil, macrophage and eosinophil $[Ca^{2+}]_i$ levels remained at 87 ± 5nM n=18, 72 ± 3nM n=14, 44 ± 2nM n=12, respectively, following the addition of cell culture supernatants collected 24 hours following the stimulation of the cell cultures with either a single human cytokine or cytokine combinations. However, increases in $[Ca^{2+}]_i$ levels were observed when fura-2 loaded eosinophils were stimulated with supernatant from JH-4 cells incubated with guinea-pig macrophage derived gpTNFα (1μg/ml) for 24 hours. The addition of supernatant (100μl) induced elevations of $[Ca^{2+}]_i$ in eosinophils by 76nM ± 21nM, n=3. In each case the fura-2 loaded cells were shown to be responsive to 10nM human chemokine standards (human RANTES or human IL-8) or PAF (table 4.1).

Table 4.1: Agonist induced elevations of $[Ca^{2+}]_i$ in fura-2 loaded guinea-pig peritoneal leukocytes.

Leukocyte	**Purity of preparation**	**10nM agonist**	**Increase in $[Ca^{2+}]_i$ (nM)**	**n**
eosinophils	92%±3%	PAF	265.1 ± 24	12
neutrophils	82%±7%	human IL-8	236.3 ± 23	18
macrophages	86%±5%	human RANTES	184.2 ± 19	14

4.2 Summary of the induction of IL-8 and chemokine-like activity by TNFα

- Supernatants from JH-4 cells incubated with recombinant gpTNFα, for 4 - 48 h, induced an IL-8-like bioactivity which was maximal at 18 hours. This bioactivity in the supernatant was reduced by co-incubating the JH-4 cells with gpTNFα and rabbit anti murine TNFα antibody.
- IL-8 mRNA was detected in JH-4 cells following incubation with gpTNFα but not human TNFα.
- Supernatants from guinea-pig spleen cells, peritoneal macrophages and JH-4 cells incubated with human TNFα either alone or in combination with IL-1 and IFNγ, for 24 h, did not induce increases in $[Ca^{2+}]_i$ in fura-2 loaded peritoneal leukocytes.
- Supernatants from JH-4 cells incubated with guinea-pig macrophage derived TNFα for 24 h, induced increases in $[Ca^{2+}]_i$ in fura-2 loaded peritoneal eosinophils.

5. *In vivo* bioactivity of TNFα in the guinea-pig lung

5.1 In vivo bioactivity of gpTNFα

For all of the following *in vivo* studies tracheal instillation was used. The major advantages of this method were that a known amount of agent could be instilled directly to the trachea and as the animals were allowed to recover following instillation this enabled time course studies to be performed. To monitor the delivery and distribution of the instilled agents pilot experiments were performed using a protein dye, Evans Blue (50μg per animal), and an eosinophil chemoattractant, PAF (5nmoles per animal). Examination of the intact lungs following instillation of Evans Blue indicated that this method evenly distributed the instilled agents within the lung. Furthermore, the efficiency of the method was demonstrated by the instillation of PAF which induced a pronounced BAL eosinophil and neutrophil accumulation 24 hours post instillation as shown in figure 5.1.

Instillation of gpTNFα (50 -150ng per animal) induced a significant BAL eosinophil accumulation ($p<0.01$) at 24 hours (shown in plate 5.1 a and b) compared with vehicle instilled time matched controls. This response was dose dependent reaching significance at 50 and 150ng/animal, as shown in figure 5.2. BAL neutrophil numbers were variable within experiments. No increase in macrophage/monocyte numbers was observed at 15 or 50ng gpTNFα, compared to vehicle treated animals, although the higher dose of 150ng induced macrophage/monocyte infiltration. Lymphocyte numbers were not significantly altered in any of the test groups.

Pre-incubation of rabbit anti murine TNFα sera (1 in 100) with gpTNFα prior to instillation reduced the BAL eosinophil accumulation by 79.2%±9.4%, 24 hours after instillation, as detailed in figure 5.3. Heat treatment of 50ng gpTNFα abrogated the ability of gpTNFα to recruit leukocytes to the airways, 24 hours after instillation (figure 5.4).

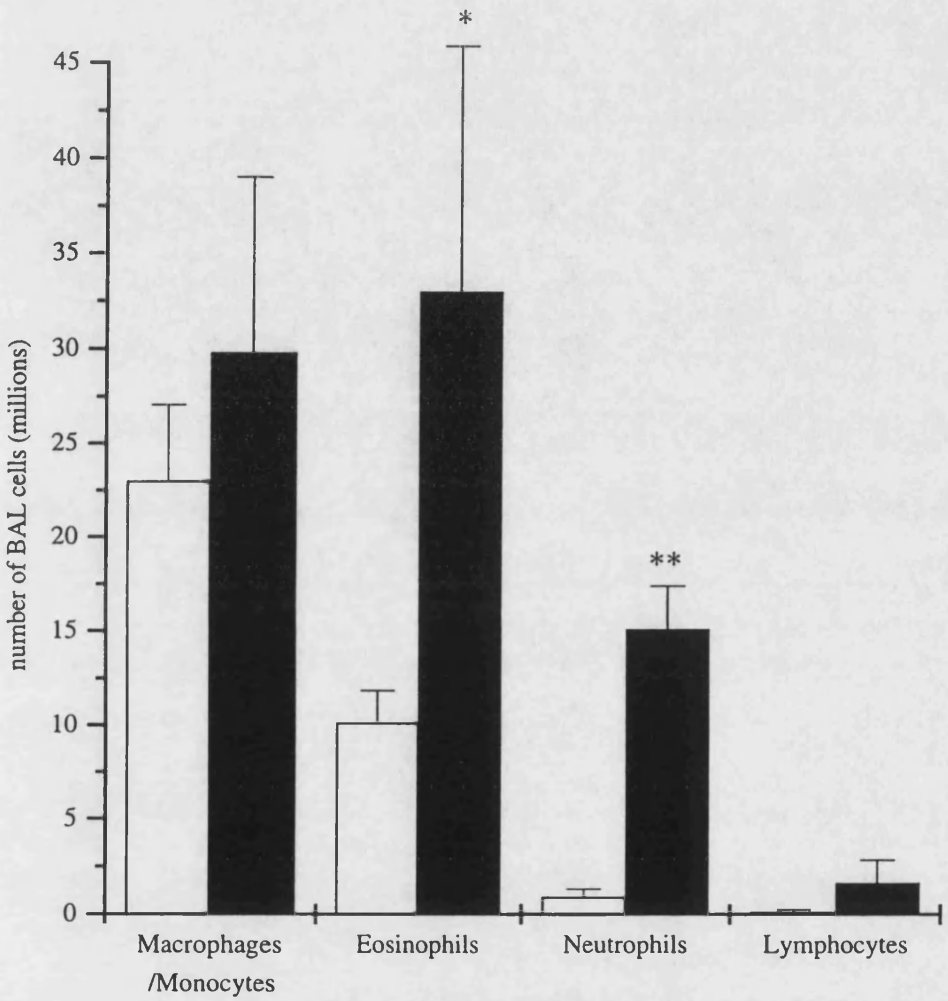
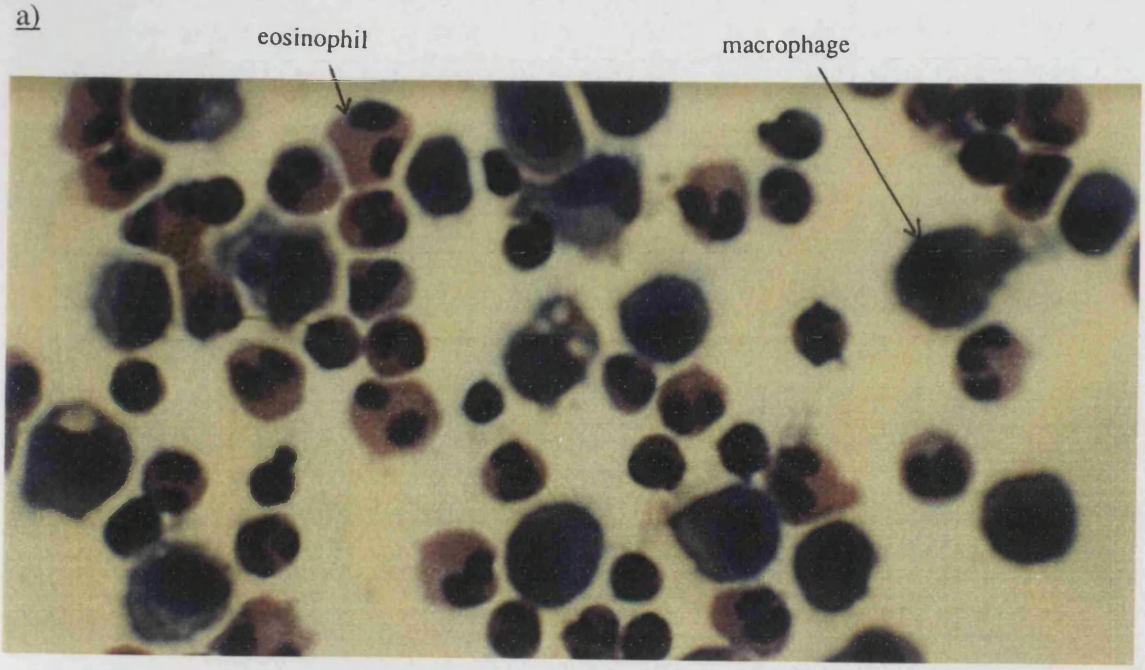


Figure 5.1: The effect of the instillation of PAF on BAL leukocyte accumulation. Guinea-pigs were instilled with either vehicle PBS 0.1% BSA (☐) or PAF (■ ; 5nmoles/animal) 24 h prior to BAL. Bars represent the mean +/- sem, n=3 animals/treatment. *$p<0.05$, **$p<0.01$ compared to vehicle treated animals.

a)



b)

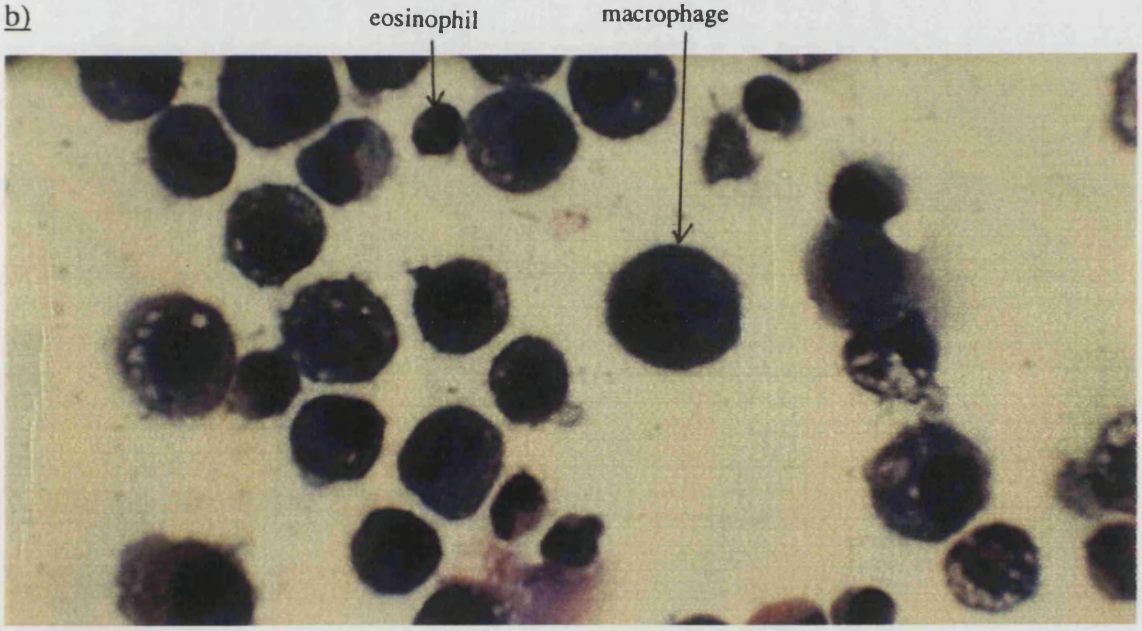


Plate 5.1 a: Photograph of gpTNFα (50ng) induced BAL eosinophil accumulation 24 hours after instillation of naive guinea-pigs, BAL from vehicle instilled animals is shown in plate 5.1 b. Magnification 2000x, cells stained with Diff-Quik.

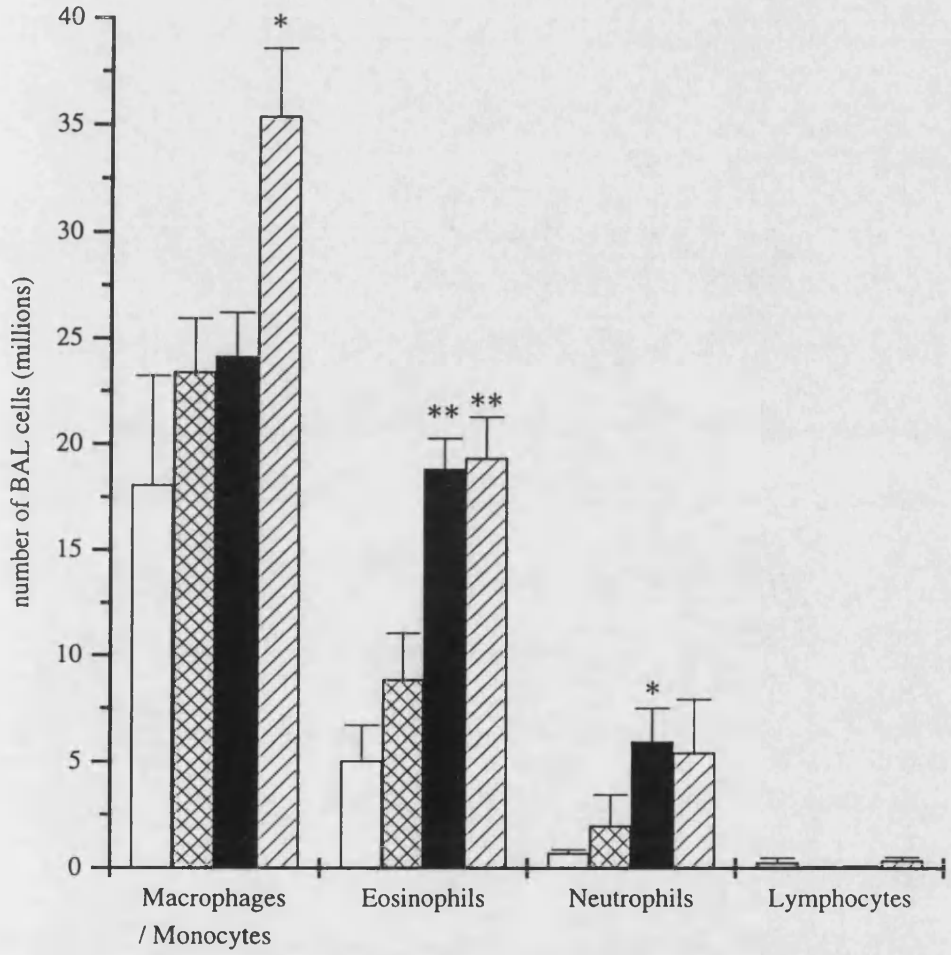


Figure 5.2: The effect of the instillation of gpTNFα on BAL leukocyte accumulation. Guinea-pigs were instilled with either vehicle (PBS 0.1% BSA ☐), 15 (▨), 50 (■) or 150ng (▨) gpTNFα/animal 24 h prior to BAL. Bars represent the mean +/- sem, n=3 animals/dose. *p<0.05, **p<0.01 compared with vehicle treated animals.

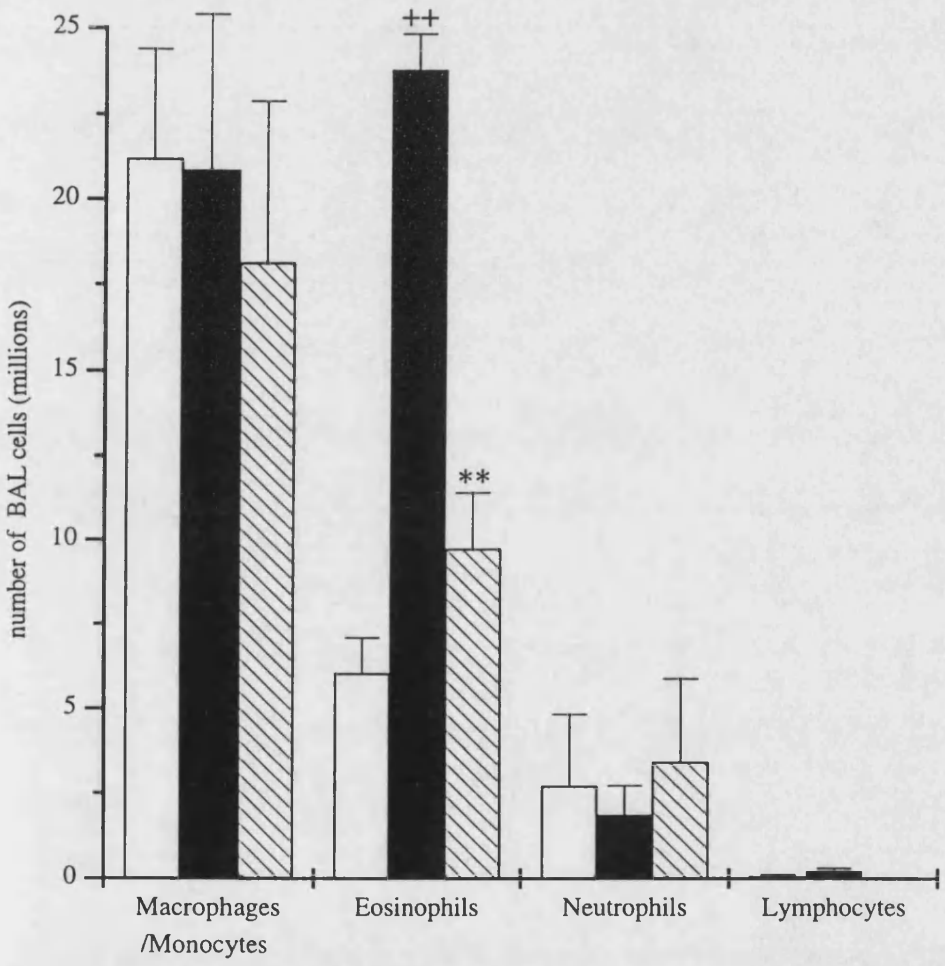


Figure 5.3: Neutralisation of gpTNFα induced BAL leukocyte accumulation by anti TNFα antibody. Guinea-pigs were instilled with either vehicle (PBS 0.1% BSA, ☐), 50ng gpTNFα (■) or 50ng gpTNFα preincubated with 1 in 100 rabbit anti murine TNFα serum (▨) 24 h prior to BAL. Bars represent the mean +/- sem, n=3 animals/treatment. ++p<0.01 compared with vehicle treated animals, **p<0.01 compared with animals treated with gpTNFα alone.

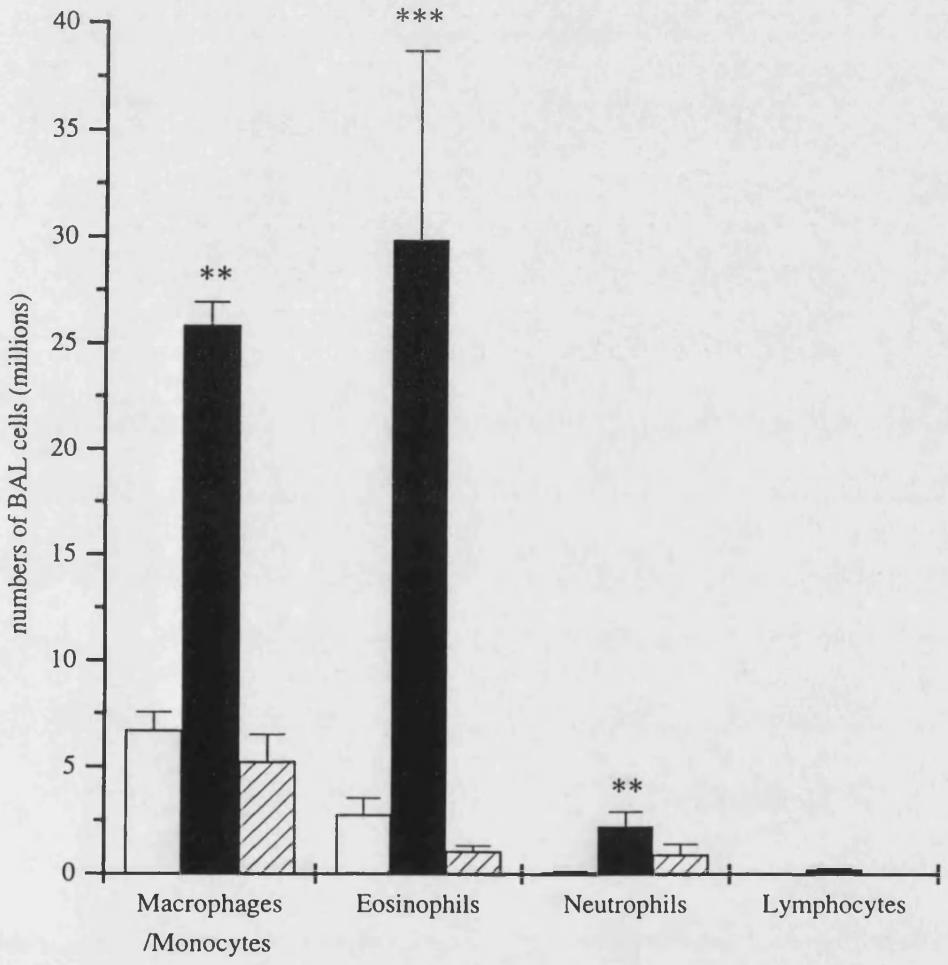


Figure 5.4: The effect of heat treating gpTNFα on BAL leukocyte accumulation. Guinea-pigs were instilled with either vehicle (PBS 0.1% BSA ☐), gpTNFα (50ng ■) or heat treated gpTNFα (50ng, 80°C 1 h, ▨). Bars represent the mean +/- sem, n=3 animals/treatment. **p<0.01, ***p<0.001 compared with vehicle treated animals.

Time course studies of BAL leukocyte accumulation, presented in figure 5.5, following the instillation of gpTNFα (50ng) indicated that maximum leukocyte accumulation occurred 24 hours after instillation. A slight increase in total BAL leukocyte numbers occurred 6 hours after instillation which did not reach significance ($p=0.051$) possibly due to the small sample size ($n=3$). A pronounced BAL eosinophil infiltration was observed 24 hours and maintained significantly above vehicle treated time matched controls at 48 and 72 hours after instillation. In these time course studies significant increases in BAL macrophage/monocyte and neutrophil numbers was only observed at 72 hours after instillation. BAL lymphocyte numbers were not significantly elevated at any of the time points studied.

Further experiments were performed to assess the effect of the instillation of gpTNFα (50ng) on *in vivo* bronchial hyperreactivity 24 hours after instillation. As can be seen in figure 5.6, although gpTNFα did cause BAL leukocyte accumulation in these animals, bronchial hyperreactivity was not induced. Administration of either 1.8-10μg/kg histamine or methacholine 3.2 and 10μg/kg caused a dose response effect increasing lung resistance in both the control vehicle instilled animals and in the gpTNFα instilled animals but differences between the groups were not statistically significant.

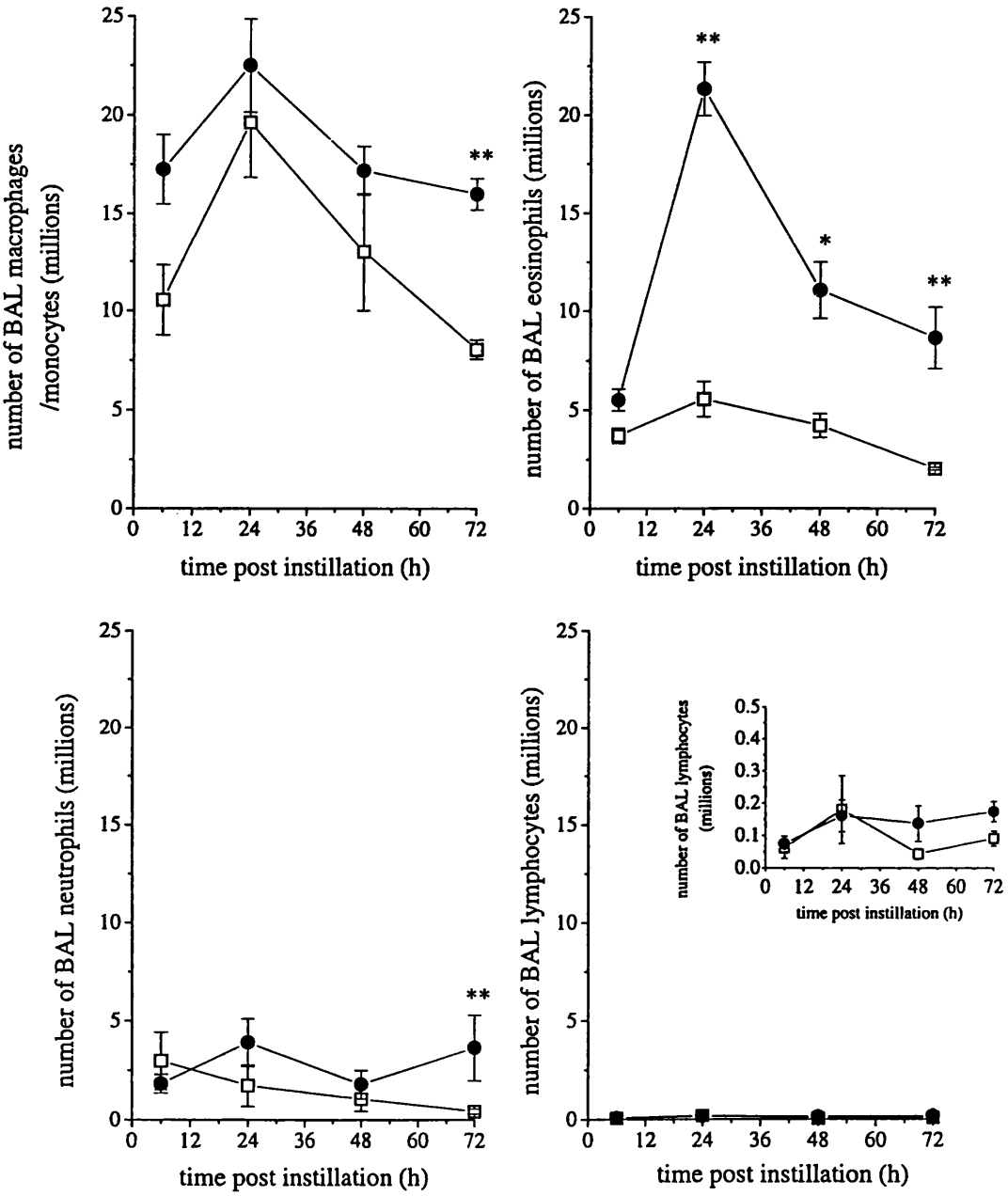


Figure 5.5: Time course of gpTNFα induced BAL leukocyte accumulation.
Guinea-pigs were instilled with either vehicle (PBS 0.1% BSA, —□—) or 50ng gpTNFα (—●—) at 6, 24, 48 or 72 h prior to BAL. Points represent the mean +/- sem, n=3 animals/treatment at 6, 48, and 72 h, n=6 animals/treatment at 24 h.
*$p<0.05$, **$p<0.01$ compared with animals treated with vehicle treated animals.
Lymphocyte data insert shows enlarged scale.

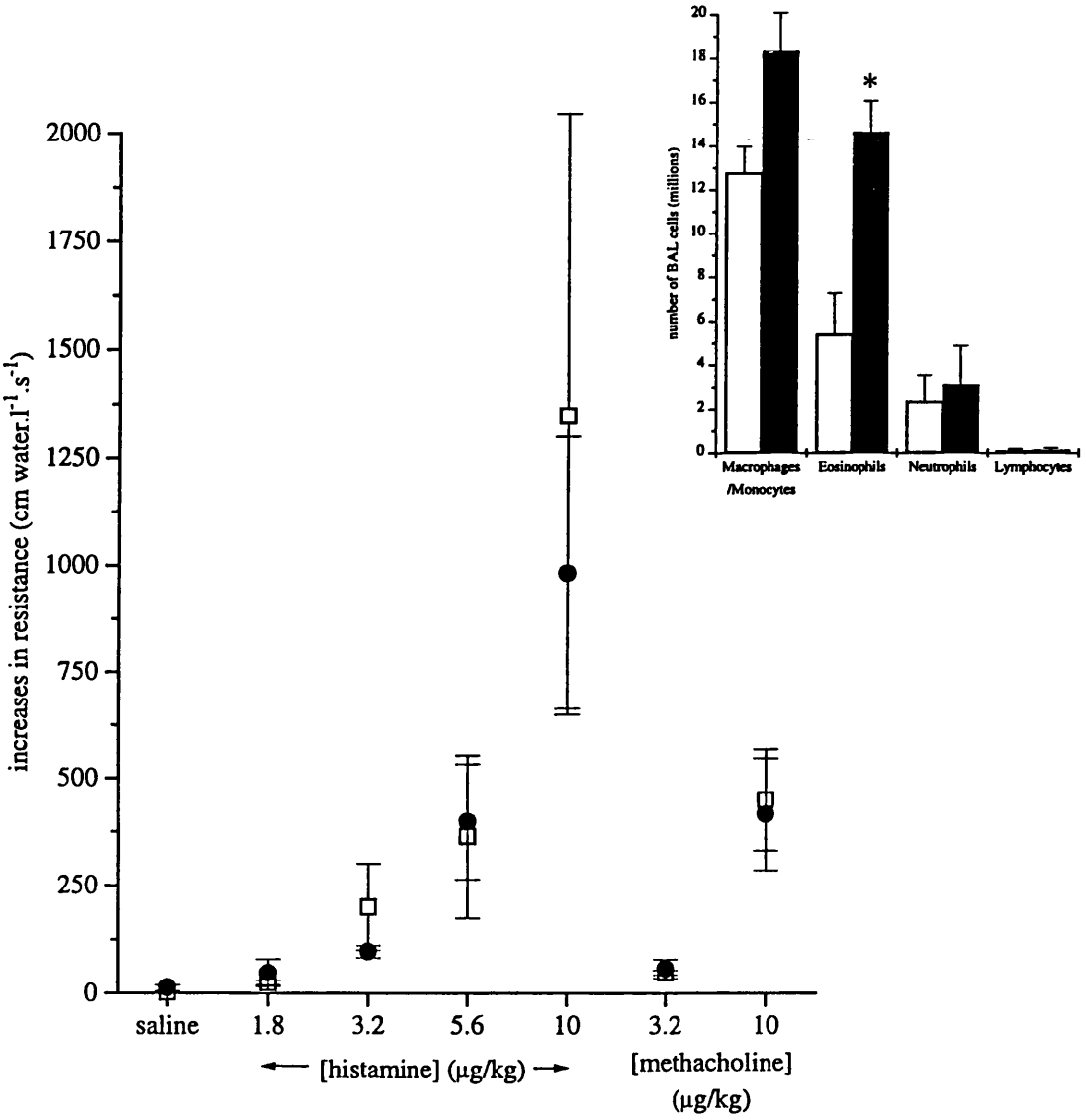


Figure 5.6: The effect of instilling 50ng gpTNFα on *in vivo* bronchial responsiveness induced by histamine and methacholine. Guinea-pigs were instilled with either vehicle (PBS 0.1% BSA □) or gpTNFα (50ng ●) 24 h prior to lung function tests being performed. Points represent the mean +/- sem, n=3 animals/treatment.

Figure 5.6 insert: The effect of instilling 50ng gpTNFα on BAL leukocyte accumulation, following lung function tests, indicating that the gpTNFα was biologically active. Guinea-pigs were instilled with either vehicle (PBS 0.1% BSA □) or gpTNFα (50ng ■) 26 h prior to BAL. Bars represent the mean +/-sem, n=3 animals/treatment. *p<0.05 compared with vehicle instilled animals.

5.2 *In vivo bioactivity of human TNFα*

In comparison with gpTNFα, the instillation of human TNFα (15-150ng) did not induce BAL eosinophil infiltration by 24 hours, figure 5.7. At the highest dose examined (150ng) human TNFα induced elevations in BAL macrophage/monocyte (which increased by approximately 2 fold) and neutrophil numbers, but only the increase in neutrophils reached statistical significance. A photograph of BAL leukocytes following instillation of 150ng human TNFα is shown in plate 5.2.

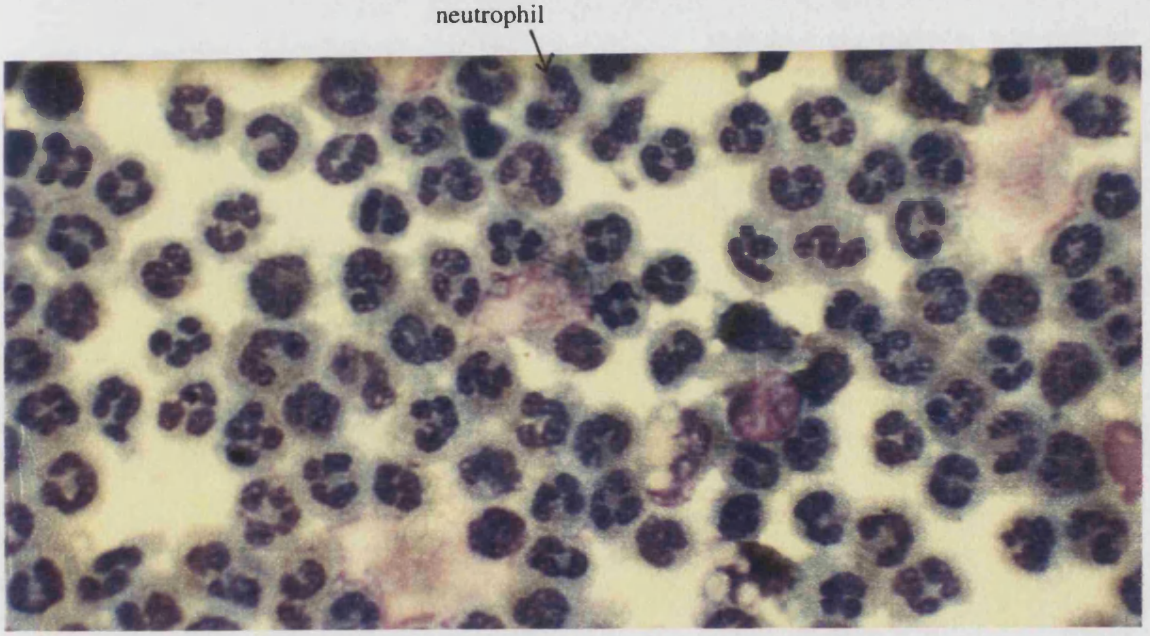


Plate 5.2: Photograph of human TNFα (150ng) induced BAL neutrophil accumulation 24 hours after instillation of naive guinea-pigs. Magnification 2000x, cells stained with Diff-Quik.

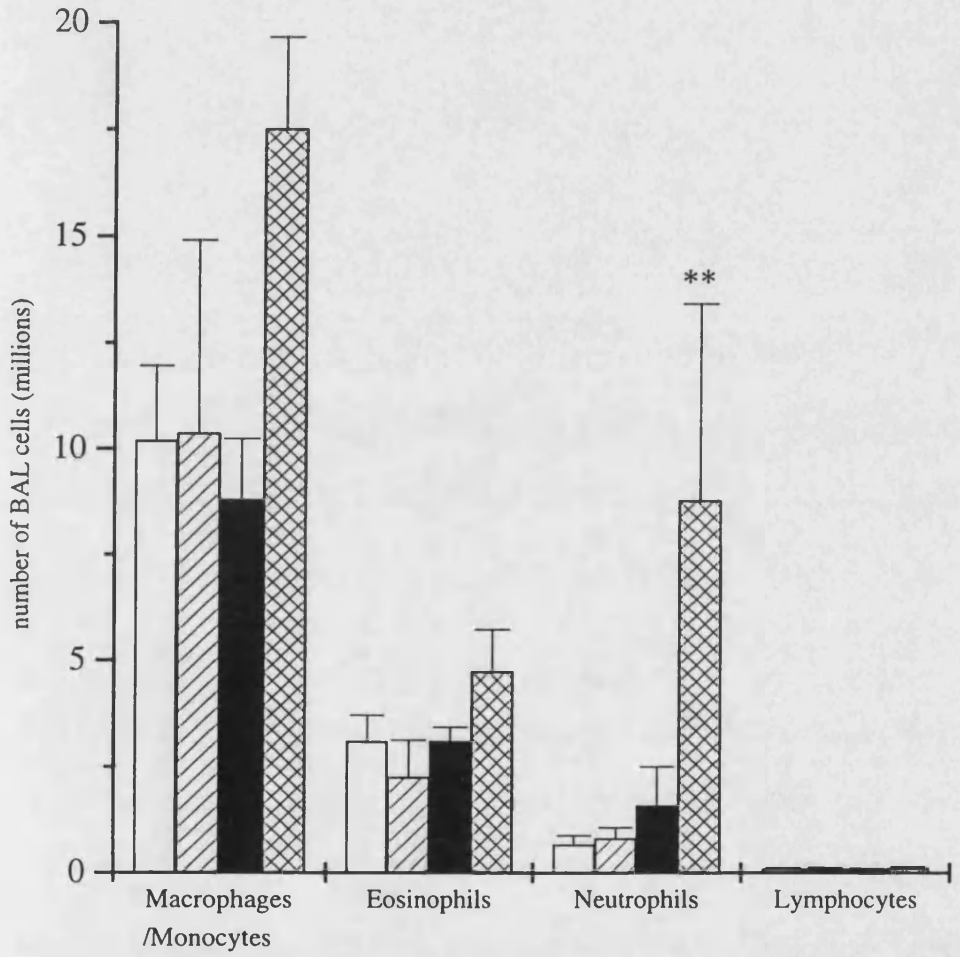


Figure 5.7: The effect of instilling human TNFα on BAL leukocyte accummulation. Guinea-pigs were instilled with either vehicle (PBS 0.1% BSA, ☐, n=6), 15 (▨, n=3), 50 (■, n=3) or 150ng (▩, n=6) human TNFα/animal 24 h prior to BAL. Bars represent the mean +/- sem for each treatment. **p<0.01 compared with vehicle treated animals, where no symbols are indicated the differences were not significant (p>0.05) compared with appropriate controls.

In addition, experiments were performed to assess the effect of administering aerosol human TNFα to naive guinea-pigs. The aerosols were delivered either via a nose-cone or via an in-line nebulizer connected between the ventilator and the trachea cannulae. As shown in tables 5.1 and 5.2 and figures 5.8 and 5.9, at the time points examined the administration of human TNFα, by either method, did not induce airway hyperreactivity or BAL leukocyte accumulation. Figures 5.8 and 5.9 show the effect of administering human TNFα, by aerosol challenge using a nose cone, on airway reactivity and BAL leukocyte infiltration 6 hours after challenge. Furthermore, visual observation of the basal lung resistance and compliance levels over the 30min TNFα (15μg) challenge, using the in-line ultrasonic nebuliser, indicated that aerosol administration of human TNFα had no immediate overt effect on *in vivo* lung function.

Table 5.1: Changes in *in vivo* guinea-pig bronchial reactivity following the aerosol administration of human TNFα. When using the in-line tracheal nebuliser each animal served as its own control with the increase in lung resistance induced by 10μg/kg histamine assessed immediately prior to challenge (before TNFα) and 30 min after TNFα (15μg) challenge. 50μg human TNFα was administered via the nose-cone with vehicle challenged animals being exposed to an equal volume of saline 0.1% BSA.

Human TNFα administered via	**Time after challenge**	**Treatment (n)**	**Increase in lung resistance (cm $H_2O.l^{-1}.s^{-1}$) induced by histamine (10μg/kg)**
in line ultrasonic nebuliser	30 min	beforeTNFα (3)	483.3±239
		after TNFα (3)	451.7±284
nose-cone jet nebuliser	2 hours	vehicle (4)	393.2±68
		TNFα (6)	405±67
nose-cone jet nebuliser	6 hours	vehicle (4)	612.6±196
		TNFα (4)	428.1±219

Table 5.2: Human TNFα induced changes in BAL leukocyte numbers when administered via a nose-cone (50μg) or an in-line tracheal nebuliser (15μg) Vehicle challenged animals were exposed to an equal volume of saline 0.1% BSA.

Human TNFα administered via	Time after challenge	Treatment (n)	BAL leukocyte numbers (millions)			
			macrophages /monocytes	eosinophils	neutrophils	lymphocytes
in-line ultrasonic nebuliser	2 hours	vehicle (3)	5.69±1.7	1.28±0.7	1.09±0.6	0.21±0.06
		TNFα (3)	8.40±0.9	1.73±0.2	1.18±0.3	0.26±0.2
nose-cone jet nebuliser	4 hours	vehicle (4)	11.46±1.3	5.93±1.5	0.38±0.05	1.0±0.2
		TNFα (6)	7.98±1.7	4.23±1.2	0.30±0.08	0.61±0.2
nose-cone jet nebuliser	8 hours	vehicle (4)	10.31±2.5	4.11±1.3	0.17±0.04	0.45±0.1
		TNFα (4)	11.46±4.9	6.05±1.9	0.28±0.1	0.51±0.1

Experiments were performed to determine whether aerosol generation affected the bioactivity of TNFα (table 5.3). TNFα WEHI cytolytic activity was assessed in samples before and after ultrasonic nebulisation. This treatment was found not to change TNFα bioactivity.

Table 5.3: The bioactivity of TNFα was assessed by WEHI bioassay. The cytolytic activity prior to aerosolisation was equated to represent 100% TNFα bioactivity.

TNFα treatment	Cytolytic activity (%)
Before aerosol generation (n=3)	100
After aerosol generation (n=3)	94 ± 5

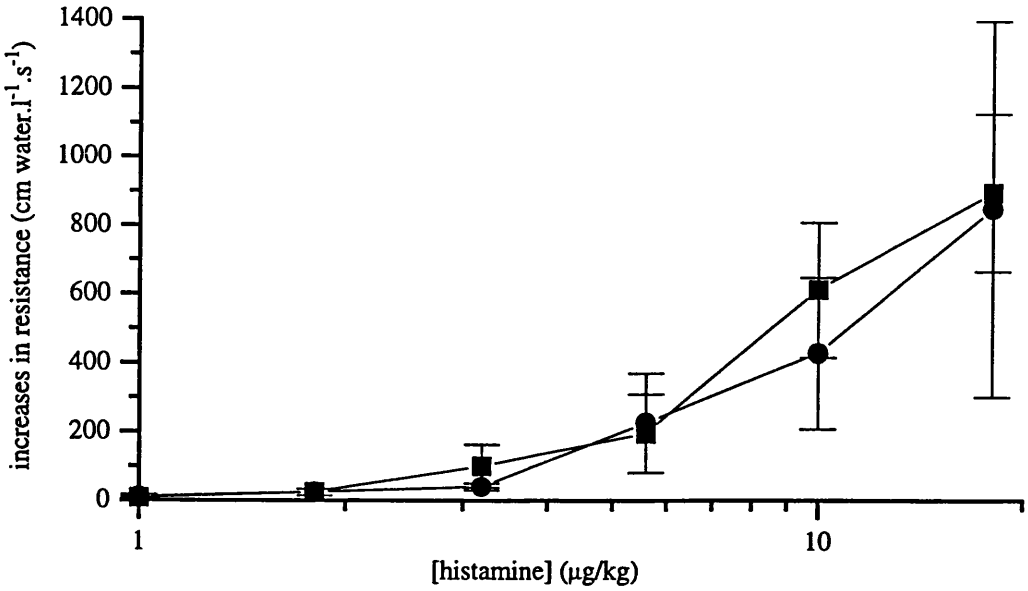


Figure 5.8: The effect of aerosol huTNFα, administered using a nose cone, on *in vivo* airway responsiveness to histamine. Guinea-pigs were exposed to 5ml of either vehicle (PBS 0.1% BSA, —■—) or huTNFα (—●—; 50μg) 6 h prior to lung function tests being performed. Points represent the mean +/- sem, n=4 animals/treatment.

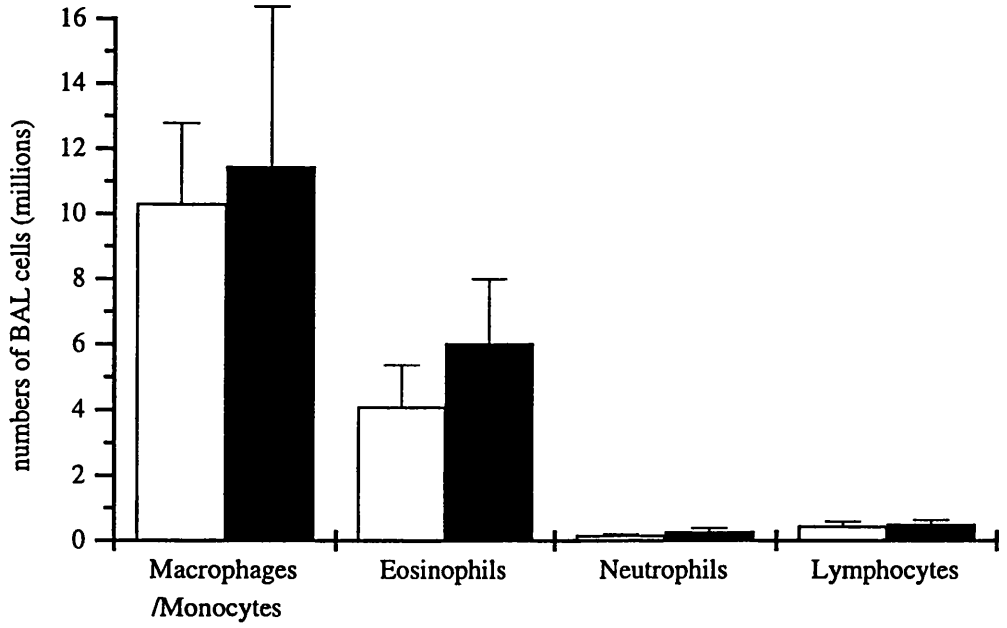


Figure 5.9: The effect of aerosol huTNFα, administered using a nose cone, on BAL leukocyte accumulation. Guinea-pigs were exposed to 5ml of either vehicle (PBS 0.1% BSA, □) or huTNFα (■; 50μg) 8 h prior to BAL being performed.
Bars represent the mean +/- sem, n=4 animals/treatment.

5.3 Summary of the in vivo activity of TNFα in the guinea-pig

gpTNFα

- gpTNFα (15-150ng) induced a dose dependent increase in BAL leukocyte accumulation which was maximum 24 hours after instillation and substained above control levels at 72 hours.
- 50ng gpTNFα induced a pronounced BAL eosinophil infiltration.
- gpTNFα induced BAL leukocyte accumulation was abrogated by pre-incubating gpTNFα with rabbit anti murine TNFα sera or by heat treating the gpTNFα prior to instillation.
- gpTNFα did not induce bronchial hyperreactivity 24 hours after instillation.

Human TNFα

- Human TNFα (150ng) induced a significant elevation in BAL neutrophil numbers 24 hours after instillation. Macrophage/monocyte numbers were slightly enhanced, but eosinophil numbers were not increased.
- Aerosol delivery of human TNFα did not induce bronchial hyperreactivity or BAL leukocyte accumulation at 30 min - 8 hours after challenge.

✪ Following the instillation of human TNFα the leukocytes recruited to the airways were predominantly neutrophils. This was in contrast to the eosinophilic leukocyte accumulation induced by gpTNFα.

6. Leukocyte accumulation, airway reactivity and TNFα generation in guinea-pig allergic airway inflammation

In order to investigate the involvement of TNFα in allergic airway inflammation an animal model was first established and characterised. The ovalbumin (OA) sensitised guinea-pig is a classical model of allergic inflammatory lung diseases. Before settling on the method used for the other studies shown in this chapter (method number 4) which demonstrated bronchial hyperreactivity and lung eosinophilia in the studies shown below, several variations of the sensitisation methods were tried. The bronchial reactivity and BAL eosinophil accumulation data from various sensitisation methods is shown in table 6.1.

Table 6.1: Increases in bronchial reactivity (induced by histamine i.v., 3.2μg/kg and 10μg/kg) and BAL eosinophil infiltration 24 hours following antigen challenge of naive or sensitised guinea-pigs using various sensitisation protocols. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with naive guinea-pigs, where no symbol is shown the difference was not statistically significant ($p>0.05$). PV: *Bordetella pertussis* vaccine, BV; *Bordetella bronchoseptiemia* vaccine.

Sensitisation protocols	**Injected (1ml, i.p.)**	**Aerosol antigen challenge**	**Treatment**	**Increases in lung resistance (cm $H_2O.l^{-1}.s^{-1}$) induced by i.v. histamine**		**BAL lung eosinophil numbers**
No.	**on days:**	**on days:**	**(n)**	**3.2μg/kg**	**10μg/kg**	**(millions)**
1 Cyclophosphamide	-1	35-42	naive (6)	127± 99	180± 69	5.8±1.2
$Al(OH)_3$ 1mg	0	10ml 0.1%				
OA 10μg	14 (no PV)	OA	sensitised (6)	138± 16	388± 133	17.9±1.3 *
PV 0.25ml	28 (no PV)					
2 $Al(OH)_3$ 1mg	0	44-51	naive (4)	319± 126	916± 74	1.91±0.2
OA 1mg	14 (no PV)	10ml 0.1%				
PV 0.25ml	36 (no PV)	OA	sensitised (4)	456± 118	1244± 159	18.2±3.6**
3 Cyclophosphamide	-1	42-52				
$Al(OH)_3$ 1mg	0	10ml 0.1%	naive (5)	200± 95	850± 208	4.67±0.8
OA 10μg	14 (no PV)	OA				
PV 0.25ml	28 (no PV)		sensitised (5)	805± 226	1305± 90	18.5±2.3**
4 $Al(OH)_3$ 2mg	0	28-40	naive (8)	12± 3	115± 38	6.2±2.3
OA 10μg	14	10ml 0.1%				
BV 0.05ml		OA	sensitised (8)	180± 72***	836±152***	26.7±0.5**

6.1 The effect of antigen sensitisation on in vivo bronchial reactivity

An anti-histamine, mepyramine, was administered 15 min prior to challenging with aerosol antigen (OA) for a 60 min period. Changes in *in vivo* bronchial reactivity to known spasmogens was assessed at set time points after antigen challenge. Examples of experimental traces, 24 hours after antigen challenge, showing changes in lung resistance and dynamic compliance following the infusion of histamine in sensitised and naive animals are presented in figure 6.1 when the effects of mepyramine were assumed to be negligible. The sensitisation of animals per se had little effect on the basal lung resistance and dynamic compliance, as can be seen in table 6.2.

Table 6.2: Basal lung resistance and dynamic compliance measurements for sensitised and naive guinea-pigs. Animals were antigen challenged 2-48 hours prior to *in vivo* lung function tests, n=3 animals/time point.

time after challenge (h)	**lung resistance (cm $H_2O.l^{-1}.s^{-1}$)**		**dynamic compliance (ml. cm H_2O^{-1})**	
	naive challenged	**sensitised challenged**	**naive challenged**	**sensitised challenged**
2	151.3±18	195.1±45	0.68±0.2	0.69±0.1
6	113.3±10	184.5±23	0.87±0.1	0.78±0.1
12	143.1±16	174.6±11	0.66±0.1	0.74±0.1
18	155.9±26	143.0±13	0.77±0.1	0.74±0.1
24	160.27±35	126.4±13	0.64±0.1	1.15±0.1
48	145.1±13	158.1±19	0.69±0.1	0.67±0.1

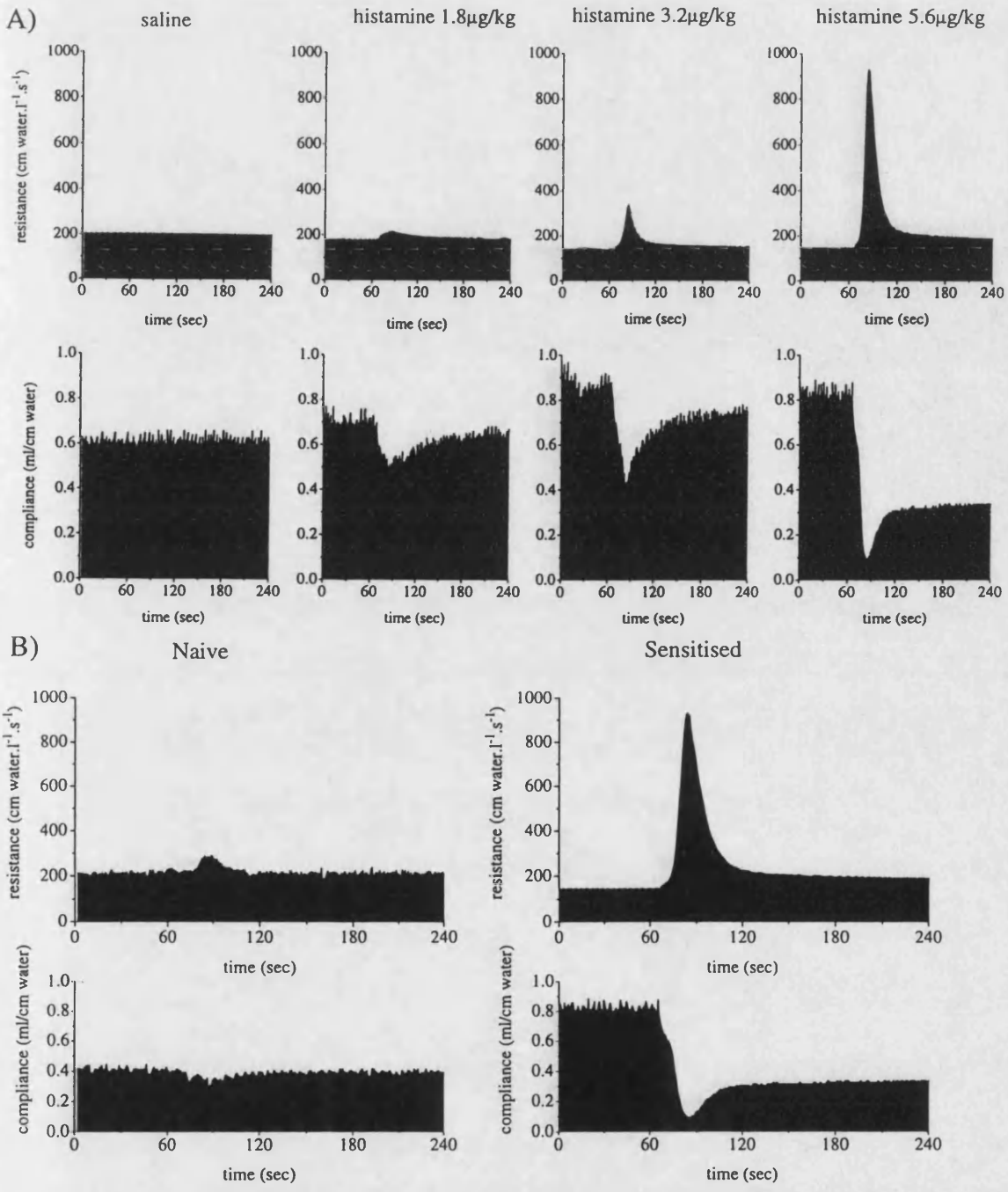


Figure 6.1 (A): Dose related increases in lung resistance and decreases in dynamic compliance induced by histamine. The experimental traces shown are from a sensitised antigen challenged, 24 h prior to assessment of bronchial reactivity, guinea-pig administered histamine (0 - 5.6µg/kg, i.v.) at the 60 second time point. 10 minutes was allowed between each infusion.

Figure 6.1 (B): Comparison of responses from a naive or sensitised guinea-pig infused with 5.6µg/kg histamine at the 60 second time point. Animals were antigen challenged 24 h prior to lung function tests being performed.

Time course studies of histamine induced increases in lung resistance at 2 - 48 hours after antigen challenge showed a biphasic pattern of hyperreactivity when comparing sensitised and naive animals as detailed in figure 6.2. Sensitised animals were hyperreactive to histamine (5.6 or 10μg/kg) 6 hours after antigen challenge, but this resolved by 12 hours. The second phase of hyperreactivity occurred at 24 hours and was sustained to 48 hours after challenge (figure 6.2). In all cases sensitised animals were compared with naive time matched controls. This pattern of hyperreactivity was also seen using 10μg/kg methacholine as the spasmogen, shown in figure 6.3, with hyperreactivity peaks at 6 hours (although this was not statistically significant, possibly due to the small numbers in the groups) and a sustained phase 24 - 48 hours. It should be noted that the degree of hyperreactivity varied considerably between different batches of sensitised guinea-pigs.

6.2 Airway leukocyte recruitment following antigen challenge of sensitised guinea-pigs

The profile of antigen induced leukocyte recruitment to the lungs between 2 - 48 hours is shown in figure 6.4. Total BAL leukocyte numbers were elevated by 6 hours and remained above the levels of time matched naive antigen challenged controls over the time course studied, as shown in table 6.3. BAL neutrophil recruitment was transient with numbers significantly elevated by 2 hours, peaking at 12 hours and returning to control levels by 24 hours. Increased recruitment of eosinophils was not observed until 12 hours after challenge of sensitised animals, with a maximum accumulation at 24 hours (plate6.1) which was sustained at 48 hours. Significant macrophage/monocyte recruitment was observed at 24 hours and sustained at 48 hours. Lymphocyte numbers remained very low throughout the time course.

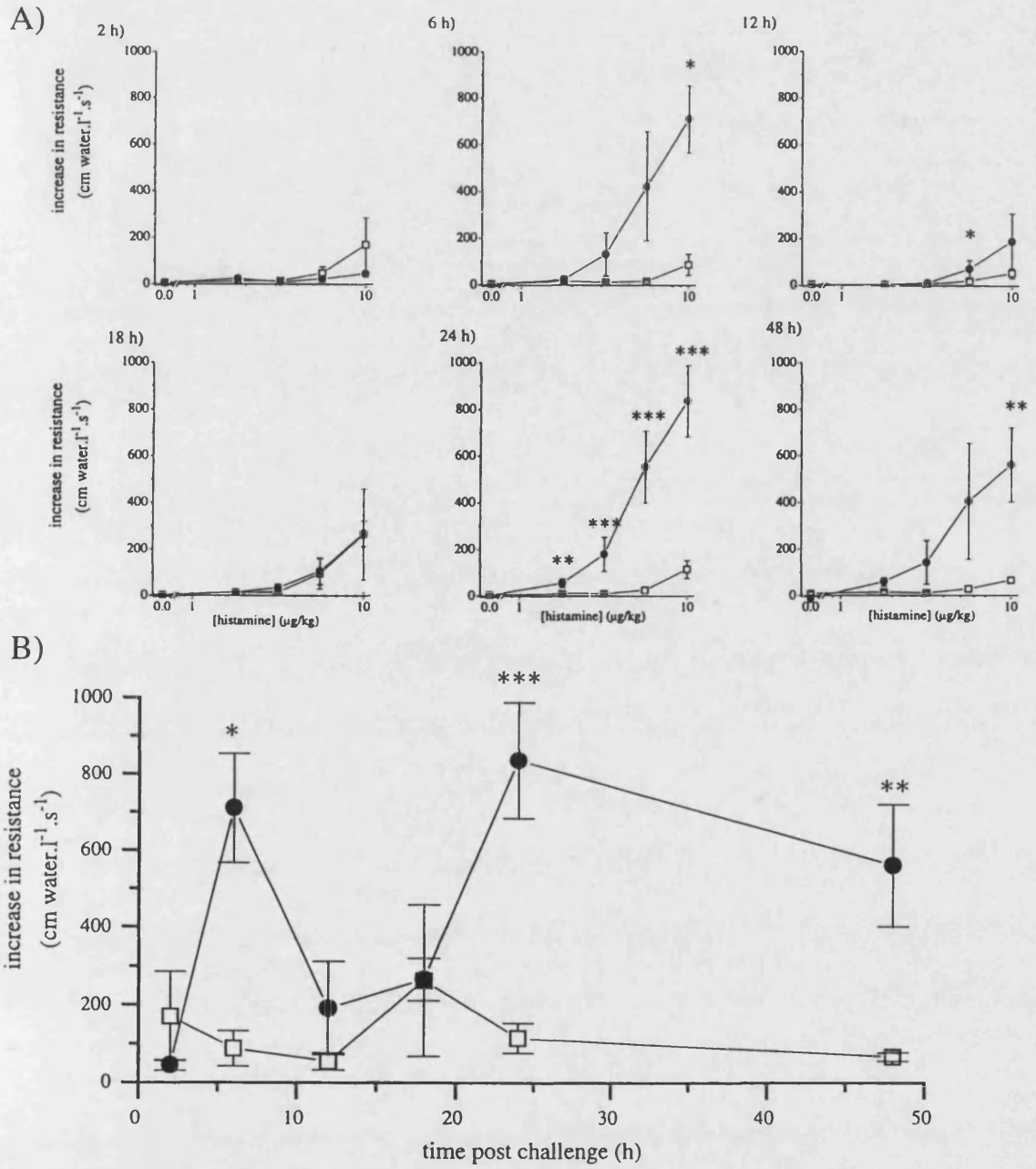


Figure 6.2: Time course studies of bronchial hyperresponsivness to histamine following antigen challenge of sensitised (—●—) or naive (—□—) guinea-pigs. Animals were aerosol antigen (0.1% OA; 10ml) challenged 2-48 h prior to lung function tests. Points represent mean +/- sem, n=3 animals/treatment for 2, 6, 12, 18, 48 h and n=8/treatment 24 h.
*p<0.05, **p<0.01, ***p<0.001 compared with naive antigen challenged animals.

Panel A shows histamine (1-10µg/kg) dose-response curves for individual time points post-challenge. Panel B shows increases in lung resistance induced by 10µg/kg histamine 2-48 h post-challenge (data taken from dose-response curves shown in panel A).

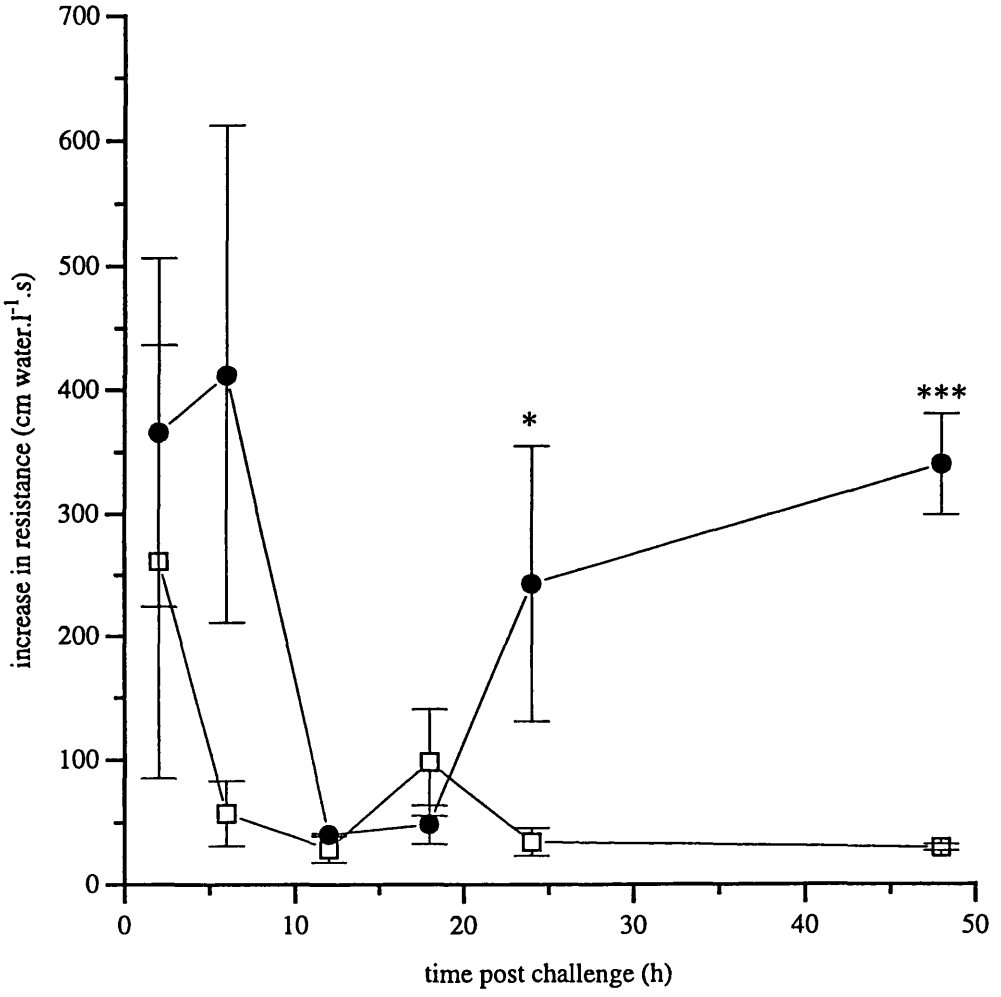


Figure 6.3: Bronchial hyperresponsiveness to methacholine (10μg/kg) following antigen challenge of sensitised (—●—) versus naive (—□—) guinea-pigs. Animals were aerosol antigen challenged (0.1% OA; 10ml) 2-48 h prior to lung function tests. Points represent mean +/- sem, n=3 animals/treatment. *$p<0.05$, ***$p<0.001$ compared with antigen challenged naive animals.

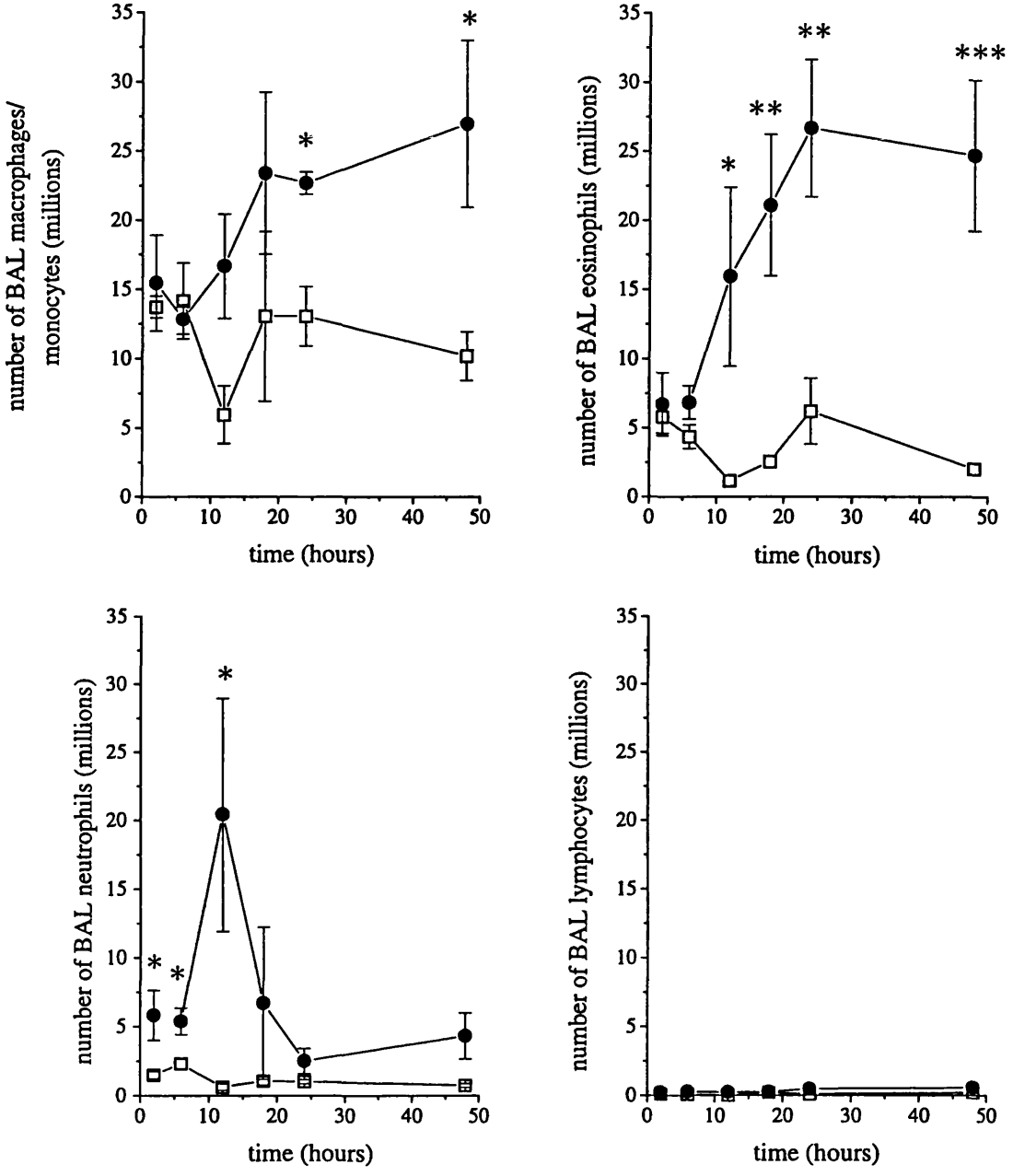


Figure 6.4: Time course of BAL leukocyte accumulation following aerosol antigen (OA) challenge of sensitised (—●—) or naive (—□—) guinea-pigs. Animals were challenged with antigen (10ml 0.1% OA) 2-48 h prior to BAL. Points represent mean +/- sem; n=3 animals/treatment for 2, 6, 12 and 18 h, n=4 animals/treatment for 24 and 48 h. *p<0.05, **p<0.01, ***p<0.001 compared with naive antigen challenged animals. Data are representative of 6 experiments.

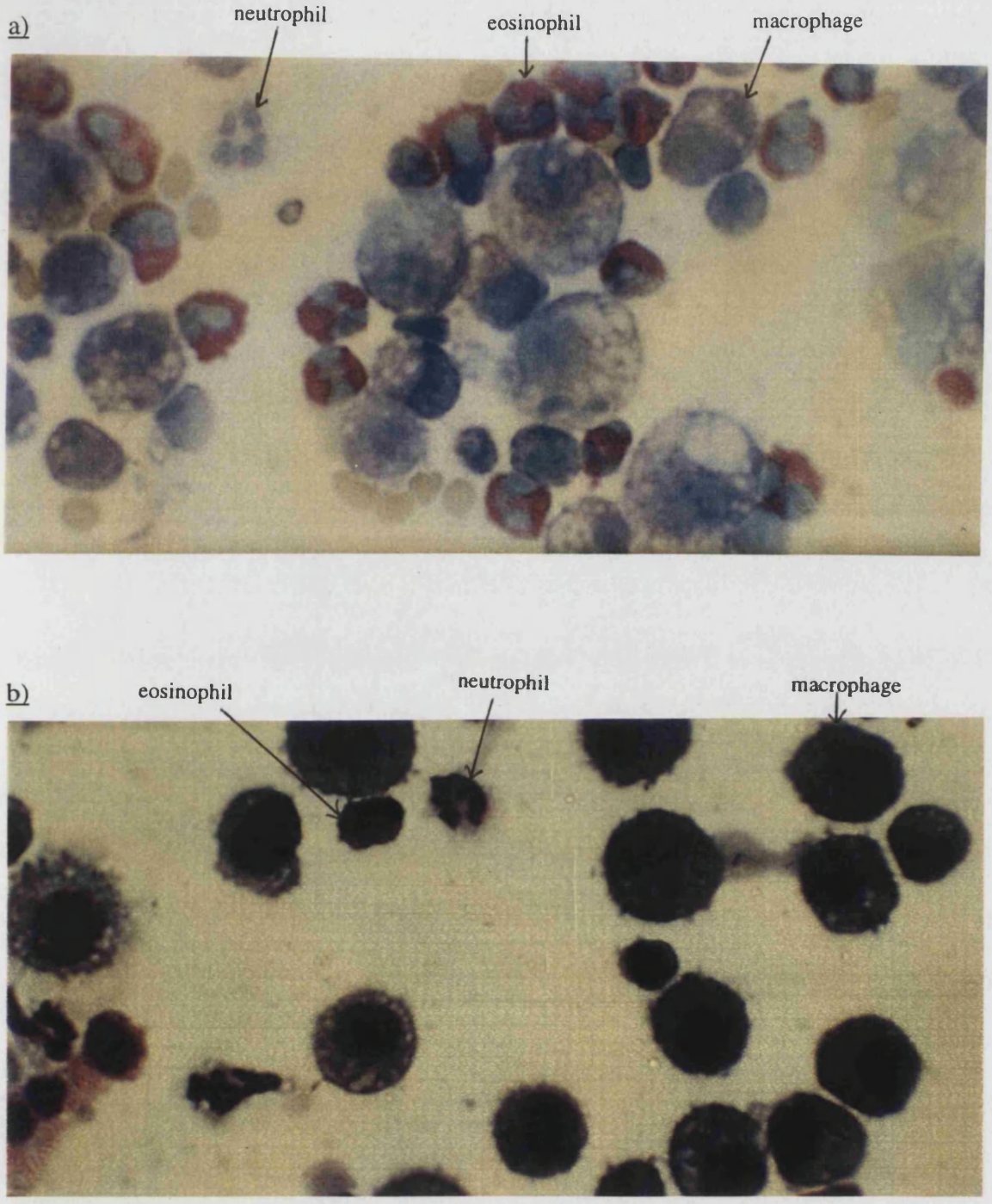


Plate 6.1 a: Photograph of antigen induced BAL eosinophil accumulation 24 hours after aerosol challenge of sensitised guinea-pigs, BAL from a time-matched control antigen challenged naive animals is shown in plate 6.1 b. Magnification 2000x.

Table 6.3: The total BAL leukocyte numbers 2 -48 hours following antigen challenge of naive or sensitised animals. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with time matched naive animals. Data are representative of 6 experiments

time after challenge (h)	total BAL leukocyte numbers (millions)	
	naive	sensitised
2 (n=3)	21.9±5.1	30.3±2.5
6 (n=3)	17.6±2.2	39.4±2.2 **
12 (n=3)	28.6±8.15	83.1±7.3 *
18 (n=3)	23.1±5.9	85.1±19.4 *
24 (n=4)	12.5±2.2	99.3±18.6 ***
48 (n=4)	26.4±2.7	81.9±8.0 ***

6.3 TNFα generation in antigen challenged sensitised guinea-pigs

TNFα generation was measured at the protein and mRNA levels at various times after antigen challenge. TNFα levels in the BAL, measured by WEHI cytotoxicity assay, were similar in both sensitised and naive antigen challenged animals, figure 6.5 (assay sensitivity >0.1IU/ml TNFα). One problem often encountered during the assay was a potentiation of WEHI growth induced by BAL samples. Experiments were performed in the presence of actinomycin D (1μg/ml) to inhibit proliferation but this also reduced WEHI adhesion and cellular viability (~50% viable) and hence was not routinely used. In some experiments, FCS was added to the BAL samples prior to the addition to wells in order to "saturate" WEHI growth, but this did not enhance the sensitivity of the assay.

Whole lung TNFα protein content was measured by western blot analysis. In the sensitised animals, 2-24 hours after antigen challenge, there appeared to be a slight increase in TNFα levels compared to the naive control animals as summarised in

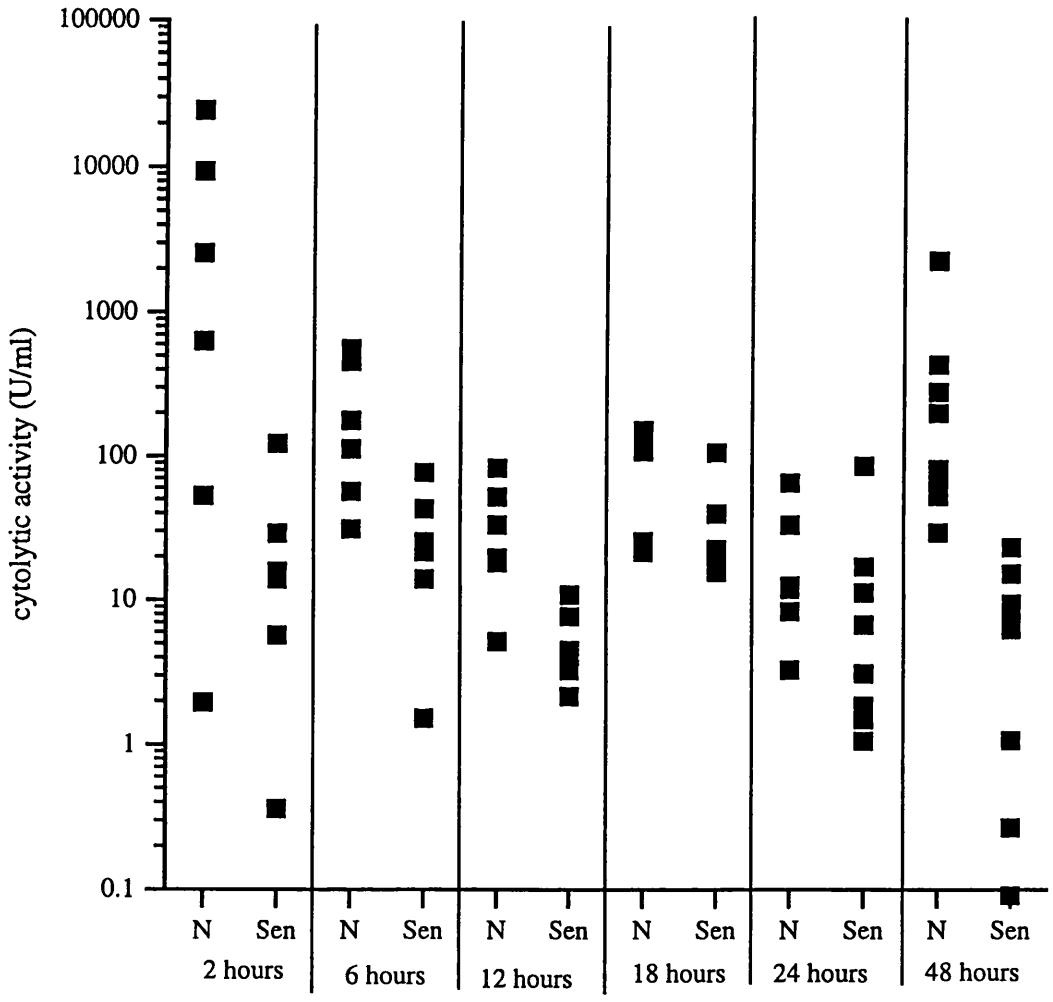


Figure 6.5: Time course of BAL TNFα content following antigen challenge of sensitised (Sen, n=5-8) or naive (N, n=6-8) guinea-pigs. Animals were challenged with antigen 2-48 h prior to BAL, and BAL TNFα content was measured by WEHI cytotoxicity assay. Each ■ represents the TNFα content of 1ml BAL from one animal. Data are expressed in cytolytic activity based on a human TNFα standards.

table 6.4. However, TNFα protein was detected in both groups at all time points examined, an example of the western blots obtained is shown in figure 6.6.

Table 6.4: Whole lung TNFα content of sensitised or naive antigen challenged guinea-pigs. Lungs were removed 2-24 hours after challenging and TNFα content determined by western blot analysis. Scale: - no TNFα, + TNFα band visible, ++ TNFα band strongly visible, each symbol represents tissue from one animal.

time after challenge (h)	**naive antigen challenge**	**sensitised antigen challenge**
2 (n=3)	-, +, +	+, +, +
6 (n=3)	+, +, -	+, +, +
12 (n=3)	-, -, +	+, +, -
18 (n=2)	-, +	+, +
24 (n=2)	-, +	+, ++

Immunohistochemical staining of BAL cytospins for each time point (2-48 h) following antigen challenge indicates TNFα in all samples. 50-75% of the BAL cells were stained positive (as shown in plate 6.2) but there was no observable difference between sensitised or naive animals and the staining was not specific for one leukocyte sub-type. However, this data does reflect an increase in overall TNFα expression, as the total BAL leukocyte numbers were increased in sensitised animals as shown in table 6.3. Due to the low number of animals in each group statistical analysis was not performed on these data. Control IgG antibody, used for each BAL sample, did not stain positive.

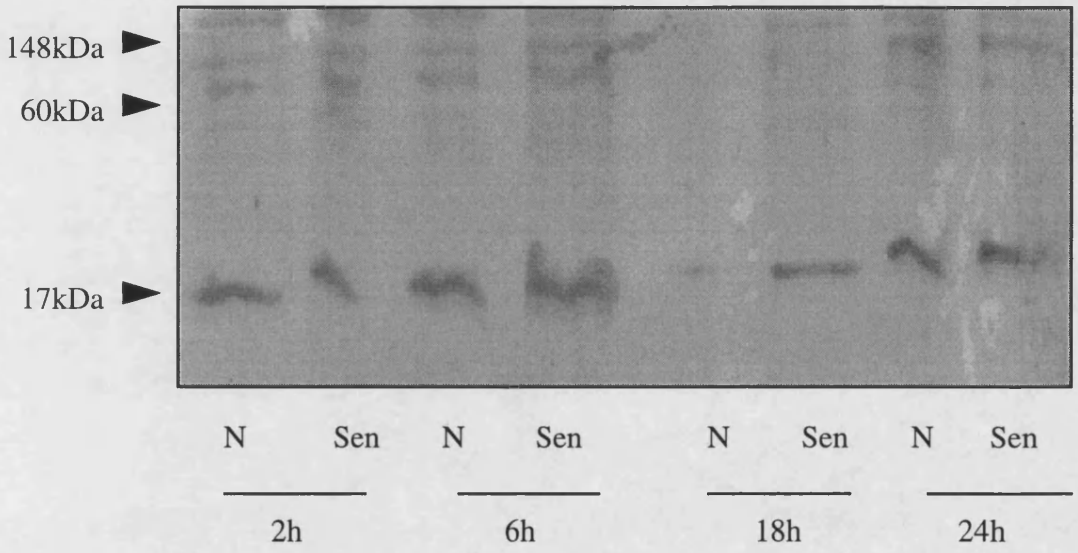


Figure 6.6: Western blot to show the presence of TNFα in guinea-pig lung tissue homogenates. Naive (N) or sensitised (Sen) animals were antigen challenged 2-24 hours prior to BAL and the subsequent removal of the lungs. TNFα was detected using rabbit anti murine TNFα antibody.

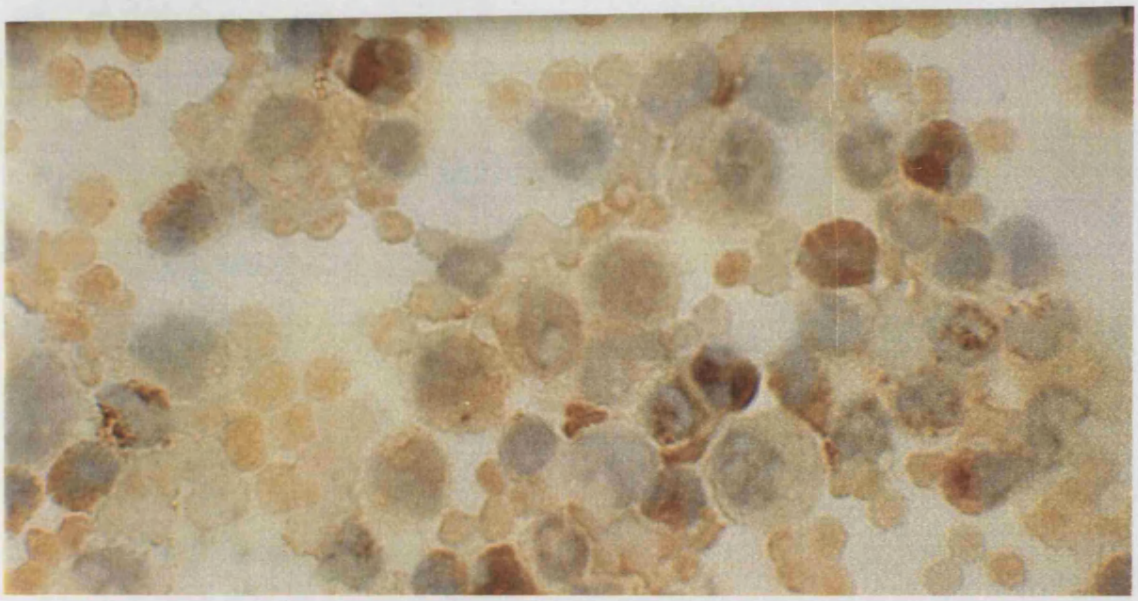


Plate 6.2: Photograph of immunohistochemically detected TNFα in BAL leukocytes from an antigen challenged sensitised guinea-pig. TNFα is detected with a brown stain and the cells counter stained with Mayers' hematoxylin (blue). BAL was collected 24 hours after aerosol challenging the animals. Magnification 2000x.

6.4 BAL induced elevation of $[Ca^{2+}]_i$ in guinea-pig peritoneal leukocytes

Elevations of $[Ca^{2+}]_i$ in fura-2 loaded peritoneal leukocytes was used to assess the chemokine-like bioactivity present in the BAL following antigen challenge of sensitised and naive guinea-pigs. It should be noted that gpTNFα does not elevate $[Ca^{2+}]_i$ in guinea-pig eosinophils, macrophages or neutrophils (M.L. Watson, University of Bath and personal observations)

BAL from sensitised antigen challenged animals induced a rapid transient $[Ca^{2+}]_i$ elevation in peritoneal eosinophils (eosinophil purity 93% ±5%, n=15), figure 6.7. Increased calcium mobilising activity in BAL from sensitised challenged animals compared with BAL from naive challenged animals was observed 2 hours after challenge but this was not statistically significant. This mobilising activity peaked at 6 hours ($p<0.001$) and was maintained above time matched controls at 12 and 18 hours ($p<0.001$, $p<0.01$ respectively), returning to control levels 48 hours after antigen challenge. The $[Ca^{2+}]_i$ elevation in eosinophils was reached 10-20s after addition of the BAL, with a lag time of ~5s.

BAL from naive challenged animals had weak macrophage (macrophage purity 85%±4% n=12) stimulating activity, and this was not significantly elevated in time matched antigen challenged sensitised animals (e.g. BAL from sensitised animals 6 hours after challenge induced a 52nM ± 10nM increase in $[Ca^{2+}]_i$ compared with 40nM ± 7nM increase with BAL from time matched controls, n=3 for each group). The profile of these $[Ca^{2+}]_i$ elevations were similar to those induced in eosinophils. BAL from sensitised or naive animals did not induce elevation of $[Ca^{2+}]_i$ above basal levels of 48nM ± 2.7nM, n=8, in peritoneal neutrophils (neutrophil purity 83%±5%, n=8).

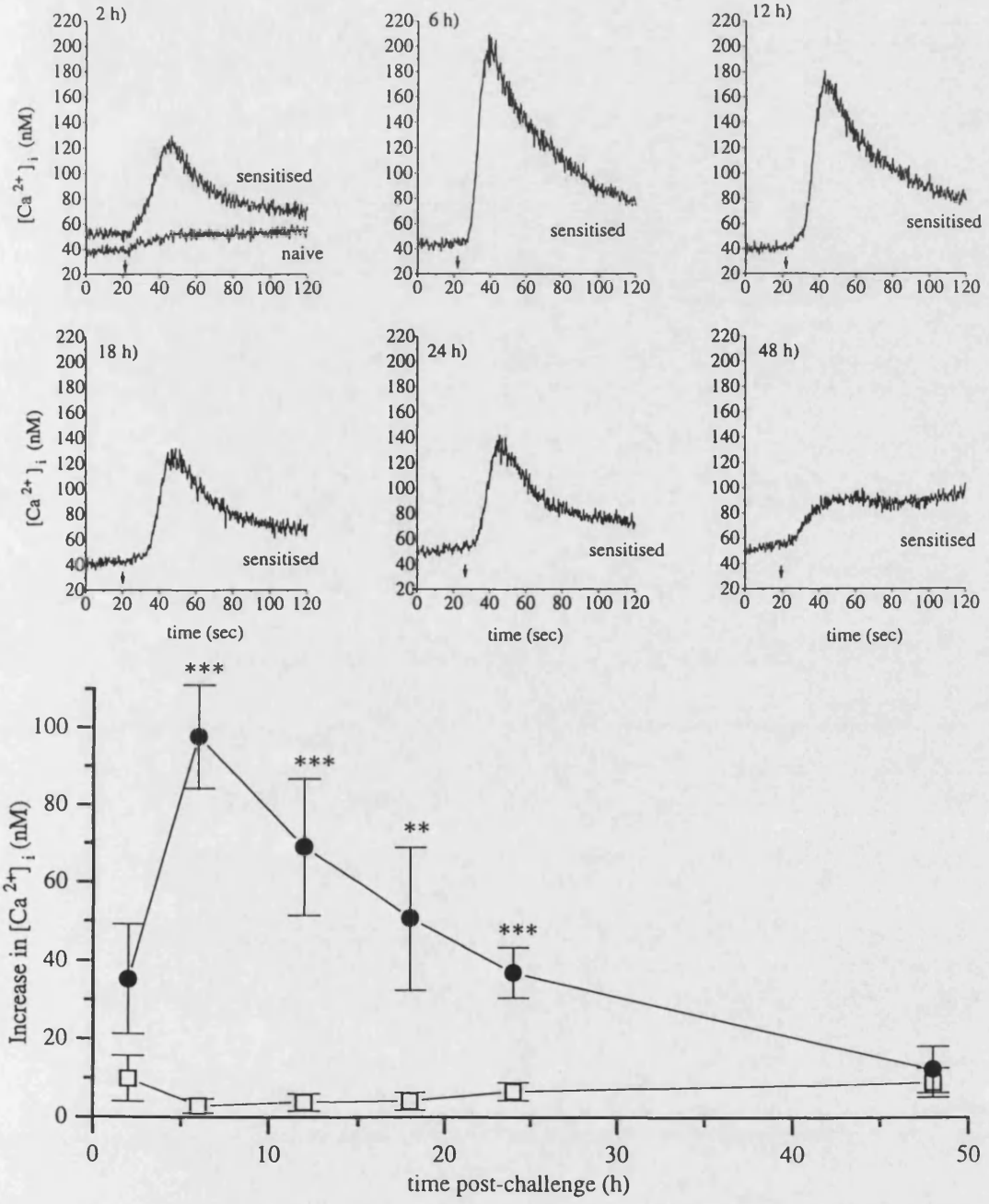


Figure 6.7: BAL induced elevation of $[Ca^{2+}]_i$ in fura-2 loaded guinea-pig peritoneal eosinophils.

Eosinophils were stimulated with 100µl BAL from either naive (—□—, n=5-9) or sensitised (—●—, n=5-9) guinea-pigs. Animals were challenged with aerosol antigen 2-48 h prior to BAL. Points represent the mean +/- sem. **p<0.01, ***p<0.001 compared with elevations induced by BAL from naive animals. Inserts show typical experimental time courses of $[Ca^{2+}]_i$ elevations following BAL stimulation at approximately 20 seconds (arrows). Only one experimental trace following the addition of BAL from a naive animal (BAL collected 2 h after challenge) is shown for clarity, this trace is shown superimposed on the 2 h trace.

6.5 Effect of instilling anti TNFα antibody 20 min prior to antigen challenge on antigen induced BAL leukocyte accumulation

The instillation of rabbit anti murine TNFα IgG in sensitised guinea-pigs reduced the eosinophil accumulation by 69.4%±3.8% (n=6) and the neutrophil accumulation by 94.1%±3.5% (n=6) 24 hours after challenge (figure 6.8) compared with sensitised guinea-pigs instilled with rabbit IgG. BAL macrophage/monocyte numbers were not significantly reduced by antibody instillation. The instillation of rabbit anti murine TNFα IgG to naive animals had no significant effect on BAL leukocyte accumulation when compared with naive animals instilled with rabbit IgG, as can be seen in figure 6.8.

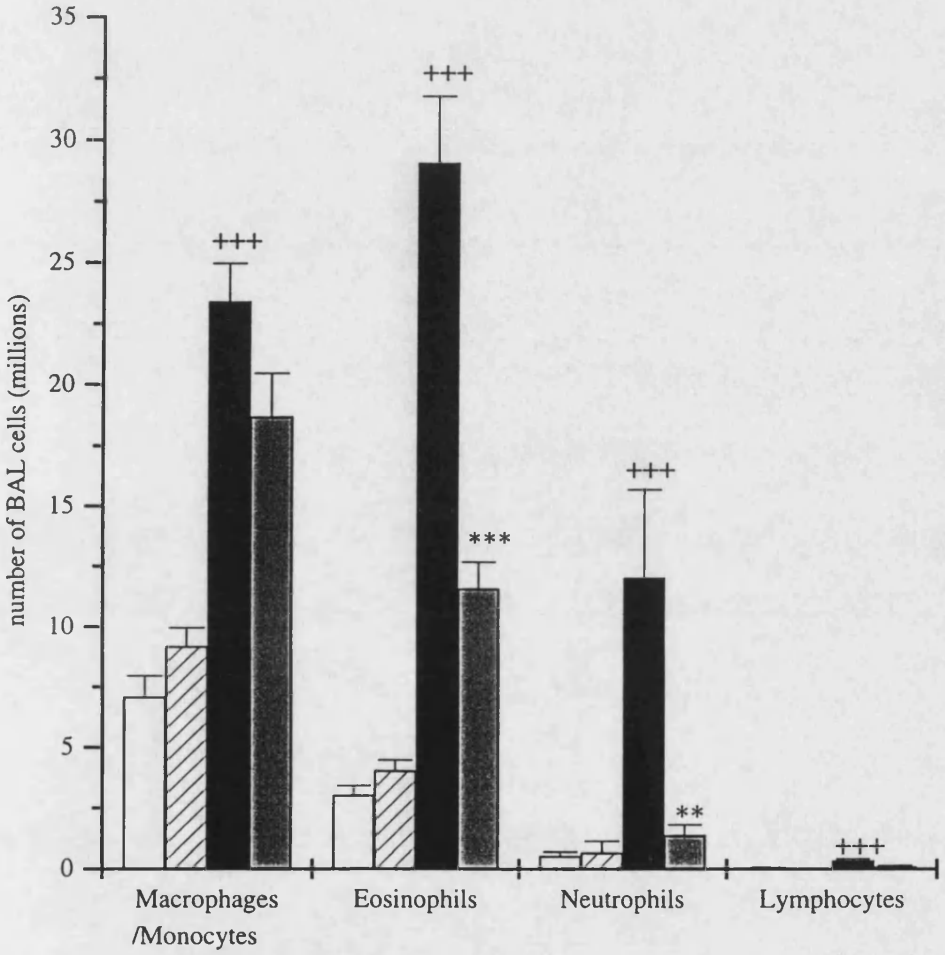


Figure 6.8: The effect of anti TNFα antibody on antigen induced BAL leukocyte accumulation. Antigen sensitised animals were instilled with either rabbit anti murine TNFα IgG (1 in 100; ▒▒▒) or control rabbit IgG (1in100; ■■■), and naive animals were instilled with either rabbit anti murine TNFα IgG (1 in 100; ▨▨▨) or rabbit IgG (1in100; □□□) as additional controls. All animals were instilled with 50µl antibody 20 min prior to aerosol antigen challenge (10ml 0.1% OA) and BAL was performed 24 h later.

Bars represent mean +/- sem, n=6 animals/treatment. +++$p<0.001$ compared with naive animals instilled with rabbit IgG. **$p<0.01$, ***$p<0.001$ compared with sensitised animals instilled with rabbit anti murine TNFα IgG. Where no symbols are indicated the differences were not significant ($p>0.05$).

6.6 Summary of the effect of antigen challenge of sensitised animals

- Antigen challenge induced biphasic bronchial hyperreactivity: The initial period of hyperreactivity occurred at 6 hours and was resolved by 12 hours, with the second period maximal at 24 hours and sustained at 48 hours.
- Antigen challenge induced BAL leukocyte accumulation: Neutrophil infiltration was transient and rapid in onset (occurring by 2 h), with maximum infiltration 12 hours after antigen challenge. The accumulation of eosinophils occurred at 12 hours, was maximum at 24 hours and remained pronounced at 48 hours. Macrophage/monocyte infiltration was later in onset, occurring at 24 hours and sustained at 48 hours.
- TNFα, mRNA and protein, was expressed in both naive and sensitised animals.
- BAL from sensitised animals induced transient increases in $[Ca^{2+}]_i$ in fura-2 loaded peritoneal eosinophils. Activity was maximum in BAL 6 hours after challenge and returned to control levels by 48 hours after challenge. BAL from both naive and sensitised animals, 2 - 48 hours after challenge, induced similar increases in $[Ca^{2+}]_i$ in fura-2 loaded peritoneal macrophages. BAL stimulation of fura-2 loaded peritoneal neutrophils did not increase $[Ca^{2+}]_i$.
- Instilling rabbit anti murine TNFα IgG 20 min prior to antigen challenge caused a marked reduction in BAL eosinophil and neutrophil accumulation, the latter being reduced to control levels 24 hours after challenge. Macrophage/monocyte accumulation was not affected.

7. Effect of human IL-13 on antigen- and gpTNFα-induced lung inflammation in vivo

To determine the effect of IL-13 on airway inflammation two guinea-pig models were used 1) gpTNFα-induced inflammation and 2) antigen induced airway inflammation.

7.1 The effect of IL-13 and IL-4 on gpTNFα induced BAL leukocyte accumulation

Initial experiments demonstrated that the instillation of IL-13 (100ng) alone did not induce increased BAL leukocyte accumulation. However, by co-instilling IL-13 (1-100ng) with gpTNFα (50ng) there was a reduction in total BAL leukocyte accumulation 24 hours after instillation. gpTNFα induced macrophage/monocyte and eosinophil infiltration was reduced in a dose dependent manner by IL-13, as shown in figure 7.1. The gpTNFα induced BAL macrophage/monocyte and eosinophil recruitment was inhibited to near control (vehicle treated) levels by the co-instillation of the highest used dose of IL-13 (100ng) with gpTNFα (50ng).

Measurements of total lung EPO performed following BAL indicated that the co-instillation of IL-13 caused a reduction in EPO levels (figure 7.2) compared to levels in lung tissue from animals instilled with gpTNFα alone. Statistical significance was not reached, probably due to the small number of animals (3/group) used in this study.

Following the co-instillation of gpTNFα (50ng) with IL-4 (100ng) a slight reduction in BAL macrophage/monocyte and eosinophil numbers and a marginal increase in neutrophil numbers were observed at 24 hours, compared with the instillation of gpTNFα alone, but none of these changes were significant as shown in figure 7.3. The instillation of IL-4 (100ng) alone induced a significant increase in BAL macrophage/monocyte and neutrophil numbers compared to vehicle instilled animals. No increase in BAL eosinophil or lymphocyte numbers was induced by IL-4.

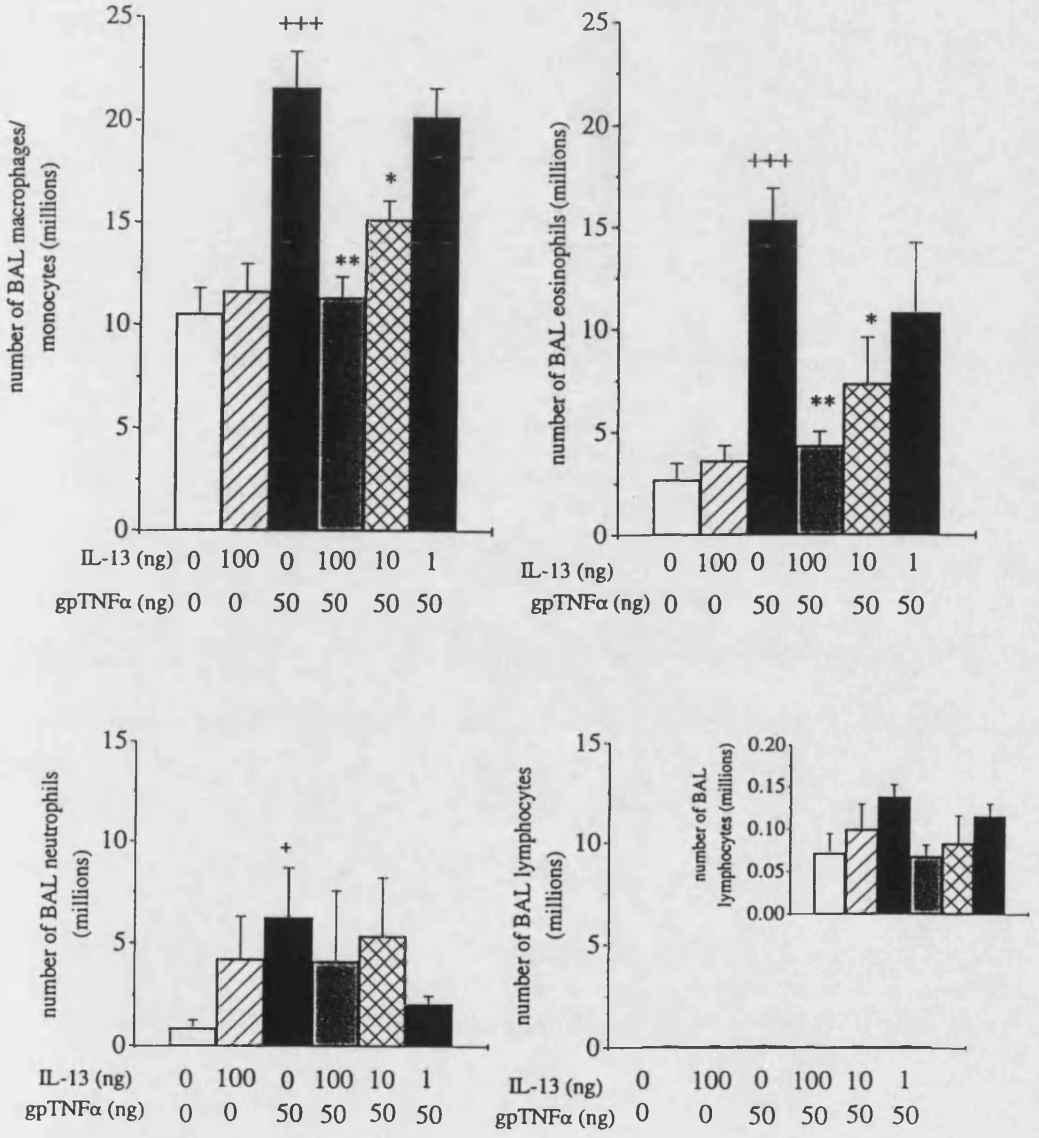


Figure 7.1: The effect of IL-13 on gpTNFα induced BAL leukocyte accumulation.
Naive guinea-pigs animals were instilled with either vehicle (PBS 0.1% BSA; ☐ ; n=5), 100ng IL-13 (▨ ; n=6), 50ng gpTNFα (■ ; n=5), 50ng gpTNFα + 100ng IL-13 (▩ ; n=6) 50ng gpTNFα + 10ng IL-13 (▩ ; n=6) or 50ng gpTNFα + 1ng IL-13 (■ ; n=6).
All animals were instilled (50µl) 24 h prior to BAL. Bars represent the mean+/- sem for each treatment. +++p<0.001 compared with vehicle treated animals *p<0.05, **p<0.01, ***p<0.001 compared with gpTNFα treated animals. Where no symbols are indicated the difference was not significant (p>0.05) compared with the appropriate controls.
Lymphocyte data insert shows enlarged scale.

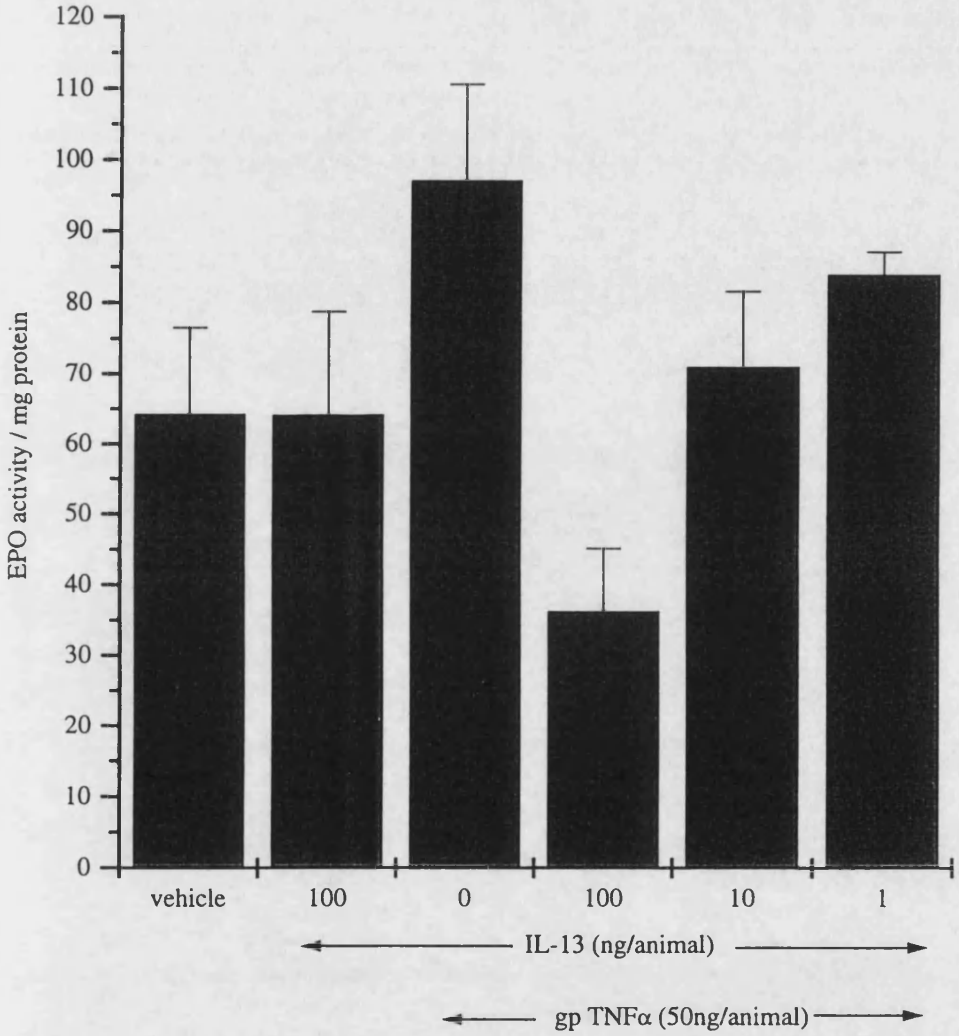


Figure 7.2: The effect of gpTNFα and IL-13 on eosinophil peroxidase (EPO) activity in guinea-pig whole lung extracts following BAL. Naive guinea-pigs were instilled with vehicle (PBS 0.1% BSA), 100ng IL-13 alone, 50ng gpTNFα alone or 50ng gpTNFα + 100, 10 or 1ng IL-13 24 h prior to BAL and the subsequent removal of whole lung tissue. Bars represent the mean +/- sem, n=3 animals/treatment.

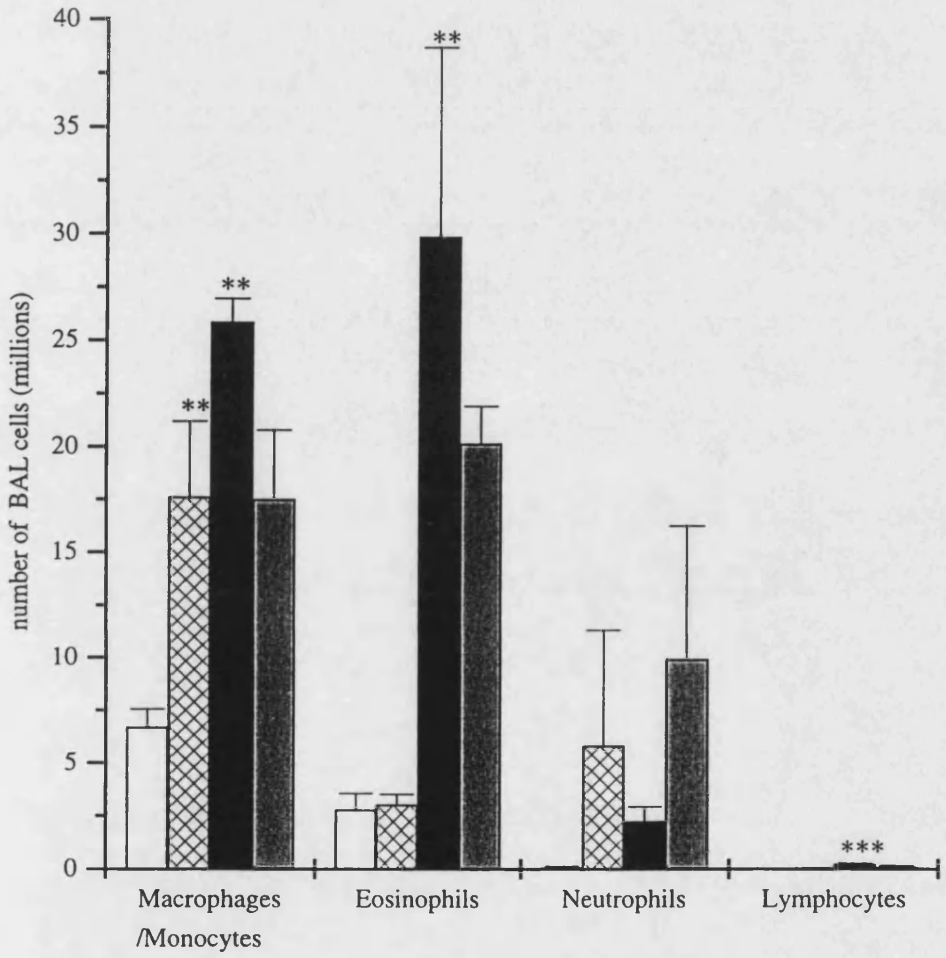


Figure 7.3: The effect of instilling IL-4 on gpTNFα induced BAL leukocyte accumulation. Guinea-pigs were instilled with either vehicle (PBS 0.1% BSA,), 100ng IL-4 ( ), 50ng gpTNFα ( ) or 50ng gpTNFa + 100ng IL-4 ( ) 24 h prior to BAL. Bars represent the mean +/- sem, n=3 animals/treatment, **p<0.01, ***p<0.001 compared with vehicle treated animals. Where no symbols are indicated the difference was not significant (p>0.05) compared with the appropriate controls.

7.2 Effect of IL-13, instilled 20 min prior to antigen challenge, on antigen-induced BAL leukocyte accumulation

The instillation of IL-13 (1-100ng/animal) 20 min prior to antigen challenge of sensitised guinea-pigs caused a dose dependent reduction in antigen induced BAL leukocyte accumulation as detailed in table 7.1. This reduced number of cells was principally accounted for by a reduction in eosinophil numbers (figure 7.4) with no significant change in macrophage/monocyte or neutrophil numbers. Lymphocyte numbers were reduced by instilling IL-13 prior to antigen challenge, which was significant at the dose of 10ng IL-13. Although the instillation of 100ng IL-13 significantly inhibited BAL eosinophil accumulation in response to antigen challenge of sensitised animals by 72% ±4% (n=6), this level was still 3 fold higher than the eosinophil numbers in naive animals instilled with IL-13.

Table 7.1: The effect of instilling IL-13 on antigen induced total leukocyte BAL accumulation. IL-13 (1-100ng/animal) or vehicle (PBS, 0.1%BSA) was instilled 20 min prior to antigen challenge of sensitised or naive animals. BAL was performed 24 hours after challenge. ††† $p<0.001$ compared with vehicle treated naive animals, * $p<0.05$, *** $p<0.001$ compared with vehicle treated sensitised animals.

Treatment	IL-13 (ng/animal)	Total number of BAL cells (millions)
Naive (n=6)	0	14.43±2.7
Naive (n=6)	100	15.79±2.9
Sensitised (n=6)	0	87.47±5.2 †††
Sensitised (n=5)	1	64.01±6.5 *
Sensitised (n=6)	10	51.69±4.0 ***
Sensitised (n=6)	100	48.67±3.7 ***

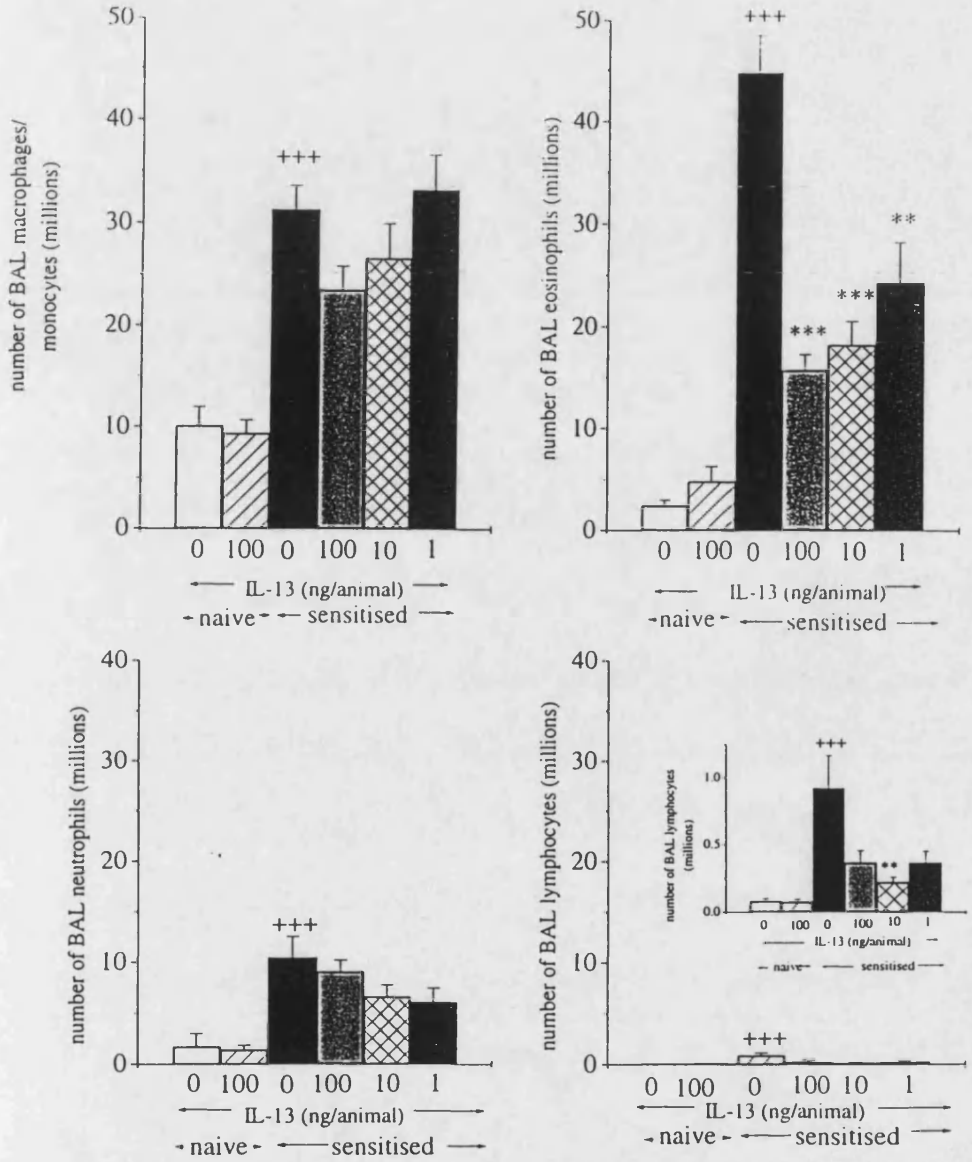


Figure 7.4: The effect of IL-13 on antigen induced BAL leukocyte accumulation. Antigen sensitised animals were instilled with either vehicle (PBS 0.1% BSA; ■ ; n=6), 100ng IL-13 (▩ ; n=6), 10ng IL-13 (▨ ; n=6) or 1ng IL-13 (■ ; n=5) prior to aerosol antigen challenge (10ml 0.1% OA). Naive animals were instilled with vehicle (□ ; n=6) or 100ng IL-13 (▨ ; n=6) as additional controls. All animals were instilled with 50µl 20 min prior to antigen challenge and BAL was performed 24 h later. Bars represent the mean +/-sem for each treatment. +++p<0.001 compared with naive-vehicle treated animals **p<0.01, ***p<0.001 compared with sensitised-vehicle treated animals. Where no symbols are indicated the difference was not significant (p>0.05) compared with the appropriate controls. Lymphocyte data inset shows enlarged scale.

7.3 Effect of IL-13 on chemokine-like activity in BAL of antigen challenged sensitised guinea-pigs

The chemokine-like activity present in the BAL was assessed by measuring changes in $[Ca^{2+}]_i$ in fura-2 loaded peritoneal eosinophils following the addition of BAL. BAL (100µl) from vehicle instilled naive antigen challenged animals induced marginal increases in eosinophil $[Ca^{2+}]_i$ (5.8±3.6 nM, n=6). However, transient increases in eosinophil $[Ca^{2+}]_i$ were induced by BAL from vehicle instilled sensitised animals, as shown in the experimental trace in figure 7.5. Following the instillation of 100ng IL-13 prior to antigen challenging sensitised animals, the calcium mobilising activity in the BAL was reduced by 60%±13% (n=6/group, compared to vehicle instilled sensitised animals) which was statistically significant ($p<0.05$).

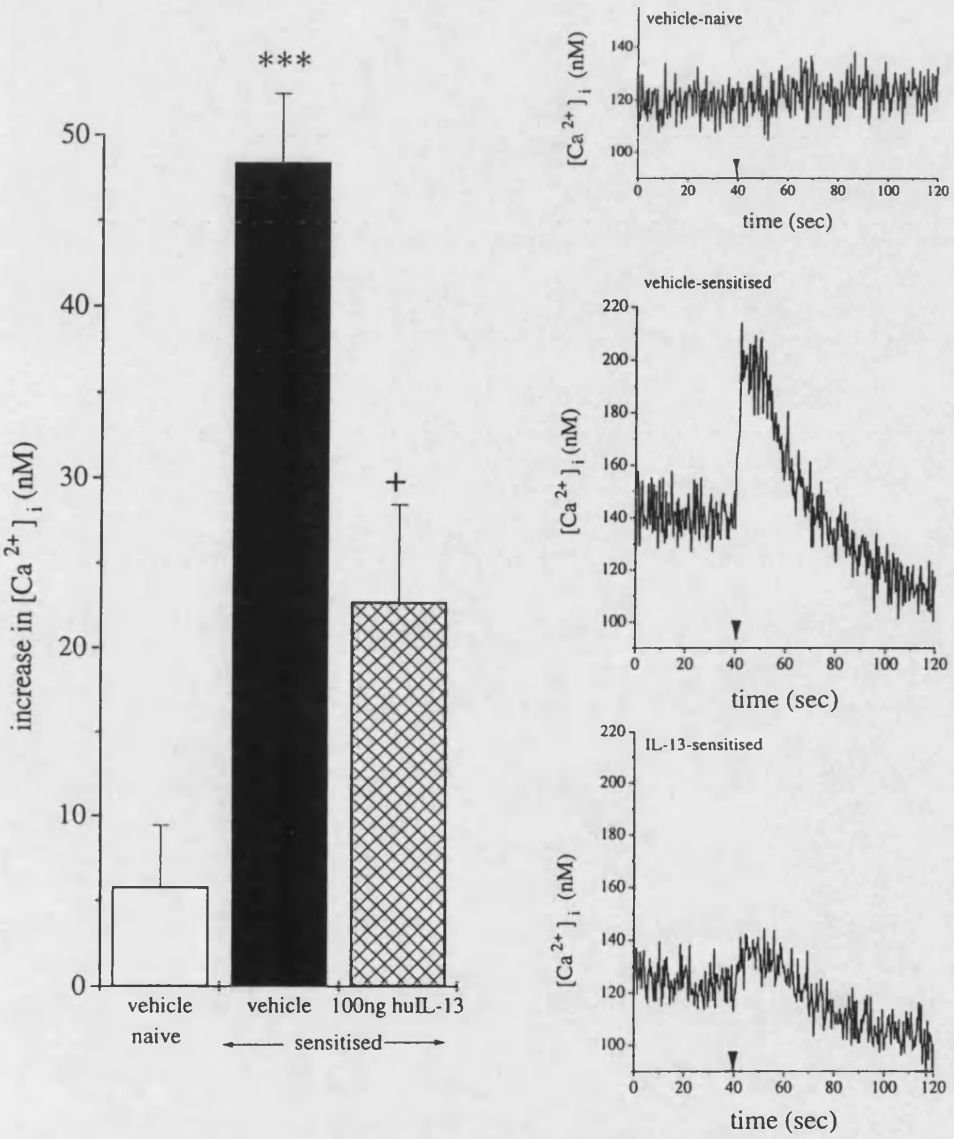


Figure7.5: Inhibition of the generation of BAL eosinophil stimulating activity by IL-13.
Fura-2 loaded guinea-pig peritoneal eosinophils were stimulated with 100µl BAL from sensitised animals instilled with either vehicle (■) or IL-13 (100ng/animal) (▨), or control naive animals instilled with vehichle (□).
Animals were instilled 20 min prior to aerosol antigen challenge (10ml 0.1% OA) and BAL was performed 24 h later. Bars represent the mean +/- sem, n=6 animals/treatment.
***$p<0.001$ compared with elevations induced by BAL from vehicle instilled naive animals,
+$p<0.05$ compared with elevations induced by BAL from vehicle instilled sensitised animals.
Inserts show typical time courses of $[Ca^{2+}]_i$ elevations following addition of BAL at the 40 second time point (arrows).

7.4 Summary of the in vivo activity of IL-13 in guinea-pig models of airway inflammation

gpTNFα induced airway inflammation

- Co-instillation of IL-13 with gpTNFα inhibited gpTNFα induced BAL leukocyte accumulation. IL-13 (1-100ng) co-instillation dose dependently reduced the gpTNFα induced BAL leukocyte infiltration with 100ng IL-13 reducing the BAL leukocyte numbers to control levels.
- Co-instillation of IL-4 (100ng) with gpTNFα did not significantly reduce gpTNFα induce BAL leukocyte accumulation.

Antigen induced airway inflammation

- The instillation of IL-13 (1-100ng) 20 min prior to antigen challenge of sensitised animals caused a dose dependent reduction in BAL eosinophil accumulation 24 hours after challenge.
- BAL from sensitised animals instilled with 100ng IL-13 prior to antigen challenge exhibited reduced ability to induce increases in $[Ca^{2+}]_i$ in fura-2 loaded peritoneal eosinophils.

8. Discussion

8.1 Initial aims of the project

At the outset of this project *in vitro* studies had provided evidence to implicate the involvement of TNFα in inflammatory diseases and this was combined with a number of clinical studies reporting elevated levels of TNFα in the asthmatic lung. However, the *in vivo* bioactivity of TNFα in the lung had been incompletely characterised. The purpose of this study was to express and purify recombinant gpTNFα and then to determine its activity *in vitro* using guinea-pig cells and in the guinea-pig airways *in vivo.* Furthermore, in order to investigate whether TNFα is expressed in antigen challenged sensitised guinea-pigs DIG-labelled DNA probes were developed and anti TNFα antibodies used. In addition, the effect on antigen-induced airway leukocyte accumulation and the generation of chemokine-like bioactivity following the administration of anti-inflammatory cytokines was examined. Agents which inhibit the actions of TNFα could be potential therapies in preventing allergic respiratory disorders.

8.2 Expression and purification of gpTNFα

The cDNA sequence predicted gpTNFα to be a 234 amino acid propeptide which, in order to correspond with the NH_2 terminus of the sequence reported by Tamatani *et al.* (Tamatani *et al.* 1989), probably forms a 154 amino acid mature protein. gpTNFα exhibited high amino acid sequence homology with published mammalian TNFα sequences. The NH_2 terminus of secreted TNFα has been predicted to result from the cleavage of the propeptide at one of two sites separated by 2 amino acid residues. In the majority of mammalian sequences there is a highly conserved area of the propeptide, shown in table 8.1 as a shaded area, in the region of the putative TNFα cleavage site. Studies of human TNFα (Pennica *et al.* 1984; Wang *et al.* 1985; Aggarwal *et al.* 1985) have identified the cleavage site to be between propeptide amino acids 76 alanine (A) and 77 valine (V), referred to as cleavage site *a* in the text and table 8.1. However, Shirai *et al.* (Shirai *et al.* 1985) predicted the cleavage site to

be between amino acids 78 arginine (R) and 79 serine (S), referred to as cleavage site *b* in the text and in table 8.1, in accordance to the sequence of TNFα derived from rabbit serum (Ito *et al.* 1986). In this study I have assumed gpTNFα is cleaved at the R-S bond, as is the case for rat (Shirai *et al.* 1989) and rabbit TNFα (Ito *et al.* 1986), and indicated by the sequence data of Tamatani *et al.*(Tamatani *et al.* 1989). This R-S bond appears to be conserved in all mammalian sequences. Furthermore, as the amino acids at the predicted cleavage site *a* (A-V) are less conserved between species than the R-S bond at cleavage site *b*, this may be of relevance when designing inhibitors of TNFα secretion and their subsequent use in animal models. However, the removal of four amino acids (VRSS) from the NH_2 terminus has little effect on the biological activity of human TNFα (Kirchesis *et al.* 1992; Gase *et al.* 1990). Gearing *et al.* (Gearing *et al.* 1994) and McGeehan *et al.* (McGeehan *et al.* 1994) have implicated a role for metalloproteinases in the processing of the propeptide and also that specific metalloproteinases inhibitors reduce *in vitro* TNFα secretion and circulating TNFα levels in rats and mice following LPS administration. A human metalloproteinase, TNFα converting enzyme (TACE), which acts at the A-V bond, has recently been cloned (Black *et al.* 1997; Moss *et al.* 1997).

Table 8.1: Alignment of a region of the TNFα propeptide amino acid sequences of various mammalian species to show the highly conserved area surrounding the putative secreted TNFα cleavage site. Cleavage is predicted to occur at one of two sites either cleavage site *a*, between the first two amino acids in the shaded area, or cleavage site *b*, between the third and fourth amino acid residues in the shaded area.

species	amino acid sequence for cleavage region of propeptide			reference
		$a\downarrow$ $\downarrow b$		
baboon	SPLAQ	AVRSSS	RTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGV	Sanjanwala,M. Genebank P33620
bovine	SPLVQ	TLRSSS	QASSNKPVAHVVADINSPGQLRWWDSYANALMANGV	(Cludts *et al.* 1993)
equine	NPLAQ	TLRSSS	RTPSDKPVAHVVANPQAEGQLQWLSGRANALLANGV	(Su *et al.* 1991)
feline	NPLPQ	TLRSSS	RTPSDKPVAHVVANPEAEGQLQRLSRRANALLANGV	(McGraw *et al.* 1990)
guinea-pig	LAQTL	TLRSAS	QNDNDKPVAHVVANQQAEEELQWLSKRANALLANGM	this report
human	SPLAQ	AVRSSS	RTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGV	(Pennica *et al.* 1984)
murine	MAQTL	TLRSSS	QNSSDKPVAHVVANHQVEEQLEWLSQRANALLANGM	(Pennica *et al.* 1985)
ovine	RPLVQ	TLRSSS	QASNNKPVAHVVANISAPGQLRWGDSYANALMANGV	(Nash *et al.* 1991)
porcine	NPLAQ	GLRSSS	QT SDKPVAHVVANVKAEGQLQWQSGYANALLANGV	(Drews *et al.* 1990)
rabbit	VAQMV	TLRSAS	RALSDKPLAHVVANPQVEGQLQWLSQRANALLANGM	(Ito *et al.* 1986)
rat	MAQTL	TLRSSS	QNSSDKPVAHVVANHQAEEQLEWLSQRANALLANGM	(Shirai *et al.* 1989)

The amplified gpTNFα cDNA was inserted into an expression system that resulted in the production of a MBP-gpTNFα fusion protein. The expressed fusion protein was shown to exhibit both cytolytic activity and TNFα immunoreactivity. Interestingly, the ability of the fusion protein to induce cytotoxicity in a WEHI

bioassay suggests that the MBP did not hinder trimer formation which is required for bioactivity (Smith & Baglioni 1987).

gpTNFα was cleaved from the fusion protein by factor Xa. Although thrombin also cleaves at R-S bonds this was not found to be efficient under the conditions used. Pilot purification experiments using reverse phase HLPC (Tamatani *et al.* 1989) and heparin columns (Lantz *et al.* 1991) were disappointing as no active product was recovered. The protein was subsequently purified using a series of hydroxyapatite, amylose and polymixin B bound agarose columns. Purification of gpTNFα resulted in the production of a single 17kDa protein which was recognised by goat and rabbit anti murine TNFα antibodies. In agreement with the findings of (Tamatani *et al.* 1989) the specific activity of gpTNFα was found to be greater than that of human TNFα. Both human and gpTNFα bioactivity could be neutralised by co-incubating with anti murine TNFα antibodies.

8.3 The in vivo bioactivity of TNFα

Inflammation induced by TNFα has been previously studied in the guinea-pig with conflicting results. *In vivo* studies by Kings *et al.* (Kings *et al.* 1990) showed that intraperitoneal administration of recombinant murine TNFα induced lung eosinophilia, whilst Collins *et al.* (Collins *et al.* 1993) were unable to induce skin eosinophilia following intradermal administration of human TNFα. Endogenous TNFα could induce eosinophil accumulation in the guinea-pig skin as the response to intradermal LPS was reduced by administration of a soluble TNFα receptor : Ig construct (sTNFαR-Fc) (Weg *et al.* 1995). Further, in isolated perfused guinea-pigs lungs, human TNFα increased the sequestration of activated human neutrophils via the upregulation of pulmonary vascular endothelial cell ICAM-1 expression (Lo, K. *et al.* 1992). Previous reports of intratracheal administration of TNFα have utilised *in vivo* rat models, where again the data have been variable. Ulich *et al.* (Ulich *et al.* 1991)

indicated that human TNFα induced a slight but reproducible lung neutrophilia which began 6 hours following instillation, peaked at 12-24 hours and subsided by 48 hours following intratracheal instillation in rats, whilst significant BAL neutrophil accumulation occurred 90 minutes following aerosol exposure human TNFα (Kips *et al.* 1992). However, Wesselius *et al.* (Wesselius *et al.* 1995) and Debs *et al.* (Debs *et al.* 1988) did not observe intra-alveolar neutrophil accumulation following intratracheal or lung-specific aerosol delivery of human TNFα respectively. These differences may reflect poor species cross-reactivity as none of the studies used TNFα from the host species.

The intratracheal instillation of gpTNFα induced a pronounced and protracted eosinophilia which was dose dependent. During the initial experiments gpTNFα appeared to selectively recruit eosinophils to the airways, however in later experiments there was also an increase in macrophage/monocyte numbers which had previously been masked by high macrophage/monocyte numbers in the control groups. The pre-incubation of rabbit anti murine TNFα sera with gpTNFα prior to instillation reduced the eosinophil accumulation, indicating this was a TNFα-driven effect. The possibility that the effect was due to LPS induced TNFα production was ruled out since both heat treatment and pre-incubation of gpTNFα with antibody abrogated the eosinophilia, in addition the gpTNFα was passed over a solid phase polymyxin B column prior to instillation. Time course studies indicated that the maximum eosinophilia was observed 24 hours following gpTNFα instillation, but significant eosinophilia was maintained at 72 hours.

The administration of human TNFα by various aerosol routes was unable to induce guinea-pig BAL leukocyte accumulation in this study, a finding also reported in a rat model (Debs *et al.* 1988). However, the administration of human TNFα by intratracheal instillation to the guinea-pig did induce an inflammatory cell recruitment which was weaker and exhibited different cellular characteristics than the

gpTNFα-induced response. Unlike gpTNFα, human TNFα did not induce eosinophil accumulation but did promote significant neutrophil infiltration and a slight increase in macrophage/monocyte numbers 24 hours after the administration of the highest dose used. The difference in leukocyte sub-type accumulation might be due to both differences in the distribution of the two TNFα receptors and their affinity for the proteins. However, only a limited number of experiments were performed using human TNFα, and further studies using anti receptor antibodies may help to clarify this. In the murine system the two TNFα receptors bind TNFα from different species with varying affinities. Whereas the murine p55 receptor binds with similar affinity to both murine and human TNFα, the murine p75 receptor shows strong selectivity for murine TNFα (Lewis *et al.* 1991; Smith *et al.* 1986). Species selectivity resulting in reduced *in vivo* bioactivity of human TNFα, compared with gpTNFα, was also demonstrated *in vitro*. *In vitro* studies indicated that gpTNFα but not human TNFα induced the production of chemokine-like bioactivity from guinea-pig cells. The stimulation of guinea-pig spleen cells, peritoneal macrophages and the guinea-pig cell line, JH-4 with either human TNFα, or a combination of human TNFα plus human IL-1 and human IFNγ was unable to induce chemokine production following 24 hours incubation. However, the incubation of JH-4 cells with either macrophage derived TNFα or recombinant TNFα induced the production of eotaxin-like bioactivity (the supernatants increased $[Ca^{2+}]_i$ in eosinophils) and IL-8 production, respectively. TNFα induced eotaxin and IL-8 expression have previously been reported in various human cell types (Westwick *et al.* 1991, Garcia-Zepeda *et al.* 1996). Similarly, human TNFα was reported to be unable to induce guinea-pig eosinophil chemotaxis or prime the eosinophils with respect to PAF or LTB_4 induced responses (Coëffier *et al.* 1991). These results further support *in vivo* studies where human TNFα did not induce skin eosinophil accumulation in the guinea-pig (Collins *et al.* 1993), suggesting species selectivity.

8.4 Is TNFα involved in allergic airway inflammation?

In order to address this question an OA sensitised guinea-pig model of airway inflammation was utilised. The sensitisation protocols used were based on those described by Andersson (Andersson 1981) and Sanjar *et al.* (Sanjar *et al.* 1990). Several variations of the methods, which included the use of cyclophosphamide and *Bordetella pertussis* vaccine, were tried in order to establish a model which demonstrated consistent bronchial hyperreactivity and lung eosinophilia. Due to the toxic nature of cyclophosphamide and the difficulty in obtaining *Bordetella pertussis* vaccine, the sensitisation method was changed to incorporate *Bordetella bronchiseptica* vaccine (P. McCabe, Glaxo-Wellcome, personal communication) and this methodology was used in all sensitisation data shown. In order to avoid fatal anaphylactic shock during antigen provocation all animals were routinely administered an anti-histamine, mepyramine, prior to challenging.

The model exhibited airway hyperreactivity, with peak responsiveness 6 and 24 hours following antigen challenge. Both an early and late phase airway hyperreactivity have previously been demonstrated in the OA sensitised guinea-pig however the time of onset of the early response is variable, reported to occur immediately (Ishida *et al.* 1989) or up to 4 hours after antigen challenge (Hutson *et al.* 1988; Boichot *et al.* 1991). The differences in onset times are in part due to variations in sensitisation methods and the time of administration of an H_1-receptor antagonist. In addition to the biphasic increase in bronchial hyperreactivity, airway leukocyte infiltration was also observed in two phases, an early infiltration of neutrophils peaking by 12 hours was followed by a delayed and protracted eosinophil infiltration, which peaked at 24 hours and the plateau was maintained at 48 hours. Unlike lung inflammation, airway hyperreactivity was not consistently observed following antigen challenge of sensitised animals. This is in agreement with the concept that cellular influx may be important but is not sufficient to induce airway hyperresponsiveness (Folkerts *et al.* 1988; Chapman *et al.* 1993). In support of this theory the instillation of gpTNFα alone did not induce airway hyperresponsiveness although these animals did

exhibit a pronounced airway eosinophilia. However, the administration of an anti TNFα antibody is reported to reduce LPS induced hyperreactivity in a rat model (Kips *et al.* 1992).

Increased TNFα levels in the airways of sensitised guinea-pigs have previously been demonstrated at 1-2 hours (Kelly *et al.* 1993) and 24 hours (Watson *et al.* 1993) after antigen challenge. Although no significant antigen-induced increases in TNFα expression were demonstrated in this report, the trends shown suggest that TNFα protein levels in whole lung tissue and BAL leukocytes were increased following antigen challenge. Pilot experiments indicated the presence of TNFα mRNA in lung tissue following antigen challenge. Unfortunately, repeating these experiments proved problematic due to RNA degradation during the extraction of message from the tissue. This may be due to high RNase activity of the infiltrating eosinophils. However, increased TNFα mRNA in murine lung tissue has been reported by others 1, 8, and 24 hours following antigen challenge (Lukacs *et al.* 1995b). Elevated TNFα levels in the BAL were variable, this may possibly have been due to the multitude of mediators collected in the BAL influencing the growth of the WEHI cells. These mediators may therefore have masked the cytolytic activity of TNFα present in the BAL. Additionally it is possible that these mediators were not released in the airways of naive animals and hence the apparent decrease in BAL TNFα levels following antigen challenge of sensitised animals compared to antigen challenged naive animals. In an attempt to resolve this problem a murine TNFα ELISA was tested using purified gpTNFα. The ELISA did not detect gpTNFα and the adaptation of the ELISA to measure the protein was hindered by the lack of specific monoclonal antibodies for gpTNFα.

Since the start of this project the involvement of TNFα in various models of inflammation has been demonstrated using anti TNFα antibodies or soluble receptors. The data presented show that the administration of an anti TNFα antibody prior to antigen challenge reduced both eosinophil and neutrophil airway inflammation 24

hours after challenge. These results are in accordance with those of Lukacs *et al.* (Lukacs *et al.* 1995b) where the administration of sTNFαR-Fc in a murine model of allergic airway inflammation reduced both the early neutrophil and later eosinophil airway infiltration. Additionally, to assess the potential use of sTNFαR-Fc as a therapeutic agent it was administered to the mice 24 hours after antigen challenge and this inhibited subsequent eosinophil influx. In contrast to inhibiting eosinophilia, in a rat model of sephadex-induced lung injury the administration of sTNFαR-Fc reduced BAL neutrophilia but slightly increased eosinophil infiltration (Gater *et al.* 1996).

The complexity of the role of TNFα has been demonstrated in studies indicating that both cytokines and chemokines which are themselves induced by TNFα may also regulate TNFα expression. Our lab has reported that the *in vivo* administration of IL-1ra reduced BAL TNFα levels and eosinophil infiltration in a guinea-pig model of antigen-induced airway inflammation (Watson *et al.* 1993). Furthermore, studies relating to the regulation of chemokine expression report that in a murine model of endotoxemia inhibition of TNFα reduced MIP-1α expression by 60% (Standiford *et al.* 1995), whilst administration of an anti MIP-1α antibody decreased BAL TNFα levels in two neutrophil-dependent models of lung inflammation (Shanley *et al.* 1995).

The development of human TNFα mutants which bind selectively to one of the two receptors (Loetscher *et al.* 1993) has promoted further investigation into the mechanisms by which TNFα induces an inflammatory response. The subcutaneous administration of a p55 agonist in the baboon resulted in neutrophil accumulation which was not seen with p75 agonist (Welborn III *et al.* 1996). Here a p75 agonist was unable to elicit an inflammatory response but the authors did not establish if p55 alone is sufficient to mediate a full inflammatory response as the p55 agonist has been shown to induce endogenous TNFα release (Van Zee *et al.* 1994) which can activate both p55 and p75.

8.5 Mechanisms by which TNFα induces airway inflammation.

The recruitment of leukocytes in response to TNFα is likely to result from a series of orchestrated events which includes increased adhesion molecule and chemokine expression. *In vitro* TNFα is a very weak inducer of eosinophil chemotaxis (Bittleman *et al.* 1996). gpTNFα does not induce eosinophil migration in chemotaxis chambers with artificial filters or induce changes in $[Ca^{2+}]_i$ in fura-2 loaded guinea-pig peritoneal eosinophils (White *et al.* 1997). However, TNFα induces the expression of VCAM-1 on endothelial cells which would facilitate the recruitment of eosinophilic and mononuclear leukocytes. Further VCAM-1 expression might favour a protracted response as VCAM-1 mRNA persists for 72 hours following *in vitro* TNFα stimulation of endothelial cells (Osborn *et al.* 1989). In addition, the *in vitro* preincubation of ^{111}In-labeled guinea-pig eosinophils with anti VLA-4 antibodies reduces eosinophil accumulation in guinea-pig skin following LPS administration (Weg *et al.* 1993).

The migration of eosinophils from the microvasculature to the lung tissue is reported to be primarily via VLA-4/VCAM-1 interactions and to a lesser extent LFA-1, Mac-1 / ICAM-1, ICAM-2 interactions (Nakajima *et al.* 1994). Several studies have demonstrated that the inhibition of VLA-4 reduces antigen-induced BAL eosinophil accumulation and bronchial hyperreactivity (Pretolani *et al.* 1994; Milne *et al.* 1995; Abraham *et al.* 1994). In contrast, a previous study reported that the inhibition of ICAM-1 reduced antigen-induced eosinophil accumulation in the BAL of monkeys (Wegner *et al.* 1990). In addition, the administration of an anti CD18 antibody, the β2 integrin sub-unit of ICAM-1 ligands LFA-1 and Mac-1, also inhibited BAL eosinophil accumulation. However, the anti CD18 antibody did not reduce the accumulation of eosinophils in the lung tissue (Milne *et al.* 1994), indicating that the subsequent migration into the airway lumen might involve LFA-1 or Mac-1 interactions with ICAM-1 and ICAM-2. This theory is supported by the distribution of

the adhesion molecules as, unlike ICAM-1 which is expressed on the endothelium and epithelium, VCAM-1 is not expressed on airway epithelial cells (Bloeman *et al.* 1993).

The identification of chemokines has helped to advance our understanding of the mechanisms underlying the accumulation of a predominant leukocyte sub-type in a disease condition. TNFα is a potent inducer of chemokine expression *in vitro* from both resident tissue cells e.g. fibroblasts and epithelial cells, and leukocytes (Lukacs *et al.* 1995a; Westwick *et al.* 1991; Cromwell *et al.* 1992). TNFα induced eosinophil migration involves, at least in part, the upregulation of chemokine expression (Watson *et al.* 1988).To date, one of the most potent chemokines with respect to eosinophil chemotaxis is eotaxin (Jose *et al.* 1994b), a C-C chemokine recently cloned from allergen-challenged guinea-pig lungs (Jose *et al.* 1994a). TNFα induces eotaxin expression in endothelial and epithelial cells (Garcia-Zepeda *et al.* 1996), and it is possibly a very important mediator of TNFα-induced eosinophil accumulation. In addition, the C-C chemokines RANTES, MCP-2, -3,-4 and the recently discovered peptide eotaxin-2 (Rot *et al.* 1992, Noso *et al.* 1994; White J. *et al.* 1997) are capable of stimulating human eosinophils. RANTES, although found to activate guinea-pig macrophages not guinea-pig eosinophils (Campbell *et al.* 1996), is increased in BAL from asthmatic subjects (Teran *et al.* 1996). A further C-C chemokine which activates eosinophils and regulates eosinophil accumulation is MIP-1α (Rot *et al.* 1992). In a model of antigen-induced airway inflammation, MIP-1α mRNA and protein expression was increased after challenge with mononuclear cells, eosinophils and airway epithelial cells expressing the protein. The inhibition of MIP-1α reduced lung and airway eosinophil infiltration by 50% (Lukacs *et al.* 1997). In addition to the direct actions of MIP-1α on eosinophil recruitment, MIP-1α can potentiate the inflammatory response by modulating macrophage function, increasing TNFα and IL-1 production (Fahey *et al.* 1992), and inducing mast cell degranulation (Alam *et al.* 1994) and the release of preformed mediators including TNFα (Gordon *et al.* 1990).

The incomplete inhibition of antigen-induced lung eosinophil accumulation following the blockade of MIP-1α alone suggests that eosinophil recruitment is unlikely to be as a result of the expression of a sole chemokine. Eosinophils have the capacity to synthesis and release certain cytokines and chemokines including TNFα, eotaxin, IL-8 and MIP-1α (Costa *et al.* 1993; Finotto *et al.* 1994; Garcia-Zepeda *et al.* 1996; Yousefi *et al.* 1995; Kita *et al.* 1995). IL-8 is a potent neutrophil activator (Baggiolini *et al.* 1989) which induces lung eosinophilia following aerosol (Smith *et al.* 1991) or intraperitoneal administration (Burrows *et al.* 1990; Burrows *et al.* 1991a) and induces eosinophil accumulation in guinea-pig skin (Collins *et al.* 1993). The pre-incubation of eosinophils with priming agents influences the biological response, for example the weak IL-8 chemotactic response is enhanced by pre-incubation with IL-3 GMCSF and IL-5 (Burrows *et al.* 1991b; Schweizer *et al.* 1994). Furthermore, eosinophils from antigen-challenged allergic individuals display enhanced transendothelial migration, compared with non-allergic individuals, and the potentiated response is similar to that seen following the priming of eosinophils from non-allergic individuals (Ebisawa *et al.* 1994).

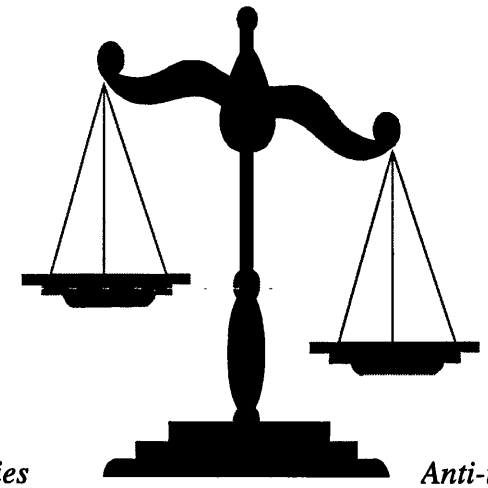
8.6 Effect of IL-13 on antigen and gpTNFα-induced airway inflammation

IL-13, originally described as a cytokine derived principally from Th2 lymphocytes, exhibits some anti-inflammatory properties (Minty *et al.* 1993). IL-13 is also expressed by activated B lymphocytes, basophils and mast cells (Krishnaswamy *et al.* 1993; Li *et al.* 1996; Burd *et al.* 1995). IL-13 inhibits the expression of mRNA for certain cytokines in LPS-stimulated monocytes, including IL-1, TNFα, IL-8 and MIP-1α (Minty *et al.* 1993; De Waal Malefyt *et al.* 1993; Cosentino *et al.* 1995), whilst it increases IL-1ra expression (Vannier *et al.* 1996). The inhibition of TNFα and IL-1, and increased production of IL-1ra by IL-13 have also been demonstrated using stimulated alveolar macrophages (Yanagawa *et al.* 1995). As IL-13 alters

mRNA levels it acts at a pre-translational level. In the case of IL-1ra, IL-13 enhances IL-1ra mRNA steady-state levels by increasing transcriptional activation of the gene, but does not affect mRNA stability, conversely, IL-13 reduces IL-1 gene transcription and mRNA stability (Vannier *et al.* 1996). Many, but not all, of the biological activities of IL-13 are similar to IL-4, the exceptions include T lymphocytes stimulation by IL-4 but not IL-13 (Zurawski *et al.* 1994).

IL-13, like IL-4, also exhibits pro-inflammatory activities such as isotype switching in B lymphocytes increasing IgE and IgG4 production (Punnonen *et al.* 1993). Furthermore, IL-13 inhibits the production of IL-12 and IFNγ by LPS stimulated monocytes (de Waal Malefyt *et al.* 1993). Inhibition of IL-12 could affect the regulation of Th1 and Th2 development, since both IL-12 and IFNγ have been shown to direct the generation of Th1 lymphocytes (Manetti *et al.* 1993). Hence, this suggests that IL-13 would favour Th2 responses with the production of IL-4 and IL-5, which are both upregulated in asthma. In addition, IL-13 upregulates the expression of MHC class II antigens which may result in more efficient antigen-presentation. As summarised in figure 8.1 the net effect of IL-13 in an *in vivo* situation is dependent on a balance between the anti-inflammatory and pro-inflammatory activities.

The data presented in this thesis report a novel *in vivo* action of IL-13 which was not shown with IL-4. IL-13 inhibited gpTNFα-induced BAL eosinophil and macrophage/monocyte infiltration when co-instilled with gpTNFα. The inhibition of antigen-induced BAL eosinophil infiltration, following the instillation of IL-13 prior to antigen challenge, was pronounced although the numbers were not reduced to those of control animals. As IL-13 produced a dose-dependent reduction maximum inhibition may not have been achieved even with the highest dose of IL-13 used. Interestingly, a low dose of IL-13 (1ng per animal) reduced antigen-induced eosinophil influx by >50%, suggesting IL-13 was an effective inhibitor of eosinophil accumulation. IL-13 did not significantly inhibit antigen-induced macrophage/monocyte BAL influx.



Pro-inflammatory activities

↑ B cell proliferation

↑ IgE and IgG4 production

↑ VCAM-1 expression

↑ MHC class II and antigen presentation

Favours Th2 production

Anti-inflammatory activities

Inhibits IL-1, TNFα, IL-8, MCP-1 and RANTES production

Inhibits nitric oxide production

↑ IL-1ra synthesis

Figure 8.1: IL-13, good or bad? IL-13 exhibits both anti-inflammatory and pro-inflammatory activites *in vitro*, *in vivo* the net effect of IL-13 is dependent on the balance between these properties.

To address whether IL-13 prevented the migration of eosinophils from the lung tissue into the airway lumen whole lung EPO measurements were performed as an indication of the relative eosinophil numbers. Lung tissue of animals co-instilled with IL-13 and gpTNFα contained lower EPO levels than animals instilled with gpTNFα alone. This indicates that the eosinophils were not retained in the lung tissue and

prevented from migrating to the lumen which might be expected if IL-13 was increasing VCAM-1 expression (Bochner *et al.* 1995).

In addition to inhibition of BAL eosinophil accumulation, IL-13 also reduced the chemokine-like bioactivity in the BAL. The antigen-induced calcium mobilisation activity in the BAL was leukocyte specific, activating only eosinophils. The C-C chemokine RANTES is a major mediator of human eosinophil activation including chemotaxis and transient changes in $[Ca^{2+}]_i$ (Kameyoshi *et al.* 1992; Rot *et al.* 1992; Bourne *et al.* 1995). However, neither guinea-pig nor human RANTES induce calcium mobilisation in guinea-pig peritoneal eosinophils, but both activate guinea-pig peritoneal macrophages (Campbell *et al.* 1996 and own observations). This chemokine-like activity is possibly due to eotaxin. Eotaxin induces calcium mobilisation and aggregation of eosinophils *in vitro*, and the administration of aerosol eotaxin *in vivo* induces the selective accumulation of BAL eosinophils (Griffiths-Johnson *et al.* 1993). The peak expression of eotaxin mRNA in lung tissue and bioactivity in BAL occurs 3-6 hours following antigen-challenge and returns to basal levels by 24 hours (Rothenberg *et al.* 1995; Jose *et al.* 1994b). The time course described is similar to the profile of eosinophil specific chemokine activity demonstrated in this thesis. Moreover the reduction in chemokine-like activity induced by IL-13 further suggests the involvement of chemokines such as IL-8 and MIP-1α, the synthesis of which may be inhibited by IL-13. To date, the effect of IL-13 on antigen- or TNFα-induced eotaxin expression have not been published. However as previously discussed others have demonstrated a significant increase in lung TNFα expression, and this report indicates enhanced TNFα levels, during antigen-induced airway inflammation, it is feasible that in the allergic model IL-13 inhibits both TNFα and chemokine expression. Furthermore, the enhancement of IL-1ra by IL-13 is an additional pathway by which IL-13 may reduce antigen-induced eosinophil accumulation. *In vivo* studies have demonstrated that the administration of IL-1ra prior to antigen challenge reduces BAL eosinophil accumulation, bronchial hyperreactivity and BAL TNFα levels 24 hours after challenge (Watson *et al.* 1993).

Although IL-4 shares many biological activities with IL-13, including inhibiting the synthesis of certain cytokines and chemokines, it did not affect gpTNFα induced BAL eosinophil infiltration when co-instilled with gpTNFα. The instillation of IL-4 alone to naive animals did increase BAL macrophage/monocyte accumulation, which was not observed when IL-13 was administered to naive animals. The differences in the IL-13 and IL-4 responses may be due to variation in the expression and affinity of the IL-13 and IL-4 receptors for the ligands. In this study both cytokines were recombinant human proteins and as previously noted with TNFα species selectivity may favour the binding of either IL-13 or IL-4. At present the receptors have yet to be fully characterised. It is believed that there are multiple receptors and certain sub-unit(s) are shared by both IL-13 and IL-4 receptors (Callard *et al.* 1996). One of the IL-4 receptors is independent of the IL-2 receptor γ sub-unit and this sub-unit is not the shared IL-13/IL-4 receptor component (He *et al.* 1995). Furthermore, the expression of the human eosinophil chemoattractant RANTES by endothelial cells was inhibited by both IL-13 and IL-4 (Marfaing-Koka *et al.* 1995), but only IL-4 reduced RANTES expression in human airway epithelial cells (Berkman *et al.* 1996), indicating that the effects of IL-13 and IL-4 may be cell type dependent. In addition, our group has recently shown that IL-13 is a potent inhibitor of MCP-1 and RANTES but not IL-8 in colonic epithelial cells, further indicating cell type dependant effects of IL-13 (Kolios *et al.* 1997)

It is clear that in order to understand the mechanism by which IL-13 inhibits antigen-induced eosinophil accumulation further research is required. Recent findings by members of the group have demonstrated that IL-13 can modulate not only chemokine expression but also reduce inducible nitric oxide synthase (iNOS) and nitric oxide production (Jordan *et al.* 1996; Kolios *et al.* 1996b; Kolios *et al.* 1996a). Exhaled nitric oxide levels are increased in asthmatic patients, suggesting that nitric oxide may have a pro-inflammatory role in asthma (Kharitonov *et al.* 1994). In addition, epithelial cells in the asthmatic, but not normal, lung express iNOS (Hamid

et al. 1993). The effects of nitric oxide on airway function have been reviewed by Barnes (Barnes, 1995), and include increased airway blood flow and plasma exudation. Nitric oxide has an inhibitory action on Th1 but not Th2 lymphocytes (Taylor-Robinson *et al.* 1994), which would favour Th2 proliferation.

To my knowledge this is the first report of IL-13 inhibiting airway inflammation and these results are contrary to the reported involvement of IL-4. Inhibition of IL-4 in a murine model of antigen-induced airway inflammation reduced BAL eosinophil accumulation (Lukacs *et al.* 1994) and IL-4 transgenic mice exhibited eosinophilia (Tepper *et al.* 1993). However IL-10, which exhibits anti-inflammatory properties similar to that of IL-13 (Fiorentino *et al.* 1991), reduces antigen-induced eosinophil infiltration and IL-5 generation in the peritoneal cavity of mice (Zuany-Amorim *et al.* 1996).

8.7 Cytokine networking in allergic lung inflammation

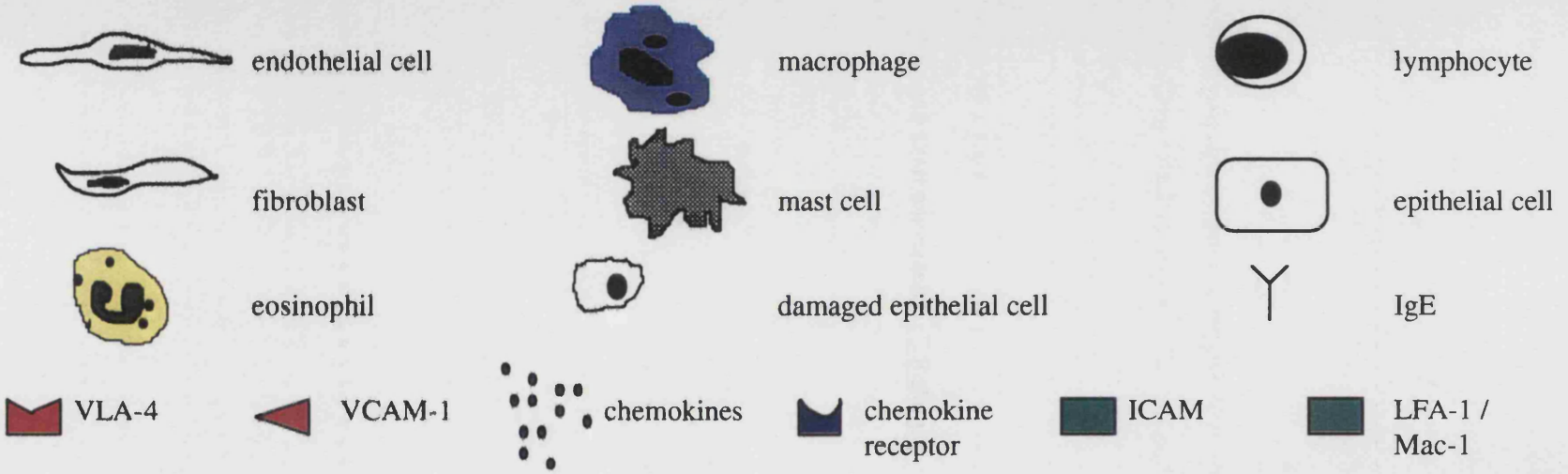
TNFα is released at an early stage of the allergic response following antigen activation of mast cells (Plaut *et al.* 1989). The potential central role for TNFα in allergic lung inflammation is summarised in figure 8.2. The upregulation of adhesion molecules is a crucial step in leukocyte accumulation. TNFα is a potent non-selective inducer of adhesion molecule expression increasing E-selectin, ICAM-1 and VCAM-1 (Kyan-Aung *et al.* 1991), whereas IL-4 and IL-13 selectively increase VCAM-1 expression on endothelial cells. Concomitantly, TNFα induces the production of chemokines from surrounding cells including endothelial, epithelial, smooth muscle cells and fibroblasts. Elevated levels of eosinophil chemoattractants including eotaxin, MIP-1α, IL-16, RANTES and IL-8 have been demonstrated in BAL from animal models or antigen-challenged asthmatics (Jose *et al.* 1994b; Lukacs *et al.* 1997; Teran *et al.* 1996; Cruikshank *et al.* 1997). As described in the introduction, chemokines are thought to aid transendothelial migration by the formation of hapatotactic and chemotactic gradients. In addition to the induction of chemotaxis RANTES and

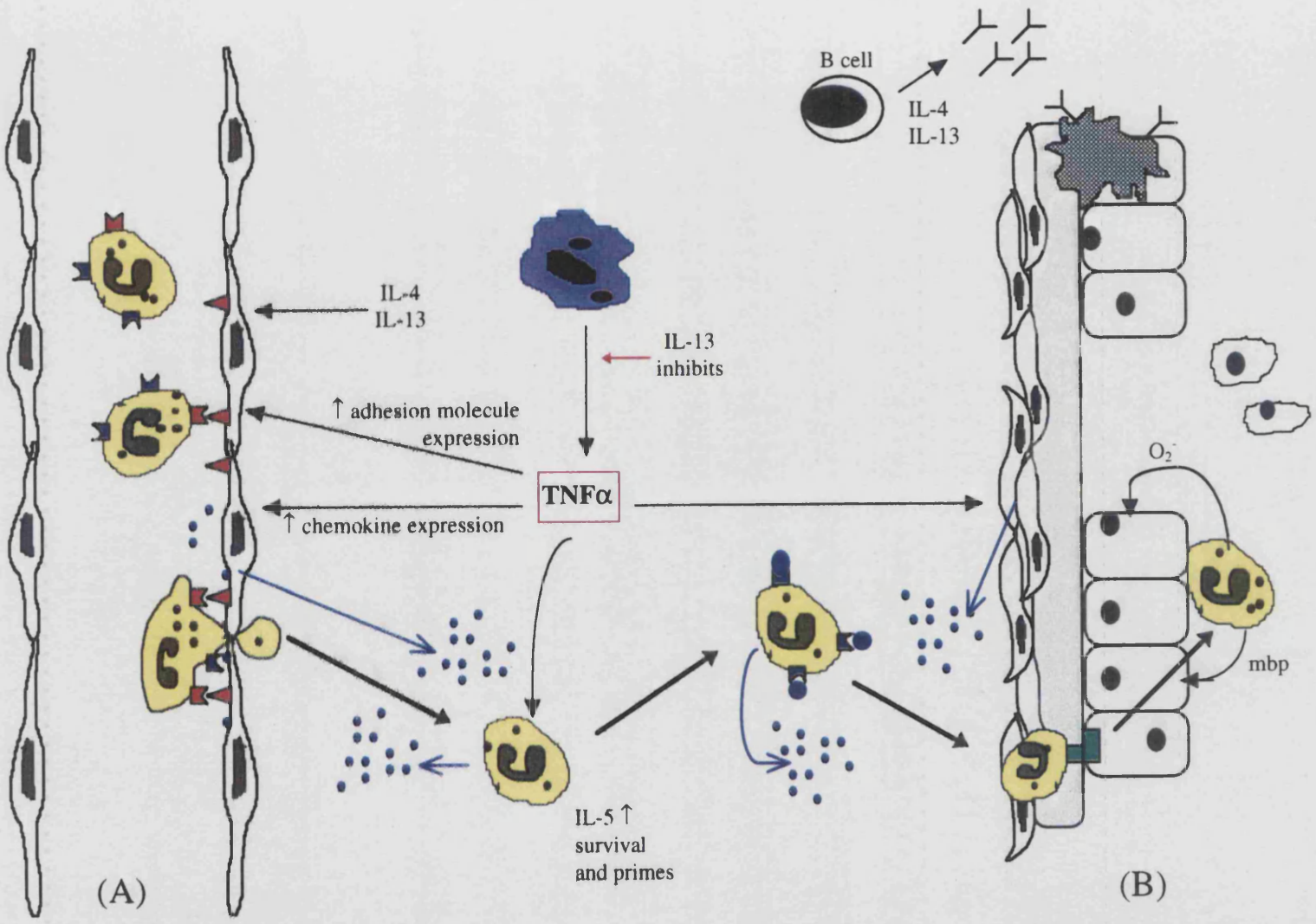
MIP-1α promote isotype switching increasing B lymphocyte production of IgE and IgG4 (Kimata *et al.* 1996). However, unlike the IL-4 and IL-13 response, RANTES and MIP-1α induced isotype switching is not enhanced by TNFα (Aversa *et al.* 1993; Kimata *et al.* 1996).

Following the initial eosinophil accumulation the process could be self-perpetuating as eosinophils themselves are known producers of and have receptors for TNFα (Costa *et al.* 1993; Roubin *et al.* 1987), IL-4 (Nonaka *et al.* 1995; Baskar *et al.* 1990), IL-5 (Yamaguchi *et al.* 1988; Broide *et al.* 1992), eotaxin (Garcia-Zepeda *et al.* 1996), MIP-1α (Costa *et al.* 1993; Rot *et al.* 1992) and IL-8 (Yousefi *et al.* 1995; Kita *et al.* 1995; Erger *et al.* 1995). The inflammatory response may be resolved by anti-inflammatory cytokines such as IL-13, IL-10 and by the production of receptor antagonists (e.g. IL-1ra, soluble TNF receptors).

Figure 8.2: (please see next page)

Figure 8.2: (on the facing page) The potential cytokine network involved in allergic airway inflammation. Antigen activation of mast cells induces degranulation and cytokine release including IL-4, IL-13 and TNFα. TNFα activation of resident and infiltrating cells in the lung induces the synthesis and release of eosinophil chemoattractants especially eotaxin, IL-8, RANTES and MIP-1α. TNFα also upregulates the expression of adhesion molecules on the endothelium and epithelial cells. IL-4 and IL-13 selectively increase VCAM-1 expression. Eosinophils migration from the microvasculature (A) into the lung tissue is directed by adhesion molecule and chemokine expression. The release of IL-5 from T lymphocytes promotes eosinophilia and the survival and activation of eosinophils. Activated eosinophils degranulate to release toxic mediators such as major basic protein (mbp) and super oxide anions (O_2^-), which contribute to epithelial destruction (B). Although IL-13 inhibits the production of TNFα and chemokines from macrophages, smooth muscle, endothelial and epithelial cells (anti-iflammatory activities) it also promotes isotype switching in B cells increasing IgE production (pro-inflammatory activity).




B cell
IL-4
IL-13
IL-4
IL-13
IL-13
inhibits
↑ adhesion molecule
expression
TNFα
↑ chemokine expression
O_2^-
mbp
IL-5 ↑
survival
and primes
(A)
(B)

8.8 Implication as possible therapeutic agents

The present treatments for asthma, most importantly β_2 agonists and glucocorticosteroids, are highly effective at alleviating the visible symptoms of the disease and thus improving the patients quality of life, but they do not cure asthma. Furthermore, the frequent and long term use of many anti-asthmatic drugs increases the side-effects associated with the therapies, e.g. frequent use of the β_2 agonist fenoterol was linked to increased mortality (Crane *et al.* 1989). Effective new therapies are required which relieve not only the bronchospasm but also the inflammatory reaction occurring within the lung. By inhibiting the eosinophil influx into the lung permanent architectural damage to the tissue should be prevented. From this report it is clear that TNFα is an important mediator of eosinophil accumulation and that specific inhibitors of TNFα would potentially be of therapeutic benefit. IL-13 also demonstrated anti-inflammatory activity suppressing antigen-induced eosinophil infiltration. Interestingly, as IL-13 inhibited TNFα-induced eosinophil accumulation this suggests that IL-13 may be a useful pharmacological tool in modulating cytokine-driven inflammatory reactions. Further investigation is required into the mechanisms of action of IL-13.

8.9 Final conclusions

In this thesis I report the expression, purification and *in vivo* bioactivity of gpTNFα. Mature gp TNFα was expressed as a 154 amino acid, 17kDa, protein which was approximately 80% homologous with other mammalian TNFα sequences. The *in vivo* data presented in this thesis indicate the importance of TNFα in the generation of lung eosinophilia in the guinea-pig. Following intratracheal instillation of gpTNFα there was a pronounced and protracted eosinophilia. This response was inhibited by pre-incubating gpTNFα with anti TNFα antibody and by heat inactivating the protein prior to instillation. The instillation of human TNFα induced only a weak leukocyte accumulation with different cellular characteristics than the gpTNFα-induced response

further indicating the potential danger of evaluating the activities of proteins when not using the host species. In a guinea-pig model of allergic lung inflammation, which demonstrated antigen-induced lung eosinophil infiltration and bronchial hyperreactivity, enhanced TNFα levels were detected after antigen challenge. The role of TNFα in generating allergic airway eosinophilia was reinforced by the administration of an anti TNFα antibody which reduced BAL eosinophil numbers.

A novel finding presented in this report is the inhibitory action of IL-13. IL-13 inhibited both gpTNFα- and antigen-induced airway eosinophilia. Although the mechanisms by which IL-13 induced this effect require further characterisation, IL-13 inhibited migration of eosinophils from the microvasculature into the lung tissue and thus the effect was not simply as a possible consequence of an increase in the expression of adhesion molecules retaining the eosinophils in the lung tissue. Furthermore, IL-13 reduced the chemokine bioactivity present in the BAL of antigen challenged animals.

In conclusion, this report demonstrates that TNFα is an important mediator of airway eosinophil accumulation *in vivo*. Furthermore, agents which inhibit the actions or production of TNFα may be potential therapies in the treatment of diseases which are characterised by airway eosinophilia. In addition, the ability of IL-13 to prevent antigen and gpTNFα-induced eosinophil accumulation suggests that IL-13 represents an important therapeutic lead in the development of agents to modulate allergic airway inflammation.

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