UNIVERSITY OF LEICESTER

THE IMMUNE RESPONSE TO NON-SMALL CELL LUNG CANCER

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A thesis submitted for the degree of

Doctor of Medicine

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January 2010

University of Leicester

Abstract

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Non-small cell lung cancer (NSCLC) is responsible for more deaths worldwide than any other cancer. Currently, 5-year survival for patients with stage IA disease is just 67%. Chemotherapy offers no cure at present for patients with NSCLC. It is now recognised that the immune system plays a significant role in both tumour modulation and progression. Therefore, a better understanding of immune responses to NSCLC may lead to the development of novel therapies.

In these studies, I have investigated, using immunohistochemistry, the microlocalisation of macrophage and mast cell phenotypes (as well as mast cell degranulation), $TNF\alpha$ expression, non-macrophage expression of markers associated with macrophage phenotypes, chemokine receptors and markers of apoptosis and cellular proliferation in surgically resected NSCLC tissue.

These studies have demonstrated for the first time in NSCLC that there are two major macrophage phenotypes, M1 and M2, and that the cytotoxic M1 phenotype predominates in the islets of patients with extended survival. The presence of high numbers of mast cells in the islets, irrespective of phenotype, also predicts extended survival. In addition, $TNF\alpha$ expression in tumour islets was found to be an independent predictor of increased survival but its expression in tumour stroma was an independent predictor of poor survival. Also, patients with increased tumour islet expression of markers associated with cytotoxic macrophages (HLA-DR, iNOS, MRP 8/14 and $TNF\alpha$) by non-macrophage cells was associated with extended survival. Patients with increased expression of CXCR3 and CCR1 in their tumour islets also had extended survival suggesting that these chemokine receptors may be involved in a pathway attracting cytotoxic components of the immune system into tumour islets.

In summary, these studies highlight the importance of microlocalisation and phenotype of immune cells in determining whether they play a pro- or anti-tumorigenic role in NSCLC.

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ACKNOWLEDGEMENTS

ABBREVIATIONS

AMS	above median survival
APC	antigen presenting cell
BMS	below median survival
CD4	Helper T cell
CD8	Cytotoxic T cell
CD163	cluster of differentiation 163
CEA	carcinoembryonic antigen
CK18	cytokeratin 18
COX2	cyclooxygenase 2
CSF-1	colony stimulating factor-1
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
ES	extended survival
GM-CSF	granulocyte macrophage colony-stimulating factor
HCL	hydrochloric acid
HIF-1	hypoxia-inducible factor-1
HLA-DR	human leukocyte antigen DR, class II histocompatibility antigen
IFN-γ	interferon gamma
IL	interleukin
IMS	international methylated spirit
iNOS	inducible nitric oxide synthase
LC	lung cancer
LPS	lipopolysaccharide

M0	precursor monocytes
M1	cytotoxic macrophage phenotype
M2	chronic inflammatory macrophage phenotype
MAGE	melanoma antigen
MC_T	mast cell expressing tryptase
MC_{TC}	mast cell expressing tryptase and chymase
MHC	major histocompatibility complex
MIF	macrophage inhibitory factor
MIP	macrophage inflammatory protein
MRP 8/14	myeloid related protein dimerisation 8/14
NK	natural killer
NSCLC	non-small cell lung cancer
NF-kβ	nuclear factor kappa beta
PBS	phosphate buffered saline
PET	positron emission tomography
PS	poor survival
ROI	reactive oxygen intermediates
SEM	standard error mean
TAM	tumour associated macrophage
TBS	tris buffer solution
TGFβ	transforming growth factor-beta
TLR	toll-like receptor
TNFα	tumour necrosis factor alpha
Treg	FoxP3+ regulatory cells
VEGF	vascular endothelial growth factor

PUBLICATIONS

The following peer-reviewed original publication has arisen from the work described in this thesis:

Ohri, C.M., Shikotra, A., Green, R., Waller, D. & Bradding, P. "Macrophages within NSCLC tumour islets are predominantly of a cytotoxic M1 phenotype." *Eur Respir J* 2009; 33: 118-126

The following published abstracts have arisen from the work described in this thesis:

Ohri, C.M., Shikotra, A., Green, R., Waller, D. & Bradding, P. "Tumour necrosis factor-alpha expression in tumour islets confers a survival advantage in non-small cell lung cancer". *Thorax* 2009; 64(suppl 4): 61

Ohri, C.M., Shikotra, A., Green, R., Waller, D. & Bradding, P. "Chemokine receptor expression in tumour islets and stroma in non-small cell lung cancer". *Thorax* 2009; 64(suppl 4): 60

Ohri, C.M., Shikotra, A., Green, R., Waller, D. & Bradding, P. "The microlocalisation of non-macrophage expression of markers associated with M1 and M2 macrophages in non-small cell lung cancer". *Thorax* 2008; 63(suppl 7): 57

Ohri, C.M., Shikotra, A., Green, R., Waller, D. & Bradding, P. "Infiltration of tumour islets by M1 macrophages is associated with extended survival in non-small cell lung cancer." *Journal of Thoracic Oncology* 2008; 3, (suppl 7):239-240

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Ohri, C.M., Shikotra, A., Welsh, T., Waller, D.A. & Bradding, P. "Degranulation of stromal mast cells of the tryptase-only phenotype is associated with improved prognosis in non-small cell lung cancer." *Thorax* 2007; 62(suppl 3):11

Chapter 1

Introduction: The immune response

to non-small cell lung cancer

Non-small cell lung cancer

Lung cancer (LC) is the most common cause of cancer-related death worldwide (Parkin et al. 2005). Non-small cell lung cancer (NSCLC) accounts for 75-80% of all lung cancer cases (Juretic, Sobat, & Samija 1999). In 2002, approximately 1.35 million people were diagnosed with LC worldwide (1.18 million of those died of LC) (Youlden, Cramb, & Baade 2008), with 29,000 new cases diagnosed in England and Wales (NICE 2005). 21.4% of men and 21.8% of women diagnosed with LC in England between 1993 and 1995 were alive 1 year after diagnosis and only 5.5% survived for 5 years (NICE 2005). Survival from cancer has been shown to be lower in the United Kingdom (UK) than in most other European countries as a result of patients in the UK presenting with more advanced stages of disease (Berrino et al. 2001). It is known that LC patients presenting with early stage cancer have improved prognosis (Mountain 1997) and it has been demonstrated that cancer patients in the UK have had considerable delays in their diagnosis (Allgar & Neal 2005). However, even with early presentation (stage 1A) LC patients have a five-year survival of only 67% (Mountain 1997) which is lower than that for other solid tumours (Sant et al. 2003).

NSCLC can be divided into three major histological subtypes: adenocarcinoma, squamous and large cell (Donati et al. 2007). LC occurs more often in smokers (Peto et al. 2000). Adenocarcinoma of the lung is associated with smoking but also often occurs in patients who have never smoked (Charloux et al. 1997). The distribution of these histological subtypes has changed worldwide in the last few decades, marked by a growing incidence of adenocarcinoma and a concurrent decline in the incidence of squamous cell carcinoma (Gabrielson 2006). A recent review of the predictive role of histology

in advanced NSCLC concluded that some studies have suggested a correlation between histology and survival, however, other studies provide conflicting results (Hirsch et al. 2008). The incidence of lung cancer is generally declining in developed countries (Jemal et al. 2006) but is increasing in developing countries due to the rise in cigarette smoking in the developing world (Youlden, Cramb, & Baade 2008). It has been proposed that smoking can impair innate immunity (Green et al. 1977) and subsequently impair macrophage, mast cell and natural killer (NK) cell responses. This may be a contributing factor as to why the incidence of LC is higher in smokers. However, other factors including genetic and environmental are also important (Cassidy et al. 2009).

The tumour stage is an essential part of determining treatment options as well as prognosis for patients with NSCLC (Mountain 1997). The TNM staging system has been used for this purpose but has recently been revised (Goldstraw et al. 2007). Currently, the only options for the cure of NSCLC are surgical resection and continuous hyperfractionated accelerated radiotherapy (Smythe 2001) but these depend on early stage of the tumour and good performance status of the patient. At present, chemotherapy does not offer a cure for NSCLC but it can improve survival and quality of life for more advanced stages of disease (Spiro et al. 2004). Current regimens include gemcitabine in combination with platinum based chemotherapy (Sculier & Moro-Sibilot 2009) - cisplatin-based treatment has a survival advantage compare to carboplatin-based treatment for patients with advanced NSCLC (Hotta et al. 2004). The median survival of patients with metastatic NSCLC treated with such regimens is approximately 8 to 10 months (Bunn, Jr. 2002). Novel targeted therapies, including epidermal growth factor tyrosine kinase inhibitors, such as erlotinib and gefitinib, and bevacizumab, a monoclonal antibody against vascular

endothelial growth factor (VEGF), have showed some value as second line agents in advanced NSCLC in terms of slight survival benefit (Kim et al. 2008;Sandler et al. 2006;Tsao et al. 2005). Similarly, pemetrexed has also proven to be useful as a second line agent (Hanna et al. 2004).

Ultimately, however, the overall survival from NSCLC remains extremely poor, especially in comparison to other solid tumours (Sant, Aareleid, Berrino, Bielska Lasota, Carli, Faivre, Grosclaude, Hedelin, Matsuda, Moller, Moller, Verdecchia, Capocaccia, Gatta, Micheli, Santaquilani, Roazzi, Lisi, & the EUROCARE Working Group 2003). There is increasing interest in the role played by the innate and adaptive immune systems in the regulation of tumour development and progression (Bingle, Brown, & Lewis 2002;O'Byrne & Dalgleish 2001). Thus, novel therapies will be vital in the future and it is anticipated that an increased understanding of the molecular and cellular immunology of NSCLC will lead to the identification of novel targets for immunotherapy.

The role of inflammation and immunity in cancer

The immune surveillance theory (Burnet 1957) highlighted that the immune system can play a role in cancer prevention and progression. However, in 1863 Virchow was the first to note the presence of leucocytes in neoplastic tissues and made a connection between inflammation and cancer (Balkwill & Mantovani 2001). He suggested that the ―lymphoreticular infiltrate" reflected the origin of cancer at sites of chronic inflammation. However, more recently, studies have suggested that the immune infiltrate may actually benefit the tumour by producing a pro-tumorigenic microenvironment (Bingle, Brown, & Lewis 2002).

Rous was the first to recognise that cancers may develop as a result of viral or chemical carcinogens that induce somatic changes (Rous 1947). This is known as ‗initiation‘ and involves DNA alterations which are irreversible and can persist in otherwise normal tissue indefinitely until the occurrence of a second type of stimulation or hit (now referred to as ‗promotion‘). Promotion can result from exposure of initiated cells to chemical irritants, factors released at wound sites, partial organ resection, hormones or chronic inflammation (Coussens & Werb 2002). Clinical and epidemiological studies have suggested a strong association between chronic infection, inflammation, and cancer (Coussens & Werb 2002;Fox & Wang 2007;Hussain, Hofseth, & Harris 2003;Lin & Karin 2007;Shacter & Weitzman 2002). For example, there are strong associations between alcohol abuse, which leads to inflammation of the liver and pancreas, and cancers of these organs (Go, Gukovskaya, & Pandol 2005;McKillop & Schrum 2009). Cigarette smoking, asbestos exposure, and silica exposure are each associated with inflammation of the lung and subsequently lung cancer (Dostert et al. 2008;Mossman & Churg 1998;Peto, Darby, Deo, Silcocks, Whitley, & Doll 2000); inflammatory bowel disease is associated with colon cancer (Yang, Taboada, & Liao 2009); infection with *Helicobacter pylori* is associated with gastric carcinoma (Hatakeyama 2009); chronic viral hepatitis is associated with liver cancer (Lee 1997); infection with *Schistosoma* is associated with bladder and colon carcinoma (Cheever 1978); infection with some strains of Human Papilloma Virus is associated with cervical cancer (Monk & Tewari 2007); and infection with Epstein Barr Virus is associated with Burkitt‘s lymphoma and nasopharyngeal carcinoma (Deyrup 2008).

Human papillary thyroid carcinoma can be used as an example to demonstrate inflammatory processes that are involved in cancer (Mantovani et

al. 2008). Rearrangement of the chromosome on which the gene encoding the protein tyrosine kinase (RET) is located leads to the development of papillary thyroid carcinoma. This in turn triggers several inflammatory processes including the release of:

- colony-stimulating factors, which promote the survival of leukocytes and their recruitment from the blood to the tissues

- interleukin 1β (IL-1β), one of the main inflammatory cytokines

- cyclooxygenase 2 (COX2), which is frequently expressed by cancerous cells and is involved in the synthesis of prostaglandins

- chemokines that can attract monocytes and dendritic cells (chemokine ligands – CCL2 and CCL20)

- chemokines that promote angiogenesis (such as IL-8; also known as chemokine ligand CXCL8)

- the chemokine receptor CXCR4 which binds to the chemokine ligand CXCL12

- extracellular-matrix-degrading enzymes

- the adhesion molecule lymphocyte selectin (L-selectin).

It is now considered that both innate and adaptive immunity play a role in cancer development and modulation (O'Byrne & Dalgleish 2001), and that most tumours arise within an immune-permissive environment (O'Byrne & Dalgleish 2001). This is associated with upregulation of humoral immune responses, a relative suppression of cell mediated immunity, and the production of growth and cell-survival factors that induce angiogenesis and inhibit apoptosis.

The innate immune system

The innate immune system forms the first line of defense against infections (Mogensen 2009). It is also the oldest known mechanism of defense against microbes present in all multicellular organisms including plants and insects. The innate immune system comprises of mast cells, macrophages, NK cells, neutrophils, eosinophils, basophils and dendritic cells (Delves & Roitt 2000). Their function within the immune system is to identify and eliminate pathogens that might cause infection. They do this in a non-specific way and do not confer long-lasting immunity, unlike the adaptive immune system. The major functions of the innate immune system are (Medzhitov & Janeway 2000):

- To provide an epithelial barrier to microbes
- To recruit mast cells, macrophages, NK cells, neutrophils, eosinophils, basophils and dendritic cells to sites of infection and stimulate inflammation – this is mediated by cytokines such as Interleukin (IL)-1 and Tumour Necrosis Factor-alpha (TNFα)
- To activate the complement cascade
- To identify and remove microbes
- To activate the adaptive immune system by antigen presentation

Epithelial Barriers

The three major barriers in humans are the skin, gastrointestinal tract mucosa and respiratory tract mucosa (Hornef et al. 2002). Each of these barriers has a continuous epithelium to prevent microbial entry. The epithelia produce peptides, including defensins, which have an antibiotic effect against a number

of bacteria and fungi. Lymphocytes are also present in the epithelial barriers which have a cytotoxic effect against microbes.

Components of the Innate Immune System:

a) Mast cells

Mast cells are produced in bone marrow before entering tissues via the circulatory system (Galli 1993) and are predominantly found in connective tissue. They contain granules which are released during degranulation as a result of microenvironmental triggers such as trauma or chemical stimulation, or as a result of IgE binding to high-affinity receptors on the surface of mast cells (Galli 1993). In humans these granules contain histamine, the proteoglycan heparin, and proteases (Galli, Tsai, & Piliponsky 2008). Released histamine acts at H1, H2, H3 and H4 receptors on cells and tissues, and is rapidly metabolised extracellularly (Nguyen et al. 2001). The proteoglycan has two functions: it packages histamine and basic proteins into secretory granules, and it regulates the stability of the protease, tryptase (Bernfield et al. 1999). Neutral proteases, which account for the vast majority of the granule protein, serve as markers of mast cells and of different types of mast cells.

There are two major phenotypes of human mast cell based on protease expression (Irani et al. 1986). The MC_{TC} (mast cells containing tryptase and chymase) phenotype predominates in normal skin and intestinal submucosa, whereas the MC_T (mast cells containing tryptase only) phenotype predominates in normal intestinal mucosa and lung alveolar wall (Caughey 2007). Similar concentrations of each type are found in nasal mucosa.

In serum, elevated levels of tryptase are detected in systemic mast-cell disorders, such as anaphylaxis and mastocytosis (Caughey 2007). Ongoing

mast-cell activation in asthma appears to be a characteristic of this chronic inflammatory disease. Recently, investigations suggest that mast cells can be either pro- or anti-inflammatory. With respect to animal models, mast cells have been demonstrated to play a role in promoting allergy-driven inflammation (Williams & Galli 2000). However, there is also mounting evidence that not all mast cell-initiated inflammation is unfavorable. In a mouse model of septic peritonitis, release of TNFα and other factors from mast cells on exposure to colonic bacteria appeared to be important in recruiting neutrophils to control the infection (Malaviya et al. 1996).

b) Macrophages

Macrophages also originate as cells in the bone marrow and then circulate in the blood (Galli 1993), mature and subsequently become activated in various body tissues. Their primary function is phagocytosis of foreign material, for example microbes or tumour tissue (MacMicking, Xie, & Nathan 1997). The phenotype and function of the macrophage is influenced by its surrounding tissue type and microenvironment (Crowther et al. 2001).

Macrophages have a role to play in both innate and adaptive immunity. With respect to innate immunity, in normal tissues following pathogenic challenge there is local release of a number of growth factors (Pollard 2004) — colony stimulating factor 1 (CSF-1; also known as macrophage CSF), granulocyte–macrophage CSF (GM-CSF), macrophage-stimulating protein (MSP) and transforming growth factor-β1 (TGF-β1) — and chemokine ligands, which include CCL2,CCL7,CCL8 (monocyte chemoattractant protein family-1-3), CCL3 (macrophage inflammatory protein 1α (MIP-1α)), CCL4 (MIP-1β) and macrophage migration inhibitory factor (MIF). These factors, together with the

products of tissue breakdown, recruit circulating monocytes and stimulate them to differentiate into macrophages. Macrophages, in turn, mediate immune responses, kill pathogens, stimulate angiogenesis and effect tissue repair (Lingen 2001). Macrophages then phagocytose foreign material and produce cytokines, including IL-1, IL-6,   IL-10, IL-12, IL-18 and TNFα (Shibata et al. 1998), that recruit and activate other immune cells leading to an inflammatory response. In adaptive immunity macrophages ingest foreign material and present it to T cells which results in activation of the macrophage causing it to kill the foreign material. IL-1 and TNFα produced by macrophages in response to microbial contact leads to the synthesis of defensins in the epithelial barrier (Nicod 2005).

It is now well recognised that there are two major phenotypes of macrophage (Anderson & Mosser 2002;Gordon 2003;Mantovani et al. 2002;Sica et al. 2006) – M1 and M2 macrophages. The M1 phenotype (classically activated) macrophages are thought to be induced by interferon-gamma (IFNγ), with or without lipopolysaccharide (LPS) and TNFα, and exert a cytotoxic effect against cancer cells. M1 macrophages are associated with the expression of interleukin (IL)-1, IL-12, TNFα and iNOS (Mantovani, Sozzani, Locati, Allavena, & Sica 2002). The M2 macrophage phenotype, also known as the alternatively activated phenotype, comprise of macrophages which play a role in chronic inflammation (Mantovani, Sozzani, Locati, Allavena, & Sica 2002).

c) Natural Killer Cells

NK cells are a sub-type of lymphocytes that provide defense against foreign substances without the need to be activated (Ribas et al. 2003) by other components of the immune system (as opposed to CD8+ T lymphocytes). NK cells attack microbes and viruses following pathogenic contact which activates the NK cells. Class I Major Histocompatibility Complex (MHC) molecules from self cells bind to inhibitory receptors on NK cells to prevent the attack of self cells. When both activating and inhibitory receptors of NK cells are bound, the effects of the inhibitory receptors are dominant.

Despite the fact that NK cells can function independently of other components of the immune system, their numbers are increased following release of IL-15 from macrophages (Salvucci et al. 1996). Macrophage-derived IL-12 also enhances the production of IFN-γ by NK cells which enhances cytotoxicity by recruiting further phagocytic macrophages (Skeen & Ziegler 1995).

d) Neutrophils

Neutrophils are the most abundant type of white blood cell. Their major function is to identify and ingest microbes by phagocytosis (Reeves et al. 2003). Their cytoplasm contains granules filled with enzymes including collagenase, lysozyme and elastase, which can destroy microbes. Mature neutrophils have a short lifespan in circulating blood (approximately 6 hours) and are recruited to sites of infection and inflammation. Unlike macrophages, neutrophils are not capable of becoming further differentiated or undergoing further cell division at the site of inflammation.

e) Dendritic cells

Dendritic cells are leukocytes which act as antigen presenting cells and it has been suggested that they play an important role in activating T cells (Langenkamp et al. 2002). In fact, they have been described as being the most powerful type of antigen presenting cell (APC) due to the fact that they have the highest surface density of MHC molecules as well as being motile and able to release a stimulatory repertoire of cytokines and chemokines (Banchereau & Steinman 1998;Ribas, Butterfield, Glaspy, & Economou 2003).

Identification of Microbes

Neutrophils and macrophages express surface markers which recognise microbes (Medzhitov & Janeway, Jr. 2002). This leads to ingestion and microbial killing (phagocytosis). Macrophage mannose receptors and scavenger receptors recognise microbes but not host cells (Medzhitov & Janeway 2000). Microbes are also coated in various proteins (opsonins) during opsonisation to enhance the process of phagocytosis (Jack, Klein, & Turner 2001). Opsonins include antibodies, complement proteins and lectins. Toll-like receptors also activate phagocytes in response to microbes (Underhill & Gantner 2004). Once activated, macrophages and neutrophils convert oxygen into reactive oxygen intermediates (ROIs) which are oxidising agents capable of killing microbes (Bogdan, Rollinghoff, & Diefenbach 2000). The most common ROI produced by macrophages is nitric oxide as a result of the enzyme inducible nitric oxide synthase (iNOS). iNOS is absent in resting macrophages but in activated macrophages is produced in response to microbial LPS.

The adaptive immune system

The adaptive immune system differs from innate immunity in that it relies on previous exposure to foreign substances to illicit a response (Table 1) (Hilleman 2004). The adaptive immune system comprises of lymphocytes (B and T cells) (Fearon & Locksley 1996) and their products and can itself be further sub-classified into two parts – humoral immunity and cell-mediated immunity (Hilleman 2004). Humoral immunity is mediated by antibodies released by B cells which help promote phagocytosis and the release of inflammatory cytokines. Cell-mediated immunity is mediated by T cells and it targets host cells invaded by foreign substances inaccessible to circulating antibodies. Helper T cells (CD4$^+$) respond to cytokines released by antigen presenting cells, as well as MHC Class II molecules (Ribas, Butterfield, Glaspy, & Economou 2003), and bind to host macrophages phagocytosing microbes/foreign substances and release cytokines themselves (see T helper 1 and T helper 2 responses) which then bind to the macrophage, inducing its activation and destruction of the microbe/foreign substance. Cytotoxic T cells (CD8$^+$) bind to host cells which have become infected with a microbe in response to cytokine release and also to MHC Class I molecules (Ribas, Butterfield, Glaspy, & Economou 2003) and this leads to death of the infected host cell including its microbial contents which are eliminated.

Activity	Innate	Adaptive
Origin	Primitive, ancient	More recent
Recognition	Chemical pattern	Specific epitopes
Development	Rapid, immediate	Slow (1–2 weeks)
Messengers	Cytokines	Cytokines (diverse)
Memory	None	Long, up to lifetime
Presentation	Direct recognition by toll-like and other receptors	Processing, synapse. Dual system: Humoral-antibody Cell-mediated Cytotoxic T helper
System	Very simple, direct recognition and response	Highly complex, recognition and response

Table 1. Innate versus Adaptive Immunity

The principal features of the adaptive immune system are:

- Specificity – distinct antigens elicit specific responses

- Diversity – responds to a large variety of antigens

- Memory – enhanced responses following repeated exposure

- Specialisation – optimal defence against different microbes

- Self-limitation – return to basal state by homeostasis

- Non-reactivity to self – prevents injury to the host

There is also a sub-population of CD4$^+$ helper T cells called regulatory T cells (Treg) (Lyddane et al. 2006). These cells express CD25 (the alpha chain of IL-2). They suppress autoreactivity as, in their presence, other lymphocytes specific for self antigens fail to react to them. The depletion of these cells leads to the development of autoimmune diseases (Ribas, Butterfield, Glaspy, & Economou 2003).

T helper 1 and T helper 2 responses

On the basis of cytokine profiles, two distinct populations of CD4$^+$ T helper cells have been described (Mosmann & Coffman 1989):

- T helper 1 (Th1) – IL-2, IFN-γ, TNFα

- T helper 2 (Th2) – IL-4, 5, 6, 10

Th1 cytokines are thought to be cytotoxic and Th2 cytokines are associated with antibody production and reduced cell-mediated responses (Ribas et al. 2000). It has been shown in a murine model that deviation from a type 1 to a type 2 cytokine profile is associated with decreased protection to tumours (Ribas,

Butterfield, Hu, Dissette, Meng, Koh, Andrews, Lee, Amar, Glaspy, McBride, & Economou 2000).

The immune system in cancer

It has been hypothesised that the human immune system only mounts a weak response to cancer as tumour cells may be recognised as originating from the host themselves (Rosenberg 2001). Conversely, tumours that elicit strong immune responses include those induced by oncogenic viruses in which the viral proteins are foreign antigens (Flanagan 2007). Tumours can also evade immune responses leading to cancer growth (Rabinovich, Gabrilovich, & Sotomayor 2007).

Tumours express antigens that are recognised as foreign by the host's immune system (Zou 2006). Tumour antigens can arise as a result of:

- oncogenic mutations to normal genes

- oncogenic viruses

- oncofetal antigens

- glycolipid and glycoprotein antigens

It is thought that some individuals have T cells which can adapt in response to mutated oncogenes, for example Ras, and thus limit tumour proliferation but in individuals who do not have such responses, cancers develop (Schubbert, Shannon, & Bollag 2007). Tumour antigens can also arise following exposure to oncogenic viruses, for example, Human Papilloma Virus is associated with cervical carcinoma (Monk & Tewari 2007). After viral infection of the host cell, tumour antigens can be expressed on the infected cell surface and in the absence of adequate T cell responses, cancer can develop. Oncofetal antigens

are proteins that are expressed in high levels in normal developing fetal tissue, but not in adults, and noted to be expressed by certain tumours. Examples include alpha-fetoprotein which is expressed in liver tumours and carcinoembryonic antigen (CEA), expressed in gastrointestinal and breast cancers (Lindblom & Liljegren 2000). Most tumours express increased levels or abnormal glycolipids and glycoproteins, such as CA-125 in ovarian cancer (Lindblom & Liljegren 2000).

As well as tumour cells, cancers comprise of a number of immune cell types including, NK cells, T cells and macrophages (Zhang et al. 2003). The key component of the immune response to tumours is thought to be cytotoxic CD8^{+} T lymphocytes (Gao et al. 2007). Increasing evidence suggests that tumour infiltration by T cells results in a more favourable prognosis in ovarian, oesophageal and small cell lung cancers (Eerola, Soini, & Paakko 2000;Schumacher et al. 2001;Zhang, Conejo-Garcia, Katsaros, Gimotty, Massobrio, Regnani, Makrigiannakis, Gray, Schlienger, Liebman, Rubin, & Coukos 2003) implying that the adaptive immune system plays an important role in limiting cancer growth. However, in normal immune responses T cell activation requires the release of cytokines from APC's, thus, the same principal can be proposed in responses to tumours. This implies that tumour cells are attacked firstly by macrophages and other antigen presenting cells, which recognise tumour specific antigens, before helper T cells are involved, and then cytotoxic T cells. There is also evidence that NK cell infiltration of gastric cancers is associated with favorable prognosis (Coca et al. 1997;Ishigami et al. 2000). It is thought that NK cells can effectively destroy tumour cells which do not express MHC Class I molecules as these send inhibitory signals to NK cells when present (Anfossi et al. 2006).

However, it has been suggested that innate immune cells may contribute to cancer progression by promoting angiogenesis and suppressing anti-tumour adaptive immune responses in pancreatic cancer (Esposito et al. 2004). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ) is a transcription factor and coordinator of inflammation and innate immune response. It has been shown to be a tumour promoter (Karin 2006). Part of its action is by activating anti-apoptotic genes (Karin & Lin 2002).

Tumours can also evade immune responses and subsequent destruction, a process known as tumour escape. There are several mechanisms that have been suggested (Ribas, Butterfield, Glaspy, & Economou 2003) as follows:

- down-regulation of class I MHC expression on tumour cells to avoid recognition by cytotoxic T cells
- a loss of expression of antigens by the tumour that elicit an immune response
- a loss of class II MHC expression
- tumour cells release products suppressing responses against the tumour, such as transforming growth factor-β
- tolerance to tumour antigens

It is now recognised that immunotherapy may have an important role to play in the treatment of cancer (Bradbury & Shepherd 2008;Ribas, Butterfield, Glaspy, & Economou 2003). This can be undertaken by stimulating existing immune responses with tumour vaccines, cytokine stimulation or non-specific immune system stimulation, or by adding to the existing immune responses with anti-tumour antibodies (Ribas, Butterfield, Glaspy, & Economou 2003). Tumour

vaccines have been tested in animal models but also clinically (Bradbury & Shepherd 2008) showing potential for the future. Stimulation with the cytokines IL-2, -6, -12, TNFα (Ribas, Butterfield, Glaspy, & Economou 2003) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (Salgia et al. 2003) have demonstrated promising anti-tumour effects but equally devastating systemic events including pulmonary oedema, septic shock and liver dysfunction (Caspi 2008). Anti-tumour antibody therapy has shown the most potential to date, for example Her-2/neu antibodies to treat breast cancer (Piccart-Gebhart et al. 2005).

The immune system and NSCLC

There is increasing evidence that host immunological function may influence the behaviour of LC (de Visser, Eichten, & Coussens 2006;Jassar et al. 2005;Richardson et al. 2003;Welsh et al. 2005). NSCLC's are infiltrated by host lymphocytes (Al Shibli et al. 2008;Petersen et al. 2006) but failure of the host defence mechanism to control tumour growth may involve reduced NK cell activity, inadequate lymphokine-activated killer cell function, or tumour secretion of immunomodulating factors (Pisani 1993).

The key to successful future immunotherapy for patients with NSCLC may lie with targeted treatment according to an individual patient's characteristics. For example, the IDM-2101 vaccine has shown early promise for patients who were HLA-A2 positive (Barve et al. 2008). All patients in that study had stage IIIb or IV NSCLC but following treatment with the IDM-2102 vaccine, one year survival was 60% - much higher than previously described one year survival rates (NICE 2005). The IDM-2101 vaccine has been designed to induce cytotoxic T cell responses, which are restricted by HLA-A2, against

five tumour antigens known to be over expressed in NSCLC – CEA (Slodkowska et al. 1998), p53 (Fijolek et al. 2006), HER-2/neu (Brabender et al. 2001) and melanoma antigens (MAGE) 2 and 3 (Sienel et al. 2004).

Tumour microenvironment

The tumour microenvironment, which includes immune cells, cytokines and chemokines, is a potential area for future targeted chemotherapy (Albini & Sporn 2007). Solid tumours are made up of tumour cells as well as host non-malignant cells, including adipocytes, fibroblasts, macrophages, mast cells and neutrophils. This results in a unique microenvironment for each individual tumour (Pollard 2004). There is evidence that these non-malignant cells can actually contribute towards tumour proliferation and subsequent metastases (Balkwill & Mantovani 2001;Coussens & Werb 2002).

Tumours can be divided into two microanatomically distinct areas – islets and stroma. Fig 1 demonstrates tumour islets and stroma seen after immunohistochemical staining. The islets consist of compact areas of tumour and immune cells, including macrophages, lymphocytes, and endothelial cells. The stroma also consists of these immune cells (Hakansson et al. 1997) as well as fibroblasts (Martin, Pujuguet, & Martin 1996). Eosinophils, granulocytes, NK cells and B cells are also found in the stroma of some tumour types (Coussens & Werb 2002;Pollard 2004). Kataki noted in NSCLC that the predominant cell type in tumour stroma is lymphocytes with macrophages accounting for approximately one-third of stromal cells (Kataki et al. 2002) and the dendritic and NK cells accounting for small numbers.
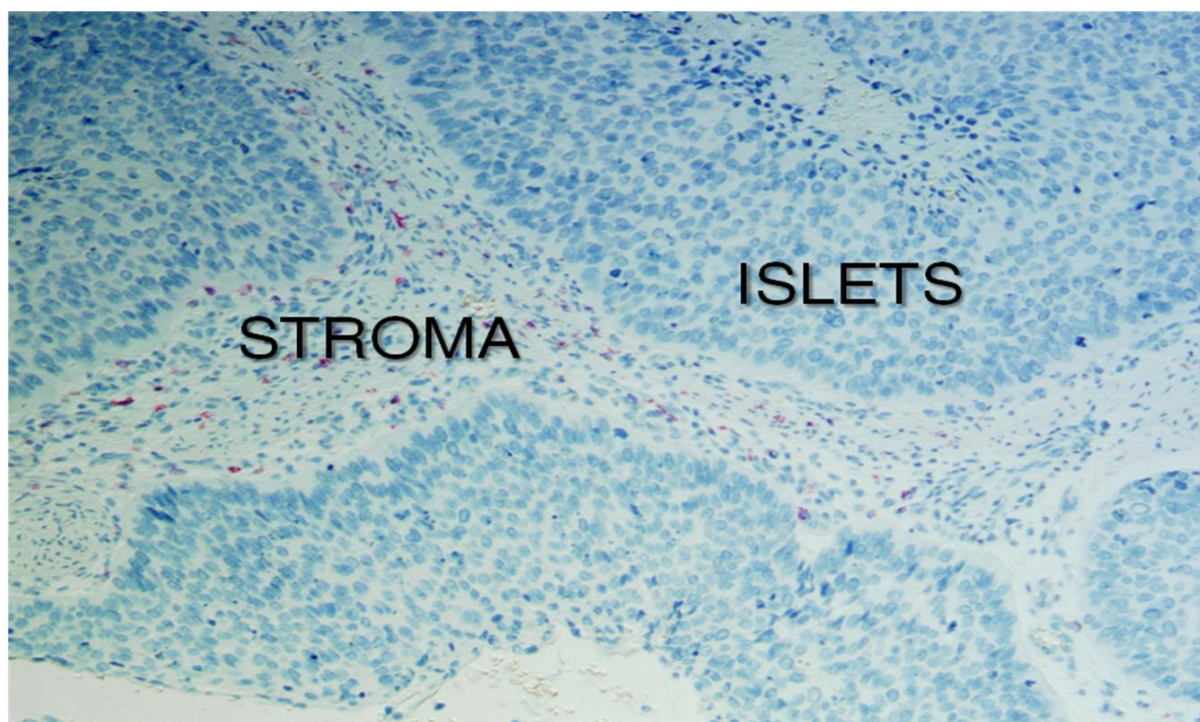
Fig 1. Immunohistology slide showing the distinction between tumour islets and stroma – tryptase positive cells (red)

Magnification x100

The phenotype of immune cells is influenced by microenvironment and one of the key differences between microenvironments is cytokine/chemokine repertoires (Lewis & Pollard 2006;Liotta & Kohn 2001;Park, Bissell, & Barcellos-Hoff 2000). It also thought that physiological factors such as hypoxia in tumour stroma can influence the phenotype of different cell types (Balkwill & Coussens 2004;Bingle, Brown, & Lewis 2002;Li, Fan, & Houghton 2007;Mantovani, Allavena, Sica, & Balkwill 2008;Pollard 2004).

Interestingly, Carraga and colleagues have shown that NK cells exist in NSCLC tissue but predominate in the tumour stroma (Carrega et al. 2008). The authors of that paper postulate that this a result of chemokines released in the tumour stroma which anchor NK cells in the stroma. Others have suggested that extracellular matrix proteins, present in the stroma, might act as a barrier preventing direct NK cell infiltration of the tumour islets (Hagenaars et al. 2000;Kuppen et al. 2001).

It has also been suggested that two types of cells found in high numbers within tumours are macrophages and FoxP3+ regulatory (Treg) cells (Curiel et al. 2004;Liyanage et al. 2002;Mantovani, Allavena, Sica, & Balkwill 2008;Pollard 2004). Treg cells release transforming growth factor-beta (TGF-β) which inhibits the actions of cytotoxic T cells (Prud'homme & Piccirillo 2000). It is also known that tumour cells and macrophages are capable of expressing TGF-β. Interestingly, on removal of Treg cells, cytotoxic T cells can become fully active again (Sutmuller et al. 2001). It has also been shown that NSCLC patients with a higher proportion of intratumoural Treg cells compared to T cells have an increased risk of tumour recurrence (Petersen, Campa, Sperlazza, Conlon, Joshi, Harpole, Jr., & Patz, Jr. 2006). Treg cells also influence differentiation of

macrophages to the M2, alternatively activated, phenotype (Tiemessen et al. 2007).

Abnormal vascularisation and hypoxia are characteristics of neoplastic tissues and have been associated with decreased therapeutic responses, malignant progression, local invasion and distant metastasis (Allavena et al. 2008;Semenza 2003). Hypoxia triggers angiogenesis in tumour cells (Pugh & Ratcliffe 2003). The transcription factor hypoxia-inducible factor-1 (HIF-1) is a major regulator of cell adaptation to hypoxic stress and has been cited as a potential target of anticancer therapies (Semenza 2003). HIF-1 also operates by allowing cancer cells to switch from aerobic to anaerobic metabolism allowing their survival in a hostile environment (Airley & Mobasheri 2007).

Macrophage phenotypes

M1, or classically activated macrophages are thought to be cytotoxic and thus, in cancer, are likely to be of benefit to patients. M2, or alternatively activated macrophages, are thought to relate to chronic inflammation (Mantovani, Sozzani, Locati, Allavena, & Sica 2002), and in cancer be related to the process of angiogenesis, and therefore tumour progression. It has been suggested that polarisation to M1 macrophages is a result of bacterial stimuli or Th1 cytokines (Sica, Schioppa, Mantovani, & Allavena 2006), which are usually not present at the tumour site. In contrast, differentiating macrophages are likely to encounter factors that most frequently polarise them toward M2 type macrophages (e.g. IL-10) (Sica, Schioppa, Mantovani, & Allavena 2006).

Macrophages in cancer

It is recognized that there is a dual role for macrophages in cancer (Kataki, Scheid, Piet, Marie, Martinet, Martinet, & Vignaud 2002;Lewis & Pollard 2006). However, tumour associated macrophages (TAM's) have been viewed as being the predominant phenotype in tumours, seen to be harmful, involved in processes such as angiogenesis (M2 macrophages) and located primarily in tumour stroma (Lewis & Pollard 2006). The link with TAM's and angiogenesis has been noted in breast (Leek et al. 1999;Tsutsui et al. 2005), prostate (Lissbrant et al. 2000), endometrial (Ohno et al. 2004) and renal cancers (Hamada et al. 2002). Conversely, a favorable prognosis has been noted when high numbers of TAM's are noted in gastrointestinal cancers (Funada et al. 2003;Ohno et al. 2003) and melanoma (Piras et al. 2005). It has been suggested that the reason for a pre-dominance of M2 / TAM's in LC may be due to the fact that pathways leading to the selection of M1 phenotypic macrophages with cytotoxic capabilities are suppressed (Kataki, Scheid, Piet, Marie, Martinet, Martinet, & Vignaud 2002).

The role of macrophages in NSCLC has been investigated in several studies which have demonstrated conflicting results (Chen et al. 2003;Johnson et al. 2000;Koukourakis et al. 1998;Takanami, Takeuchi, & Kodaira 1999). These varying results are likely to be a result of differences in the number, grade, stage, and size of tumours included in each study along with considerably different methodologies used to assess macrophage infiltration. Importantly, the microlocalisation of macrophages within tumour compartments appears to influence survival in patients with NSCLC irrespective of tumour stage and histology (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding

2005). Welsh and colleagues assessed macrophage counts in terms of microlocalisation where previous studies had not. Given the likely importance of microenvironment on tumour development (Bingle, Brown, & Lewis 2002) this was an important issue to address. Welsh noted that patients who had above median density of macrophages in their tumour islets had a significantly higher five year survival than patients with below median macrophage islet density (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005), a finding also confirmed by an independent group in a separate cohort (Kim et al. 2008). Welsh also noted that patients with high macrophage stromal counts had poorer survival than patients with low macrophage stromal counts. Kawai investigated a cohort of patients with stage IV NSCLC and also noted improved survival for patients with increased numbers of macrophages, as well as CD8^{+} T cells, in their islets (Kawai et al. 2008). Ohno noted a correlation between tumour islet macrophage count and survival in gastric carcinoma (Ohno et al. 2002).

The role of macrophage phenotypes in tumour progression has been extensively reviewed (Anderson & Mosser 2002;Gordon 2003;Lewis & Pollard 2006;Mantovani, Sozzani, Locati, Allavena, & Sica 2002;Sica, Schioppa, Mantovani, & Allavena 2006). Fig 2 illustrates a precursor macrophage passing down the phenotypic pathway and factors influencing this. The M1 phenotype (classically activated) macrophages are thought to be induced by IFN-γ, with or without LPS and TNFα, and exert a cytotoxic effect against cancer cells. M1 macrophages are associated with the expression of IL-1, IL-12, TNFα and iNOS (Mantovani, Sozzani, Locati, Allavena, & Sica 2002). Monocytes activated by tumour-derived microvesicles from pancreatic, colon and lung cancer cell lines have been found to show increased expression of HLA-DR and a resulting increase in
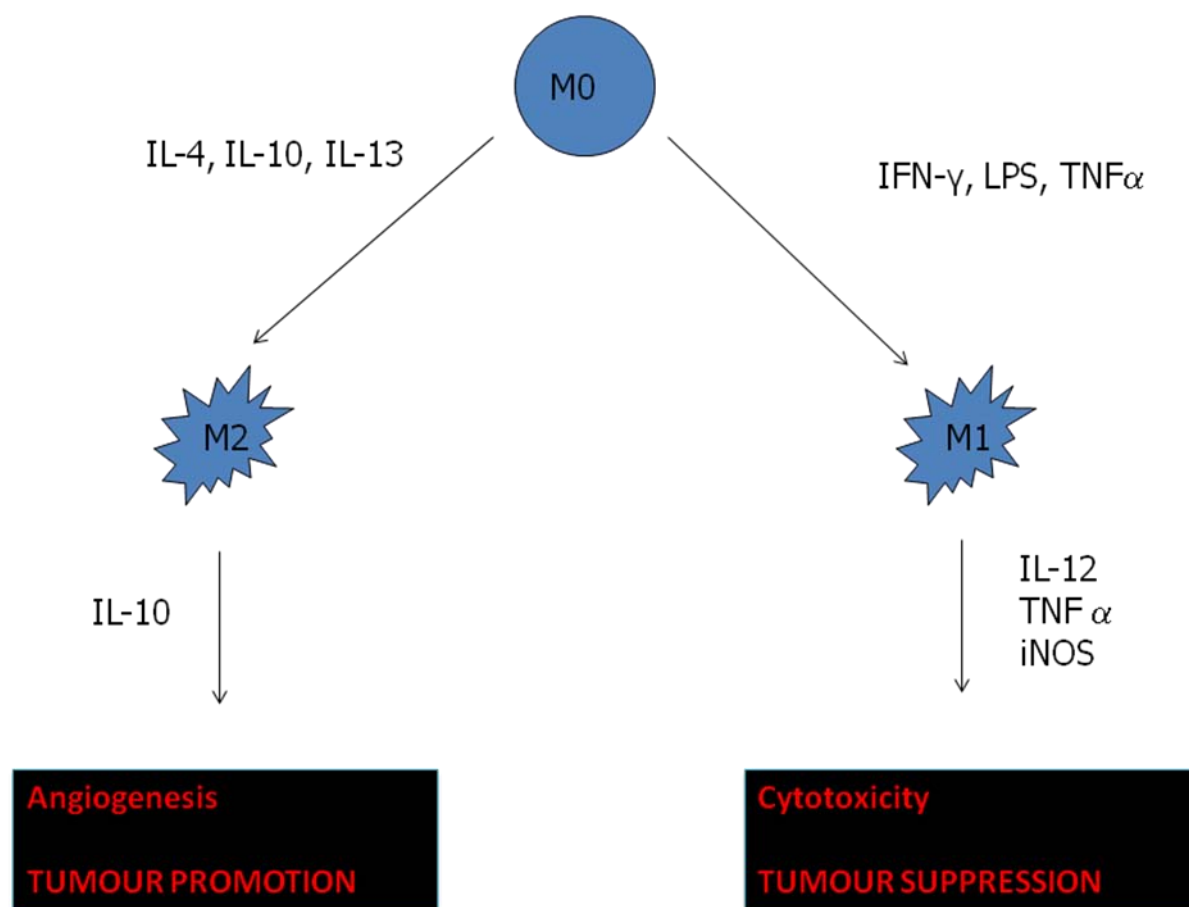
Fig 2. Factors influencing the differentiation of a macrophage precursor (M0) into either an M1 or M2 phenotype

production of reactive oxygen intermediates and TNFα (Baj-Krzyworzeka et al. 2007).

MRP 8/14 expression by macrophages is associated with the release of TNFα (Mahnke, Bhardwaj, & Sorg 1995). The antigen for MRP 8/14 has been found only in inflammatory tissues and shown to be absent from normal resident mononuclear phagocytes (Zwadlo, Schlegel, & Sorg 1986). MRP 8/14 is enhanced by IFN-γ and LPS (Bhardwaj et al. 1992;Zwadlo, Schlegel, & Sorg 1986), a characteristic of M1 macrophages, and also has bacteriostatic properties (Eue et al. 2000). Taken together these features imply that MRP 8/14 is an M1 marker.

M2 (alternatively activated) macrophages are thought to be modulated by IL-4 and -13 and associated with tumour formation. These cells are reported to express the CD163 antigen (Gordon 2003;Mantovani, Sozzani, Locati, Allavena, & Sica 2002). M2 macrophages have also been traditionally thought of as being the predominant macrophage phenotype in solid tumours (tumour associated macrophages). Recent evidence, however, supports a dual role for macrophages in the regulation of tumour proliferation and immune control (Kataki, Scheid, Piet, Marie, Martinet, Martinet, & Vignaud 2002;Lewis & Pollard 2006), and indicates that the local tissue microenvironment plays a critical role in determining cell phenotypes.

CD163 is a member of the scavenger receptor family which has been shown to be expressed by differing organ-specific macrophages, including alveolar and interstitial macrophages in the lung (Van den Heuvel et al. 1999). CD163 may play a role in chronic inflammation (Fabriek, Dijkstra, & van den Berg 2005) but its role in cancer remains unclear. VEGF has been implicated in the process of angiogenesis leading to tumour proliferation. It has also been

stated that one of the key roles of M2 macrophages is to promote angiogenesis (Lewis & Pollard 2006;Mantovani, Sozzani, Locati, Allavena, & Sica 2002). VEGF, a key component in the process of angiogenesis, is known to be produced by TAM's (Balkwill & Mantovani 2001;Barbera-Guillem et al. 2002), which have been traditionally reported as M2 macrophages. A number of cell types, including keratinocytes, macrophages, fibroblasts, and endothelial cells, are known to produce VEGF (Lingen 2001) in response to wound healing. The common factor between wound healing and tumour growth is tissue hypoxia.

There are a number of markers known to be expressed by macrophages which have been assessed previously in relation to prognosis in NSCLC but not in the context of macrophage phenotype or microlocalisation in either the tumour islets or stroma. Markers previously shown to be associated with improved survival in NSCLC include HLA-DR (Foukas et al. 2001), inducible nitric oxide synthase (iNOS) (Puhakka et al. 2003), MRP 8/14 (Endress et al. 1997) and TNFβ but not TNFα (Tran et al. 1998). It has also been suggested that reduced TNFα expression by peripheral blood macrophages in lung cancer results in cancer progression (Lopez-Gonzalez et al. 2007). Expression of the immunosuppressive cytokine IL-10 by tumour macrophages in NSCLC is also associated with worse prognosis (Zeni et al. 2007). CD163 expression has not been studied in NSCLC tissue. However, with respect to colon carcinoma co-culture models, it has been shown that macrophages expressing MRP 8/14 inhibited tumour cell proliferation but CD163 had little effect (Hauptmann et al. 1993).

These markers are also expressed by cells other than macrophages. CD163 has been shown to be expressed by dendritic cells (Maniecki et al. 2006), HLA-DR by natural killer cells (Carrega, Morandi, Costa, Frumento,

Forte, Altavilla, Ratto, Mingari, Moretta, & Ferlazzo 2008), and MRP 8/14 by neutrophils (Robinson & Hogg 2000). TNFα is expressed by lymphocytes (Scheurich et al. 1987), mast cells (Bradding, Walls, & Holgate 2006), endothelial cells (Lamas et al. 1991), adipose tissue (Fantuzzi 2005), fibroblasts (Saika et al. 2006). iNOS (Wink et al. 1998) and VEGF (Ferrara & Kerbel 2005) are also expressed by tumour cells.

Mast cell phenotypes

Human mast cells are heterogeneous, and two common phenotypes are recognised based on their protease content: mast cells which contain tryptase only (MC_T), and mast cells containing both tryptase and chymase (MC_{TC}) (Irani et al. 1986). MC_{TC} predominate in the skin and other connective tissues and are also found in significant numbers in airway submucosal tissues. MC_T predominate in mucosal epithelia, and are also present in submucosal tissues. Their roles remain unclear, but their ability to release different proteases and cytokines (Bradding et al. 1995;Caughey 2007) suggests that some actions are mutually exclusive.

Mast cells in cancer

Mast cells are innate immune cells which arise in the bone marrow, circulate as progenitors, and differentiate following migration into tissue (Galli 1993). Mast cells are found in normal and diseased tissues, and are an important component of immune-cell infiltrates in tumours (Dimitriadou & Koutsilieris 1997). Their role in tumour development and progression remains unclear. In many situations they have been linked with tumour progression and metastasis (Imada et al. 2000;Nagata et al. 2003;Takanami, Takeuchi, & Naruke 2000),

and this is proposed to be mediated through their ability to promote angiogenesis via the release of autacoid mediators and pro-angiogenic chemokines and growth factors (Coussens et al. 1999;Galinsky & Nechushtan 2008;Ribatti et al. 1999). For example, the granular products of mast cells, histamine and cannabinoids released during degranulation, have been demonstrated to enhance the migration of cervical cancer cells in co-culture (Rudolph et al. 2008). Increased histamine expression has also been shown to be associated with colorectal cancer and worsening tumour stage (Masini et al. 2005). Heparin combined to TGFβ can promote neovascularisation (Dimitriadou & Koutsilieris 1997). These studies suggest that degranulating mast cells may be associated with tumour progression. Tataroglu has suggested, however, that there is not necessarily any correlation between intratumoural mast cells and angiogenesis in NSCLC (Tataroglu et al. 2004). Dundar also assessed the presence of mast cells in lung cancer and found no correlation between mast cells and survival in NSCLC (Dundar et al. 2008), but the microlocalisation of mast cells was not assessed in that study. Mast cell phenotype has been investigated in NSCLC before by Ibaraki (Ibaraki et al. 2005), who concluded that MC_{TC} are associated with microvessel count, and thus, angiogenesis.

Interestingly, Welsh has previously shown that there is a survival advantage in NSCLC when mast cells are present in tumour islets as opposed to the tumour stroma (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005).

Tumour Necrosis Factor-alpha

TNFα is a cytokine involved in the promotion of inflammatory responses and subsequently inflammatory diseases (Bazzoni & Beutler 1996). It plays an important role in host defence and protection against cancer development as suggested by the increased incidence of cancer in patients receiving anti-TNFα therapy (Bongartz et al. 2006;Stone et al. 2006). The mechanism for this may be explained by triggering apoptotic cascades as there is some evidence that TNF may directly cause apoptosis in autoimmune disease (Beutler & Bazzoni 1998).

There is conflicting evidence as to whether or not TNF influences cancer progression or conversely prevention (Bongartz, Sutton, Sweeting, Buchan, Matteson, & Montori 2006;Shih et al. 2006;Stone, Holbrook, Marriott, Tibbs, Sejismundo, Min, Specks, Merkel, Spiera, Davis, St Clair, McCune, Ytterberg, Allen, & Hoffman 2006;Szlosarek, Charles, & Balkwill 2006). In a study investigating malignant melanoma, local administration of TNFα had a positive effect in treating metastatic disease (Lejeune, Ruegg, & Liqnard 1998). In contrast, endogenous production of TNFα may act as a tumour promoter contributing to tissue remodelling and stromal development (Balkwill 2002). It has also been shown that TNFα may have multiple roles in cancer, by inducing cell-mediated killing of certain tumours, as well as acting as a tumour promoter (Szlosarek & Balkwill 2003). Szlosarek has suggested that the tumour microenvironment is again a key factor with respect to the actions of TNFα and that it is stromal TNFα specifically which contributes towards chronic inflammatory and angiogenic pathways.

TNFα is produced by several different cell types, including macrophages and mast cells. It has been observed that lung carcinomas decrease the number of monocytes/macrophages (CD14+ cells) that produce TNFα (Lopez-Gonzalez, Avila-Moreno, Prado-Garcia, Aguilar-Cazares, Mandoki, & Meneses-Flores 2007). In other words, reduced TNFα expression by peripheral blood macrophages in lung cancer correlates with cancer progression. The mechanism for this can be explained by the fact that TNFα plays a role in apoptosis (Sidoti-de Fraisse et al. 1998). TNFα has also been evaluated as a potential serum biomarker in NSCLC (Derin et al. 2008) – elevated serum levels were noted in patients with NSCLC compared with normal controls but interestingly, TNFα serum levels rose further following chemotherapy.

TNFα is a key and proximal component of many inflammatory pathways. It plays a key role in host defence to a variety of pathogens (Mastroeni, Villarreal-Ramos, & Hormaeche 1993;Nakane, Minagawa, & Kato 1988), but is also implicated in the promotion of many inflammatory diseases (Bazzoni & Beutler 1996;Locksley, Killeen, & Lenardo 2001), including rheumatoid arthritis and inflammatory bowel disease. Its tumour biology however is complicated, with evidence of both pro-tumorigenic and anti-tumorigenic activity in many animal models (Balkwill 2006;Mocellin et al. 2005). However, perhaps the best evidence that the predominant role played by TNFα is anti-tumorigenic comes from studies of anti-TNFα strategies for the treatment of inflammatory disease in man. These show a significant increase in the rate of neoplastic disease in patients receiving active treatment, and no evidence of protection against cancer development (Bongartz et al. 2006;Furst et al. 2003;Keystone et al. 2004;Lipsky et al. 2000;St Clair et al. 2004;Stone et al. 2006;van de Putte et al.

2004). The complete resolution of NSCLC in a patient following withdrawal of anti-TNFα therapy is also described (Lees et al. 2008). In contrast administration of anti-TNFα has had no significant effect on the progression of several advanced cancers (Brown et al. 2008;Du Bois et al. 1997;Tsimberidou et al. 2003). Furthermore recombinant TNF is a useful and licensed adjunctive treatment for sarcoma and melanoma (Lejeune et al. 2006;Lejeune & Ruegg 2006).

Chemokines and chemokine receptors in cancer

Chemokines are cytokines that have chemotactic properties influencing inflammatory cell migration (Rossi & Zlotnik 2000). Chemokines themselves are released in response to cytokines and pathologic stimuli (Rossi & Zlotnik 2000). The chemokine superfamily consists of over 40 ligands and approximately 20 receptors (Rossi & Zlotnik 2000). They can be classified into four groups – CXC, CC, C and CX3C – according to amino acid position at the N terminal. It is thought that many different chemokines can regulate angiogenesis (Balkwill 2004). These include CXCL1-3 and CXCL5-8. These chemokines act through a common receptor, CXCR2. CXCL12 has been implicated in angiogenesis (Bachelder, Wendt, & Mercurio 2002;Salcedo et al. 1999) and both it and CXCR4 are up-regulated under hypoxic conditions, which is a factor influencing tumour microenvironment. The following chemokines have been demonstrated to exist in cancer tissue: CCL2, CXCL12, CXCL8, CXCL1, CXCL13, CCL5, CCL17, and CCL22 (Mantovani et al. 2004).

Chemokines and chemokine receptors in NSCLC

An important ligand/receptor pair is CXCL12 and CXCR4 which may be involved in metastasis regulation in NSCLC (Phillips et al. 2003). CCL5 expression by tumour cells in patients with stage I lung adenocarcinoma has been associated with improved survival (Moran et al. 2002). The chemokine receptor for CCL5 is CCR1. IL-8 (CXCL8) has two chemokine receptors, CXCR1 and CXCR2 (Zhu et al. 2004). IL-8 is a mediator of angiogenesis in LC (Smith et al. 1994) and correlates with angiogenesis, tumour progression and poor survival in NSCLC (Masuya et al. 2001;Yuan et al. 2000). Zhu found that cell proliferation was significantly reduced by anti-CXCR1 antibody but not by anti-CXCR2 antibody and concluded that the mitogenic function of IL-8 in LC is mediated mainly by CXCR1 receptor (Zhu, Webster, Flower, & Woll 2004). However, it has also been demonstrated in a murine model that depletion of CXCR2 inhibits tumour growth and angiogenesis in lung cancer (Keane et al. 2004).

It has been shown that CXCR3, along with IFN-γ, may play an important role with respect to NK cell infiltration of tumours (Wendel et al. 2008). With respect to CXCR5, its ligand, CXCL13, has been shown to be over expressed in breast cancer (Panse et al. 2008).

Apoptosis and cellular proliferation in cancer

Abnormalities in the control of programmed cell death or apoptosis, play an important role in tumorigenesis (Cross & Dexter 1991;Derin, Soydint, Guney, Tas, Ãamllca, DuranylldIz, Yasasever, & Topuz 2008). Apoptosis is a multistep cascade regulated by proteins that promote or counteract cell death. One of the key regulators of apoptosis is the p53 gene. p53 influences the cell cycle by

inhibiting G1 to S (Fig 3). The induction of apoptosis was found to be a common event for different classes of anticancer agents and it is believed to be one of the main cellular mechanisms by which chemotherapy and radiation therapy kill cancer cells (Kerr, Winterford, & Harmon 1994). Antineoplastic agents induce apoptosis, as DNA damage leads to the inhibition of anti-apoptotic molecules such as survivin, and also increase the production of cytokines such as TNFα (Raff 1998).

Cells undergo structural changes during apoptosis that are mediated by cellular enzymes, in particular caspases (Ulukaya et al. 2007). Caspases cleave a number of substrates including cytoskeletal proteins such as cytokeratin 18 (CK 18). A monoclonal antibody, named M30 antibody, specifically recognises CK 18 exposed after caspase cleavage. The M30 antibody detects only the caspase-cleaved fragment of CK 18 (also called M30 antigen) to enable the detection of apoptotic cells but not necrotic or non-apoptotic cells (Ueno, Toi, & Linder 2005). M30 has been found to be increased in the serum of LC patients 48 hours after they received chemotherapy compared to before treatment (Ulukaya, Yilmaztepe, Akgoz, Linder, & Karadag 2007). With respect to immunohistology in the gastrointestinal tract increased expression of M30 has been demonstrated in normal tissue at the edge of resected colorectal cancer compared with normal tissue not associated with cancer (Koornstra et al. 2004). Similarly, cellular proliferation may influence the progressive growth of a cancer. The process of cellular proliferation is determined by a number of growth factors, including TGFβ and GM-CSF (Cross & Dexter 1991). Ki-67 is a protein and marker of cellular proliferation (Scholzen & Gerdes 2000). It has been shown after cell cycle analysis that Ki-67 is present in the nuclei of
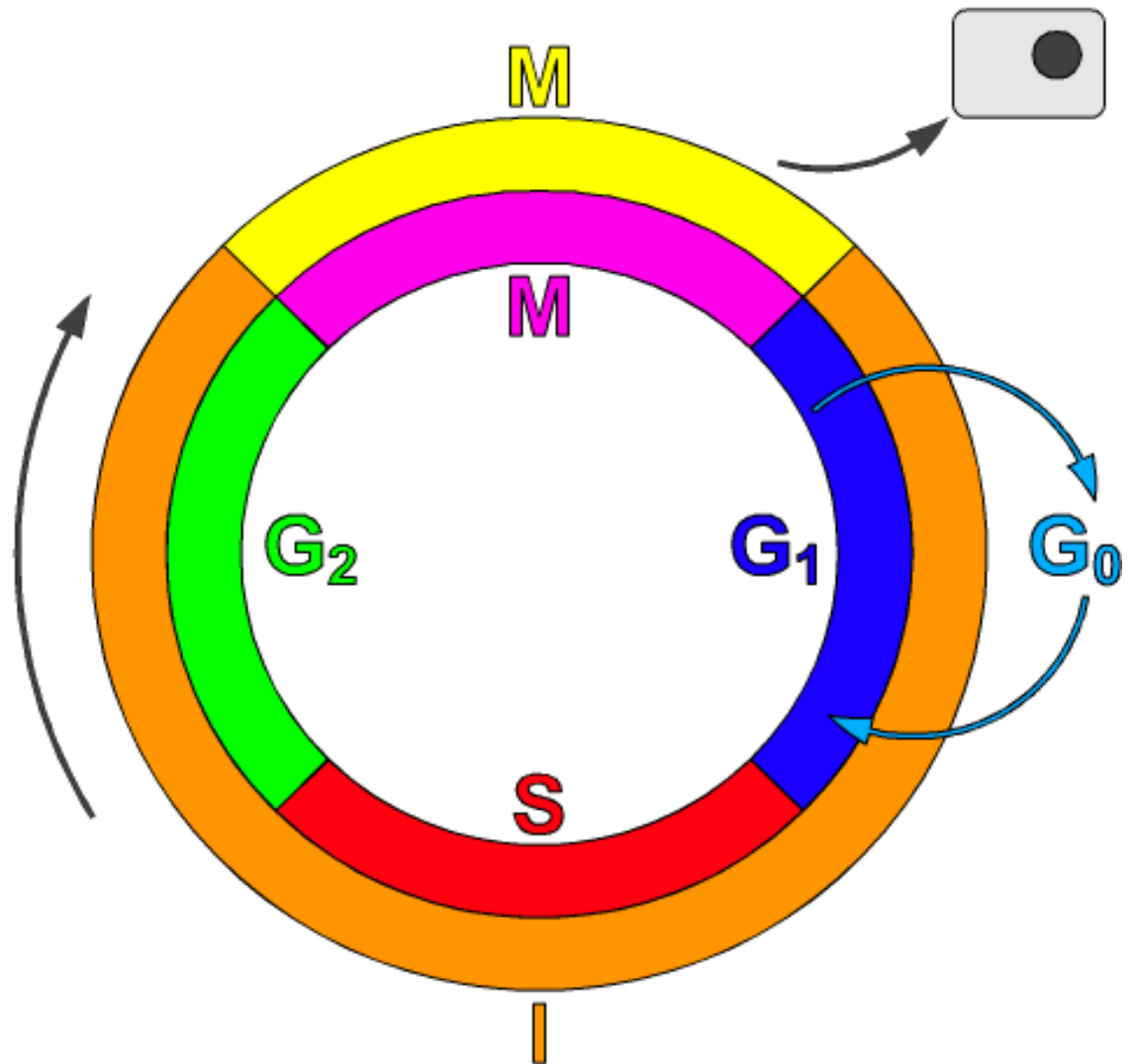
Fig 3. The cell cycle. outer ring: I=Interphase, M=Mitosis; inner ring: M=Mitosis, G_1=Gap 1 (DNA Synthesis), G_2=Gap 2 (microtubule production – required for mitosis), S=Synthesis (chromosomal replication); not in ring: G_0=Gap 0/Resting

cells in the G1, S, and G2 phases of the cell division cycle as well as in mitosis whilst resting cells in the G0 phase do not express it (Gerdes et al. 1984). The role of Ki-67 as a diagnostic marker for NSCLC remains unclear – a meta-analysis of 37 studies concluded that expression of Ki-67 is a poor prognostic factor for survival in NSCLC, however, this was seen in 15 of the studies but not in the other 22 studies (Martin et al. 2004). Interestingly, an association with fluorodeoxyglucose uptake during PET scan and Ki-67 expression has been demonstrated suggesting a possible link between tumour glucose metabolism and cellular proliferation (Vesselle et al. 2008).

<u>Aims and outline of this thesis</u>

It is now well established that the majority of cancers arise within an immune permissive environment. Our group has previously demonstrated specifically that the microlocalisation of macrophages and mast cells within NSCLC tumour tissue correlates with survival, irrespective of tumour stage, histology or other clinical characteristics. Specifically, it was found that when macrophages and mast cells are present in high numbers in the tumour islets, patients have an improved survival. The cytokine TNFα is also interesting as it has been proposed as both pro- and anti-tumorigenic. Therefore, the primary aims of these studies were to investigate in NSCLC the phenotype of macrophages and mast cells, as well as mast cell degranulation, and their association with TNFα, paying particular attention to microlocalisation in either tumour islets or stroma and the total expression and microlocalisation of TNFα irrespective of what cell type was expressing it. Additionally, I aimed to investigate the non-macrophage expression of markers associated with macrophages in NSCLC and explore any relationship between macrophage cell counts and chemokine receptor expression, as well as markers of apoptosis and cellular proliferation, again paying attention to microlocalisation.

To address this I have used immunohistochemistry to identify the presence of macrophages, mast cells, phenotypic markers, TNFα, chemokine receptors and markers of apoptosis and cellular proliferation in sections of paraffin-embedded NSCLC surgically resected tissue. Firstly, I assessed macrophage and mast cell phenotypes in a relatively small number of patients due to the large amount of analysis undertaken. Using a validated method to double-stain sections, macrophage phenotype was identified using CD68 along with CD163, HLA-DR, iNOS, MRP 8/14, TNFα and VEGF. Mast cell phenotypes

were assessed using chymase and tryptase, a degranulation index was used to assess the degree of degranulation by individual mast cells, and mast cell expression of TNFα was also investigated. To evaluate the role of TNFα in NSCLC in more detail, its expression was then assessed in our complete cohort of patients. Non-macrophage expression of markers associated with macrophages was also investigated, as well as the expression of the chemokine receptors CXCR1-5 and CCR1, and finally a marker of apoptosis (M30) and cellular proliferation (Ki-67).

It is expected that the results of these studies will increase the understanding of immune cells in the pathogenesis and modulation of NSCLC with respect to phenotypes, cytokine expression, and also provide some insight into potential chemokine receptor pathways that may potentially be exploited in the future in order to develop novel therapies.

Chapter 2

Materials and Methods

Materials

General materials and equipment

Materials were obtained from the following suppliers:

- Permanent aqueous mounting medium, xylene, industrial methylated spirits and haematoxylin: BDH (Poole, Dorset, UK)

- PAP pen: Biogenex (San Ramon, California, USA)

- Pascal Pressure cooker: Dako Cytomation (Ely, Cambridgeshire, UK)

- Dako EnVision doublestain system kit (K1395): Dako Cytomation (Ely, Cambridgeshire, UK)

- Sodium Chloride, hydrochloric acid: Fischer Scientific (Loughborough, Leicestershire, UK)

- Microscope (Olympus BX50): Olympus (Olympus, London, UK)

- Microtome: Reichert: Jung 2050: (Leica Microsystems, Wetzlar, Germany)

- Scion Image (image analysis program): Based on NIH Image for Macintosh, modified for windows by Scion Corporation, (Scion corporation, Maryland, USA)

- Tris: Roche Diagnostics (Lewes, East Sussex, UK)

- Ammonium hydroxide, trypsin tablets: Sigma (Poole, Dorset, UK)

- Vectabond reagent: Vector Laboratories Inc. (Burlingame, CA, USA)

- Trilogy Antigen Retrieval Solution: Cell Marque (Rocklin, CA, USA)

- All other reagents mentioned were provided with the EnVision doublestain kit (Dako Cytomation, UK) and used according to the manufacturer's guidelines.

Antibodies

Primary antibodies:

- Monoclonal mouse anti-human mast cell tryptase antibodies clone AA1 (IgG1 isotype). Dako Cytomation (Ely, Cambridgeshire, UK)

- Monoclonal mouse anti-human mast cell chymase antibodies clone CCL1 (IgG1 isotype). Abcam (Cambridge, UK)

- Monoclonal mouse anti-human macrophage CD68 clone PGM1 (IgG3 isotype). Dako Cytomation (Ely, Cambridgeshire, UK)

- Mouse antihuman CD163 mAb clone 10D6. Novocastra (Newcastle upon Tyne, UK)

- Mouse antihuman HLA-DR mAb clone TAL.1B5. Hycult biotechnology (the Netherlands)

- Mouse antihuman iNOS mAb clone 2D2-B2. R&D systems (Abingdon, UK)

- Mouse antihuman MRP 8/14 mAb clone 27E10. Bachem Distribution Services (Weil am Rhein, Germany)

- Mouse antihuman TNFα mAb clone P/T2. Abcam (Cambridge, UK)

- Mouse antihuman VEGF mAb clone 14-124. Abcam (Cambridge, UK)

- Mouse antihuman CXCR1 mAb clone 42705. R&D systems (Abingdon, UK)

- Mouse antihuman CXCR2 mAb clone 48311. R&D systems (Abingdon, UK)

- Mouse antihuman CXCR3 mAb clone 49801. R&D systems (Abingdon, UK)

- Mouse antihuman CXCR4 mAb clone 44716. R&D systems (Abingdon, UK)

- Mouse antihuman CXCR5 mAb clone 51505. R&D systems (Abingdon, UK)

- Mouse antihuman CCR1 mAb clone 53504. R&D systems (Abingdon, UK)

- Mouse antihuman Ki67 mAb clone MIB-1. Dako Cytomation (Ely, Cambridgeshire, UK)

- Mouse antihuman M30 CytoDeath mAb clone M30 (Roche Diagnostics Ltd, West Sussex, UK)

Isotype control antibodies:

- Monoclonal mouse anti-*aspergillus niger* glucose oxidase (IgG1 isotype). Dako Cytomation (Ely, Cambridgeshire, UK)

- Monoclonal mouse anti-*aspergillus niger* glucose oxidase (IgG2A isotype). Dako Cytomation (Ely, Cambridgeshire, UK)

- Monoclonal mouse anti-*aspergillus niger* glucose oxidase (IgG2B isotype). Dako Cytomation (Ely, Cambridgeshire, UK)

- Monoclonal mouse anti-fructosan (IgG3 isotype). BD Biosciences (Oxford, UK)

Buffers

Tris buffered saline (TBS)	final concentration
242.14g of Tris	50mM
350.64g of Sodium Chloride	150mM

These were dissolved in 1600mls of distilled water. The pH was adjusted to pH 7.65 with concentrated hydrochloric acid and made up to 2000ml with distilled water. 40g bovine serum albumin was then dissolved in the solution which was divided into two 1000ml containers. For use the solution was diluted (500ml to 10L).

Mayer's Haematoxylin Solution	final concentration
2g Haematoxylin	3.31mM
100g Potassium alum	0.11M
100g Chloral hydrate	0.3M

This was warmed and dissolved in 2000ml of distilled water. Once dissolved, the following were added:

2g Citric acid monohydrate	4.67mM
400mg Sodium iodate	1.01mM

Methods

Doublestain immunohistochemistry

Introduction

Appropriate primary antibodies were used for the target cells in question. All stains have previously been shown to be specific for their respective target cells.

Vectabond-treatment

Glass slides were placed in plastic slide racks. The slides were then washed thoroughly in 2% decontaminant detergent for 30 minutes, and then rinsed in water. They were then dried overnight at 58°C. 7ml of vectabond reagent were added to 350ml of acetone, in a fume cupboard, and mixed well in a conical flask. The slides were then immersed in acetone for 5 minutes in a glass staining dish. The slides were then removed, tapped several times to drain excess acetone, and then placed immediately in the vectabond reagent solution for 5 minutes in a glass staining dish. The slides were then removed and drained and excess reagent was eliminated by gently dipping them several times over 30 seconds in deionised water, taking care to ensure that no bubbles were created. The water was changed every 5 racks. The slides were then removed and gently agitated to decrease water droplets. The slides were then dried overnight at 37°C. Once dry they were stored until needed.

Microtomy

Formalin-fixed, paraffin embedded tissue specimens were taken and placed in crushed ice for 30 minutes. The specimen was then removed, and sections, 4µm thick, were cut using a microtome (Reichert: Jung, 2050). The sections were then floated out on a waterbath at 40°C and were then removed on vectabond-treated slides. The sections were then dried thoroughly overnight at 37°C.

Section preparation, deparaffinisation and rehydration

Tissue sections were incubated at 58°C for 15 minutes to allow full adhesion of the sections to the vectabond treated slides. The sections were then deparaffinised in xylene (2x two minutes), rehydrated through graded alcohols (ethanol for 2 minutes, 99% industrial methylated spirits (IMS) for 2 minutes, 95% IMS for 2 minutes) and then taken to distilled water. The sections were then removed and placed in a humidified chamber (all incubations were carried out in a humidified chamber). Surface tension between the reagents and the surrounding glass was maintained by encircling the section with a water repellent pen.

Antigen retrieval

10mls of Trilogy solution (concentrate) were added to 190mls of deionised water in a slide rack container. Each section was placed in the solution in a slide rack and a lid placed on the container. The container was placed in a Pascal pressure cooker (Dako Cytomation, Ely, Cambridgeshire, UK) with 900mls deionised water and another container containing 10mls Trilogy solution and 190mls deionised water but no slides. The pressure cooker was sealed and

heated to 117.5^{o}C for 60 seconds, and then cooled to 100°C for 10 seconds. The container with the slide rack in it was carefully removed and the sections were transferred to the second container with Trilogy solution and deionised water. This was left to wash for 5 minutes. The slide rack was then placed in a TBS buffer wash for 5 minutes. All washings were carried out on a rotating platform.

Blocking endogenous peroxidase

The sections were removed from the TBS and excess buffer was tapped off. Sections were then covered with 100µl of peroxidase block (0.03% hydrogen peroxide containing sodium azide 15mM) to quench endogenous peroxidase activity and incubated for 5 minutes. Sections were then directly rinsed in TBS followed by total immersion in a TBS buffer bath for 5 minutes.

Application of Primary antibody

Excess buffer was tapped off and the appropriate primary antibody was added to each section. This was incubated for 60 minutes. Sections were then directly rinsed in TBS followed by total immersion in a TBS buffer bath for 5 minutes.

Application of Secondary antibody

Excess buffer was tapped off and 100µl of horseradish peroxidase labelled polymer (conjugated to affinity purified goat anti-rabbit/anti-mouse Ig in Tris-HCL buffer containing carrier protein and anti-microbial agent) was added to each section and incubated for 30 minutes. Sections were then directly rinsed in TBS followed by total immersion in a TBS buffer bath for 5 minutes.

Visualisation of the Primary antibody

While the sections were immersed in the water bath, 1 drop of liquid 3,3'-Diaminobenzidine tetrahydrochloride (DAB) + chromagen was added to 1ml of buffered substrate (imidazole-HCL buffer, pH 7.5, containing hydrogen peroxide and anti-microbial agent) and was mixed well. Excess buffer was tapped off and the DAB-chromagen solution was added to each section and incubated for 10 minutes. Sections were then rinsed with distilled water. DAB gives a brown reaction product.

Application of 'Doublestain block'

Excess water was tapped off and 100µl of ‗doublestain block' (blocks endogenous alkaline phosphatase activity and prevents cross-reaction with previous primary antibody) was added to each section and incubated for 3 minutes. Sections were then directly rinsed in TBS followed by total immersion in a TBS buffer bath for 5 minutes.

Application of Primary antibody

Excess buffer was tapped off and the appropriate primary antibody was added to each section. This was incubated for 60 minutes and sections were then directly rinsed in TBS followed by total immersion in a TBS buffer bath for 5 minutes.

Application of Secondary antibody

Excess buffer was tapped off and 100µl of alkaline phosphatase labelled polymer (conjugated to affinity purified goat anti-rabbit/anti-mouse Ig in phosphate buffered saline containing carrier protein and 15mM sodium azide)

was added to each section and incubated for 30 minutes. Sections were then directly rinsed in TBS followed by total immersion in a TBS buffer bath for 5 minutes.

Visualisation of Primary antibody

While the sections were immersed in the buffer bath 3mg of Fast Red was dissolved in 3ml of buffered substrate (naphthol solution in Tris buffer). Excess buffer was tapped off and slide sections were covered with the fast red substrate chromagen and incubated for 10 minutes. Slides were then rinsed in distilled water. Fast red gives a red reaction product.

Counterstaining

Sections were then immersed in haematoxylin solution (3.3×10^{-3}M haematoxylin, 0.1M potassium alum, 0.3M chloral hydrate, 4.76×10^{-3}M citric acid monohydrate and 1×10^{-3} sodium iodate) for 2 minutes and then rinsed in distilled water. The sections were then dipped 10 times into a bath of 37mM ammonium hydroxide before a final rinse in distilled water. The sections were mounted and coverslipped using an aqueous-based mounting medium, as the end product formed by fast red is soluble in organic compounds. Sections were stored in the dark at room temperature.

Controls

Appropriate isotype controls were performed where the primary antibodies were replaced by irrelevant mouse monoclonals of the same isotype and at the same concentration as the specific primary antibodies.

Study population

NSCLC tissue was obtained from all available, routinely formalin-fixed, paraffin-embedded archival tissue (Leicester Lung Cancer Tissue Bank, University Hospitals of Leicester NHS Trust, UK) taken during surgical lung resections, between 1991 and 1994 and during 1999. This cohort has been described before (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005).

Full clinico-pathological information has been gathered before and following surgery to include: patient characteristics, treatment, combined clinical and surgical staging results, histological subtype, tumour grade and outcome data including peri-operative mortality and long term survival. Previous ethical approval had been given by the Leicestershire Ethics Committee.

Staining analysis and validation

Assessment of all staining was undertaken without knowledge of survival status of individual cases. Findings were correlated after all analyses had been undertaken. The ten most representative high power fields (x400) per slide were manually selected after reviewing the slide at x100 using an Olympus BX50 microscope (all subsequent counting and measuring was carried out at x400). The respective areas of stroma and tumour islets were then measured using Scion image analysis software (Based on NIH Image for Macintosh, modified for windows by Scion Corporation, (Scion corporation, Maryland, USA)). Each measurement was repeated three times to determine the repeatability of the measurements. The number of nucleated cells in each area was then manually counted and recorded. Cells single-staining for each marker and double-staining cells were recorded. The sections from ten patients were then re-examined and the two sets of data were compared to assess the repeatability

and hence the validity. In the repeat, the areas were measured only once. Having shown that the results obtained were repeatable, the rest of the slides were examined.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism v. 4.02 (GraphPad Prism Software Inc, San Diego, California, USA). To test the repeatability of measurements the intraclass correlation coefficient was calculated. For categorical analysis the median value was used as a cut point to dichotomise the series. The Chi-squared (χ^2) test was used to test for relationships between categorical variables and the Mann-Whitney non-parametric test was used to compare categorical with continuous variables. The Kruskal-Wallis one-way analysis of variance test was used to compare multiple groups. Spearman's test was used to test correlations. Kaplan-Meier survival curves were used to look for correlation with survival and were compared with the use of the log-rank statistic. A multivariate Cox proportional hazards model was used to estimate adjusted hazard ratios, 95% confidence intervals and to identify independent prognostic factors (SPSS version 13). A formal test to detect departure from the proportional hazards assumption was carried out by comparing hazard ratios in different intervals of time prior to carrying out Cox regression. The validity of the proportional hazards assumption was assessed from log(-log [Survival]) curves. A p-value of 0.05 was considered significant for all of the above tests.

Chapter 3

Experimental optimisation

There were several key steps in the methodology which required optimisation prior to processing patient samples as follows:

- Slide sample temperature after microtomy

- Deparaffinisation

- Antigen Retrieval

- Primary antibody concentration

<u>Slide sample temperature after microtomy</u>

After the paraffin embedded samples were cut, the slides were stored overnight in an incubator – a number of different temperatures were evaluated but the optimum temperature was 37°C. At higher temperatures tissue damage was noted and at lower temperatures poor adherence of tissue to the slides occurred.

<u>Deparaffinisation</u>

Two methods of deparaffinisation were assessed. Firstly, the slides were washed in Trilogy antigen retrieval solution as the manufacturer's instructions stated that it can remove paraffin and promote antigen retrieval in one step. However, using this method deparaffinisation was sub-optimal. The second method using 2x two minute xylene baths provided much better removal of paraffin from the slides.

<u>Antigen Retrieval</u>

Three methods of antigen retrieval were assessed – proteolytic digestion with trypsin, treatment with a citric acid solution, and Trilogy antigen retrieval solution. For proteolytic digestion, 1mg trypsin tablets were dissolved in 1ml

distilled water and 100µl of the solution was applied to the slide tissue sections for 6 minutes. With respect to the citric acid method, slides were placed in a container containing citrate buffer, pH 6, and placed in a microwave for various different times. The third method used Trilogy antigen retrieval solution, added to a container with the slides and placed in a Pascal Pressure Cooker (Dako Cytomation, Ely, Cambridgeshire, UK) at 117.5°C for 60 seconds, and then cooled to 100°C for 10 seconds. The trypsin method resulted in sub-optimal immunohistochemical staining, while the citric acid method was particularly damaging to the tissue sections. Excellent results, however, were noted using the Trilogy antigen retrieval solution method, with clean immunohistochemical staining and minimal tissue damage.

Primary Antibody Concentration

In order to optimise experiments, each antibody was tested using a number of different dilutions as well as different volumes of antibody solution and duration of application to sample tissue.

Chapter 4

Macrophage phenotypes in

non-small cell lung cancer

Our group has shown previously that the anatomical microlocalisation of macrophages in NSCLC strongly predicts patient survival regardless of cancer stage (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). Increasing numbers of macrophages within the tumour islets conferred a marked survival advantage, while increased numbers of macrophages in the tumour stroma were associated with worse prognosis. The association between prognosis and islet macrophage count has been confirmed recently by others in an independent cohort of patients (Kim et al. 2008), but the stromal macrophage counts in this study were not associated with survival.

Because macrophage infiltration of tumour islets in NSCLC confers a marked survival advantage, we hypothesised that macrophages expressing the cytotoxic M1 markers HLA-DR, iNOS, MRP 8/14 and TNFα predominate in the tumour islets of patients with extended survival (ES). We have tested these hypotheses in surgically resected NSCLC specimens obtained from a group of patients with extended survival versus a group with poor survival (PS). We also investigated the microlocalisation of macrophages expressing the M2 markers CD163 and VEGF.

Patients & Methods

Study Population

The tissue specimens evaluated were from 40 patients selected for this study based on their survival, without knowledge of their previous tumour macrophage counts. 20 patients had ES (mean ± SEM 92.7 ± 7.2 months), and 20 patients had PS (7.7 ± 0.7 months). Patient characteristics are summarised in Table 1.

Characteristic	Extended Survival	Poor Survival
No. of patients	20	20
Age – years	69.1±1.8	69.6±1.6
Male sex – no. (%)	16 (80)	11 (55)
Year of surgery – no. (%)		
1991	0 (0)	1 (5)
1992	3 (15)	1 (5)
1993	2 (10)	1 (5)
1994	1 (5)	1 (5)
1999	14 (70)	16 (80)
Tumour stage – no. (%)		
1	13 (65)	13 (65)
2	5 (25)	3 (15)
3a	2 (10)	3 (15)
3b and 4	0 (0)	1 (5)
Histology – no. (%)		
Squamous	14 (70)	10 (50)
Adenocarcinoma	4 (20)	5 (25)
Large cell	0 (0)	3 (15)
Other	2 (10)	2 (10)
Tumour Grade – no. (%)		
Well	2 (10)	0 (0)
Moderate	8 (40)	1 (5)
Poor	11 (55)	18 (90)
Not recorded	1 (5)	1 (5)
Adjuvant Chemotherapy (%)	0 (0)	0 (0)
Radiotherapy (%)	3 (15)	1 (5)
Palliative Radiotherapy (%)	2 (10)	1 (5)
Survival – months	92.7±7.2	7.7±0.7

Plus-minus values are means ± SEM

Table 1. Patient Characteristics

Immunohistology

Mouse antihuman macrophage CD68 mAb (clone PGM1; Dako Cytomation, Ely, Cambridgeshire, United Kingdom) was used as a specific marker for macrophages. Antibodies for phenotypic analysis were all mouse antihuman mAb as follows: i) CD163 mAb (clone 10D6, Novocastra, United Kingdom), ii) HLA-DR mAb (clone TAL.1B5; Hycult biotechnology, the Netherlands), iii) iNOS mAb (clone 2D2-B2; R&D systems, Abingdon, United Kingdom), iv) MRP 8/14 mAb (clone 27E10; Bachem Distribution Services, Germany), v) TNFα mAb (clone P/T2; Abcam, Cambridge, United Kingdom) and vi) VEGF mAb (clone 14-124; Abcam, Cambridge, United Kingdom). Immunostaining for CD68 and each individual phenotype marker was performed using the Envision double-stain kit (Dako Cytomation) according to the manufacturer's instructions and as described previously (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). Thus, six slides were prepared for each patient – CD68 versus CD163, CD68 versus HLA-DR, CD68 versus iNOS, CD68 versus MRP 8/14, CD68 versus TNFα, and CD68 versus VEGF. CD68 was developed with peroxidase and DAB (brown reaction product), and each phenotype marker with alkaline phosphatase and fast red (red reaction product). Sections were then counterstained with haematoxylin and mounted in an aqueous mounting medium (BDH Chemicals Ltd, Poole, United Kingdom). Appropriate isotype controls were performed where the primary antibodies were replaced by irrelevant mouse mAb of the same isotype and at the same concentration as the specific primary mAb.

Results

Patient Characteristics

Of the 40 patients studied, 32 had died at the time of analysis. Twenty-four tumours were squamous, 9 adenocarcinoma, 3 large cell, and 4 other. Twenty-six were stage I, 8 stage II, 5 stage IIIa, and 1 stage IV. No patients had additional chemotherapy and 4 had additional radiotherapy, 3 of whom had it for later palliation. The patient characteristics are summarized in Table 1.

Validation of Analysis

Initially, sections from five patients were stained for CD163 (M2) versus each of the M1 markers (HLA-DR (Fig 1A), iNOS, MRP 8/14, and TNFα) to assess whether these markers actually detect different cellular subsets *in vivo*. It was found that the majority of cells stained for one or other phenotype with few cells double-staining (median 6.45% [range, 2.5 to 10.2] double-stained for CD163 + HLA-DR, median 0% [range, 0 to 13.7] for CD163 + iNOS, median 0% [range, 0 to 2.9] for CD163 + MRP 8/14 and median 0% [range, 0 to 5.6%] for CD163 + TNFα).

Clear and distinguishable staining was evident for both CD68 and each phenotype marker and double-stained cells were readily identifiable (Fig 1B-F). Appropriate isotype controls were negative. Cell counts were repeated and an intraclass correlation coefficient was calculated as 0.998 (p<0.001). This method of analysis has also been validated by our group previously (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005).
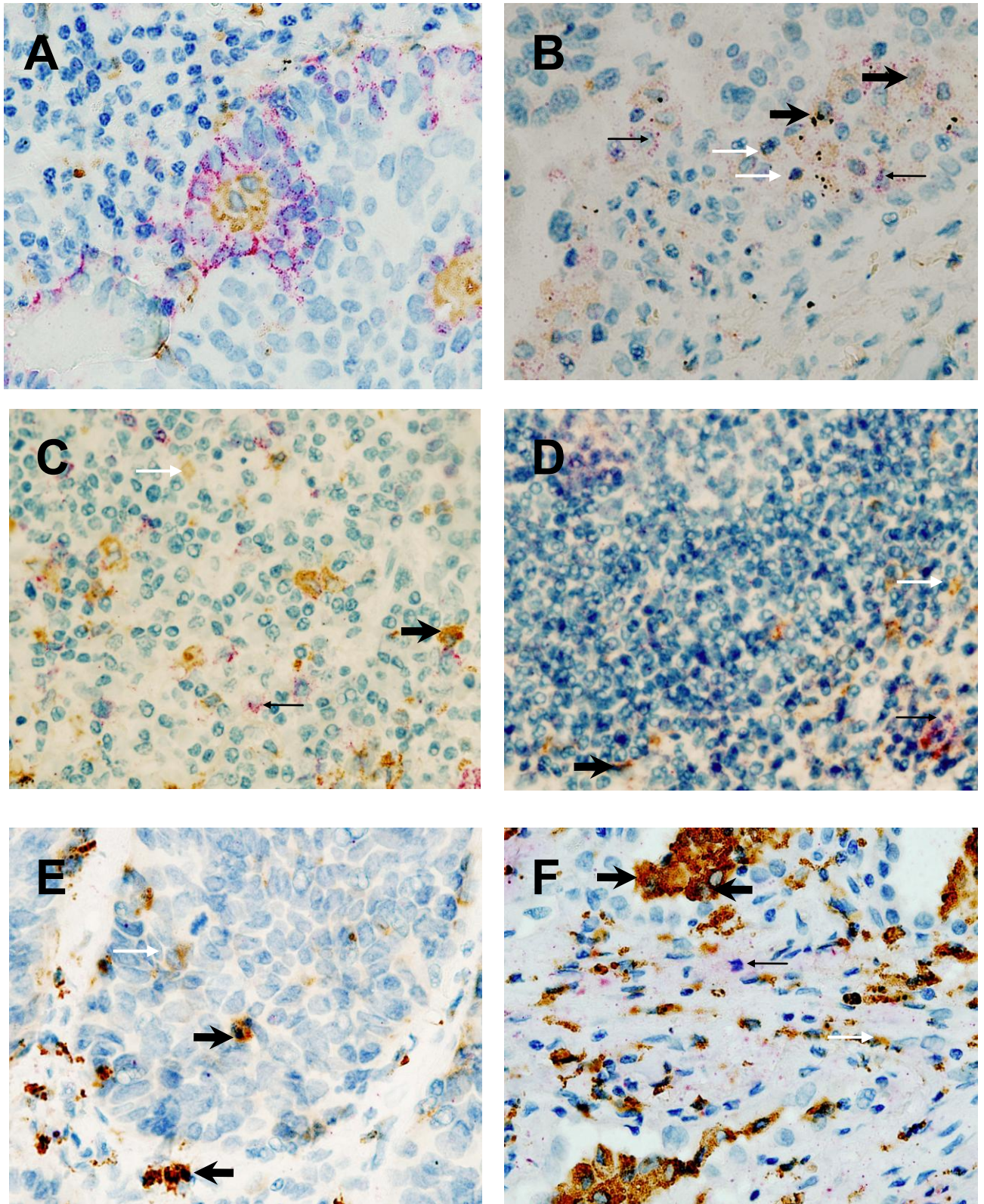
Fig 1. Immunohistology of macrophage phenotypes in NSCLC. A) Distinct cellular staining is evident between the M1 marker HLA-DR (red) and the M2 marker CD163 (brown). In (B)-(F), CD68+ cells are stained brown and (B) CD163 (red), (C) HLA-DR (red), (D) iNOS (red), (E) MRP 8/14 (red) and (F) TNFα (red). Singlestain CD68 brown (white arrowhead), Singlestain phenotypic marker red (small arrowhead), Double-stain cells contain both brown and red (large arrowhead). Magnification x400

Cellular Distribution

CD68^{+} macrophages were detected in both the stroma and tumour islets in all tumours. There were significantly more CD68^{+} cells in the tumour islets of the extended survival (ES) group (median, 33.95 cells/mm^2 [range, 14.84 to 100.5]) compared to the poor survival (PS) group (median, 4.02 [range, 0.90 to 38.03], p<0.001). In contrast, in the stroma there was no significant difference between the CD68^{+} macrophage densities in the ES group (median, 23.6 cells/mm^2 [range, 8.2 to 83.7]) and the PS group (median, 35.7 cells/mm^2 [range, 3.3 to 128.1], p=0.22). There were significantly more double-staining CD68^{+} macrophages for all six phenotypic markers in the tumour islets of ES patients compared with PS patients (Fig 2A and B)(median, 11.6 cells/mm^2 versus 3.4 for CD163 [p=0.001], 13.2 versus 3.9 for HLA-DR [p<0.001], 21.6 versus 1.8 for iNOS [p<0.001], 22.5 versus 2.9 for MRP 8/14 [p<0.001], 16.9 versus 3.7 for TNFα [p<0.001], and 13.8 versus 2.8 for VEGF [p=0.002], respectively). No differences were evident in the tumour stroma (Fig. 2C and D).

With respect to the tumour islets in the ES group, there were fewer macrophages double-staining for CD163 compared to those double-staining with HLA-DR (p=0.10), iNOS (p=0.02), MRP 8/14 (p=0.02) and TNFα (p=0.03). There were also fewer macrophages double-staining for VEGF compared to those double-staining with HLA-DR (p=0.01), iNOS (p=0.002), MRP 8/14 (p=0.004) and TNFα (p=0.003). Overall, there was a difference between all six double-staining phenotypes (p=0.002) (Fig 2A). Table 2 shows the percentage of macrophages staining for each marker and vice versa, and demonstrates that approximately 70% of macrophages in the islets of the ES group were positive for M1 markers compared to 43% that
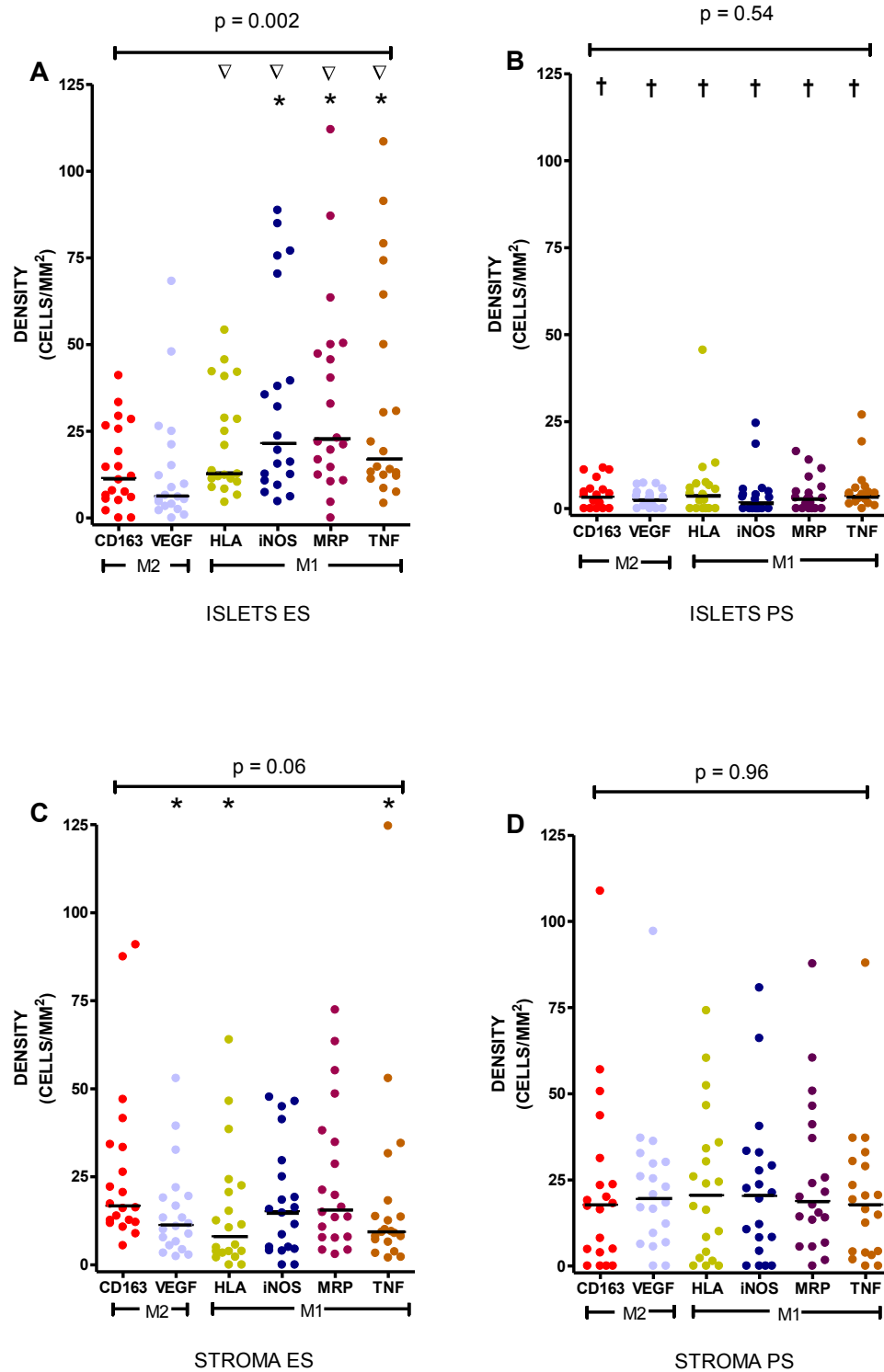
Fig 2. Macrophage double-stain densities in the islets in (A) extended survival (ES) and (B) poor survival (PS), and in the stroma in (C) ES, and (D) PS. * represents a significant difference (p<0.05) in expression compared with CD163. ∇ represents a significant difference (p<0.05) in expression compared with VEGF. ɬ represents a significant difference (p<0.05) between the corresponding macrophage marker in the ES group.

were positive for the CD163 M2 marker (p=0.04) and 33% for the VEGF M2 marker (p<0.001). No significant differences were noted between the double-staining phenotypes in the PS group in the tumour islets (Fig 2B). In the stroma of the ES group, there were significantly more macrophages double-staining for CD163 compared to those double-staining for HLA-DR (p=0.01) and TNFα (p=0.02), but not iNOS or MRP 8/14 (Fig 2C). No significant differences were noted between the double-staining phenotypes in the PS group in the tumour stroma (Fig 2D).

The density ratio of each macrophage phenotype in the islets compared to the stroma was also calculated (Fig 3). This demonstrates a marked tissue compartment-specific difference between the expression of M2 (CD163 and VEGF) macrophages compared to M1 macrophages (HLA-DR, iNOS, MRP 8/14 and TNFα) in the patients with extended survival (p < 0.001). No significant difference in ratio was noted between any of the six macrophage phenotypes in patients with PS. These patients had significantly more macrophages in their stroma compared with the islets.

Correlation between cell counts for the M1 and M2 macrophages

To address the issue of correlations between cell counts for each macrophage marker, intraclass correlations were calculated. The intraclass correlation for M1 macrophages (expressing HLA-DR, iNOS, MRP 8/14 and TNFα) was assessed in the islets (0.76, p<0.001) and in the stroma (0.72, p<0.001). This was also assessed for M2 macrophages (expressing CD163 and VEGF) in the islets (0.74, p<0.001) and in the stroma (0.78, p<0.001).
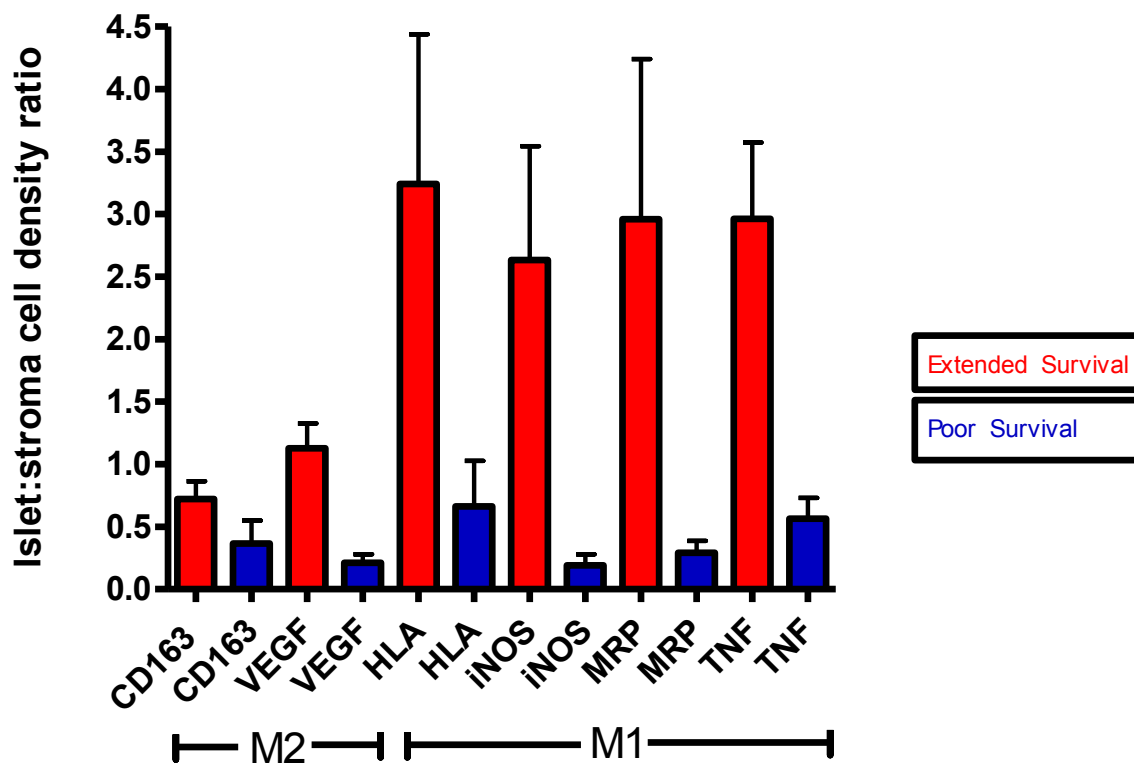
Fig 3. Islet:stroma macrophage double-stain cell density ratios.

Kaplan-Meier Survival Analysis

For further analysis, the data were divided into two groups above and below the median cell count values. Kaplan-Meier survival curves were plotted to investigate further the association of cell densities with survival. The log rank statistic was used to compare survival rates. There was a positive association between survival and tumour islet CD68/CD163 (p=0.001) and CD68/VEGF (p=0.007) macrophage density, but more marked positive associations between survival and tumor islet macrophages double-staining with the putative M1 markers - p<0.001 for HLA-DR, iNOS, MRP8/14 and TNFα (Fig 4A-F). After dichotomisation at the median cell density for macrophages expressing each marker, 5-year survival was 63.4% above the median compared with 19.0% below the median for CD163, 75.3% versus 4.3% for HLA-DR, 75.3% versus 4.3% for iNOS, 79.6% versus 0% for MRP 8/14, 80% versus 0% for TNFα, and 59.0% versus 19.0% for VEGF, respectively (Figure 4A-F). In contrast there were no survival differences evident using the same analysis on stromal cell counts (Fig 5A-F).

Multivariate Cox Proportional Hazards Analysis

Although the sample size was relatively small for Multivariate Cox Proportional Hazards Analysis, for interest we also explored whether any macrophage phenotypic markers were independently associated with survival. Only those variables that were associated with survival at a significance of p<0.1 were included in the multivariate analysis. When islet CD68 density was assessed in the model, it was again a positive independent predictor of survival as described previously (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005) (hazard ratio 0.956, 95% CI
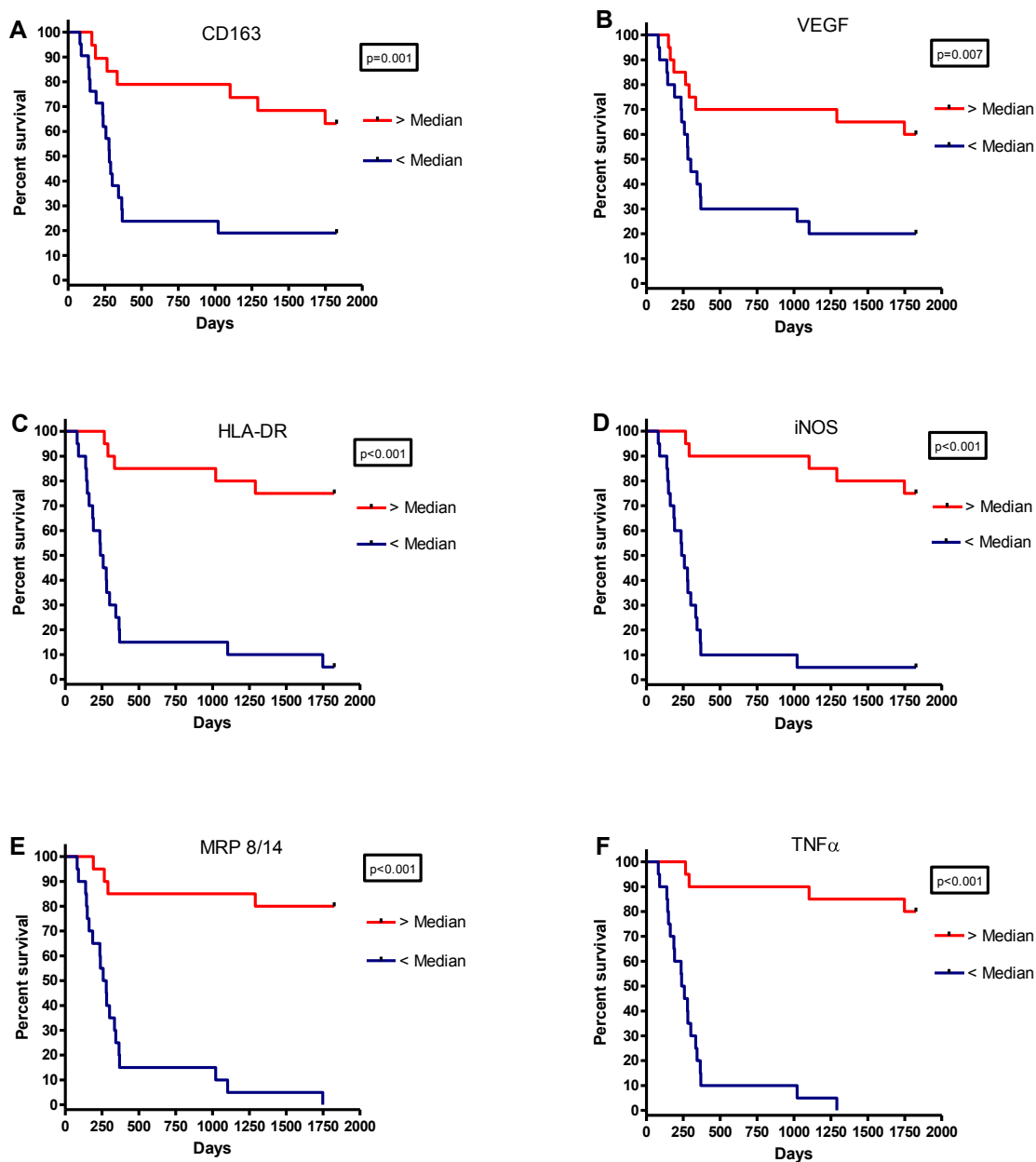
Fig 4. Kaplan-Meier five year survival curves for double-stain macrophage densities in the tumour islets for (A) CD68/CD163, (B) CD68/VEGF, (C) CD68/HLA-DR, (D) CD68/iNOS, (E) CD68/MRP 8/14 and (F) CD68/TNF.
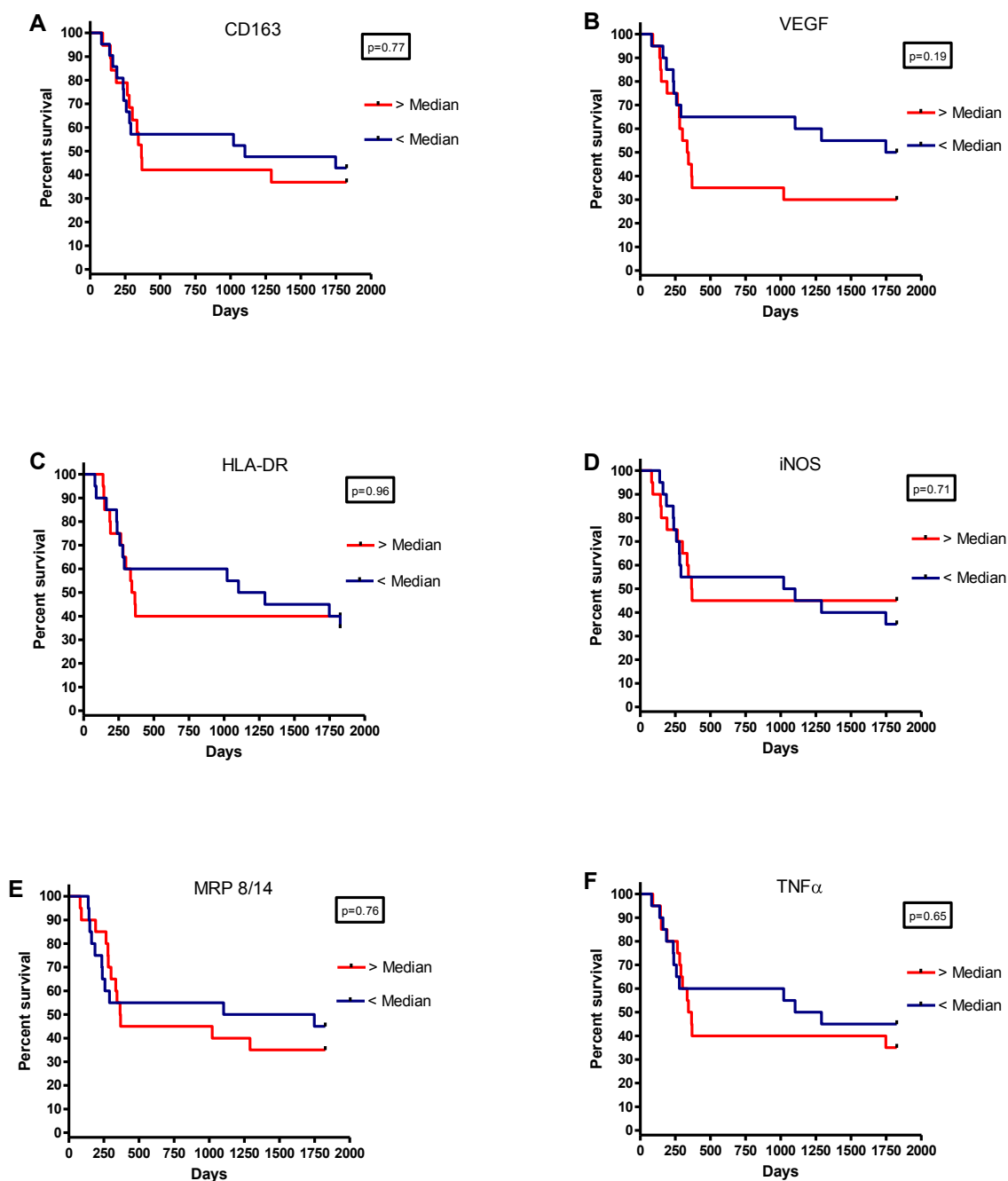
Fig 5. Kaplan-Meier five year survival curves for double-stain macrophage densities in the tumour stroma for (A) CD68/CD163, (B) CD68/VEGF, (C) CD68/HLA-DR, (D) CD68/iNOS, (E) CD68/MRP 8/14 and (F) CD68/TNF.

0.930 to 0.982, p=0.001). Because the other markers are expressed to a large extent by CD68^{+} cells, CD68 counts were included in the model when examining these markers. Interestingly, macrophages expressing MRP 8/14 in the tumour islets emerged as a highly significant positive predictor of survival (hazard ratio 0.871, 95% CI 0.800 to 0.948, p=0.001). There was no evidence of violation of the proportional hazards assumption.

DISCUSSION

We have shown previously that macrophage infiltration of tumour islets in surgically resected NSCLC confers a marked survival advantage independently of tumour stage, while increasing numbers of macrophages in the tumour stroma are associated with a worse prognosis (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). This study is the first to demonstrate that there are two distinct macrophage phenotypes in NSCLC tissue: M1 macrophages that express HLA-DR, iNOS, MRP 8/14 and TNFα, and M2 macrophages that express CD163 and VEGF.

This study tested the hypothesis that macrophages infiltrating the tumour islets of patients with extended survival following surgical resection of NSCLC are predominantly of the putative cytotoxic M1 phenotype. Macrophages expressing M1 markers, HLA-DR, iNOS, MRP 8/14 and TNFα were markedly increased in the tumour islets of patients with extended survival compared to poor survival. Macrophage expression of CD163 and VEGF, markers for the putative non-cytotoxic M2 phenotype, were also increased in the islets of the extended survival group, but to a significantly lesser extent than the M1 macrophages. The results therefore support the hypothesis that the survival

advantage conferred by tumour islet macrophage infiltration may be related to their cytotoxic potential.

The propensity for M1 macrophages to infiltrate the tumour islets in extended survival patients was particularly evident when looking at the islet: stromal ratio of cells present. This ratio (Fig 3) was markedly elevated (>2.5) for all M1 markers compared to the M2 markers (~0.75 for CD163 and ~1.1 for VEGF) in the extended survival group suggesting a predilection for the recruitment of the M1 phenotype to the islets. In contrast, in the poor survival group, the islet: stromal ratio of M1 macrophages was relatively low (<0.75), indicating that although M1 macrophages are evident in the tumour stroma in significant numbers, they cannot penetrate the tumour epithelium. In fact, M1 macrophage numbers tended to be higher in the stroma of poor prognosis patients compared to good prognosis patients. This is interesting because it demonstrates that potentially cytotoxic M1 macrophages are present in all tumours, regardless of patient survival. In some patients, M1 macrophages may be in the wrong tissue compartment for the modulation of tumour activity and the fact that survival is improved when M1 macrophages are present in the islets adds further weight to the argument that the key immune responses affecting survival in NSCLC take place in the tumour islets as opposed to the stroma. Table 2 suggests that M1 and M2 macrophages potentially play different roles, given that there are distinctly different proportions of the total numbers of macrophages for the M1 versus M2 markers in the islets of extended survival patients.

The identification of M1 and M2 macrophages has largely been made *in vitro*, with relatively little evidence for their differential expression in tissue. We were able to demonstrate that the M1 and M2 markers stained different

	ESI	ESS	PSS
CD163	**42.5** (0-100)	47.2 (15-100)	59 (0-100)
VEGF	**33.33** (0-100)	68.3 (22-100)	67.3 (0-100)
HLA-DR	**69.4** (35-100)	75 (0-100)	56 (0-100)
iNOS	**71.5** (40-100)	63.5 (0-100)	64 (0-100)
MRP 8/14	**71.3** (0-99)	79 (25-100)	57 (0-100)
TNFα	**63.9** (31-100)	50.5 (8-100)	58.5 (0-100)

Table 2. Percentage of total macrophages positive for each phenotype in extended survival patients in the islets (ESI) and stroma (ESS) and poor survival patients in the stroma (PSS)[1]. Median values are shown with (range).

cell populations although about 5% of cells were seen to stain for both markers. This provides further evidence that these represent distinct macrophage

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[1] Due to the paucity of cells in the islets of the poor survival patients, data for this group has not been included in the table.

phenotypes within the tissue. The M2 macrophage is proposed to be pro-tumorigenic (Mantovani, Sozzani, Locati, Allavena, & Sica 2002;Mantovani, Allavena, & Sica 2004;Sica, Schioppa, Mantovani, & Allavena 2006), but our demonstration of a significant increase in the islet density of M2 macrophages in the extended survival patients suggests that they may have anti-tumorigenic activity when present within the islets. Whether this is due to their own biological activity or a co-operative interaction with M1 macrophages requires further investigation.

From a biological perspective, it is logical that for macrophages to mediate anti-tumour activity they should interact intimately with the tumour epithelial cells. The mechanisms behind this remain speculative but a direct cytotoxic effect through the release of anti-tumour cytokines such as TNFα may play a role, as may the recruitment of cytotoxic T cells (Ohno, Inagawa, Soma, & Nagasue 2002). The failure of M1 macrophages to infiltrate the tumour islets also has many potential explanations which require further investigation. For example, the profile of chemoattractants expressed by the tumour islets might differ between the groups, or more subtle abnormalities in macrophage function might exist such as an inability to degrade appropriate matrix proteins for migration or expression of a dysfunctional chemokine receptor repertoire.

In summary, we have shown that there are 2 distinct macrophage phenotypes, M1 and M2, in NSCLC. The cytotoxic M1 phenotype accounts for the majority of macrophages present within the tumour islets in patients with extended survival. This supports the view that immune responses in the tumour islets play a crucial role in preventing NSCLC progression.

Chapter 5

Mast cell phenotypes in non-small cell lung cancer: correlation with survival, TNFα expression and mast cell degranulation

The anatomical microlocalisation of mast cells within NSCLC tissue seems critical to their role in disease progression (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). In particular, patients with increased mast cell expression in their tumour islets have improved survival. The granular contents of mast cells may also be influential in cancer and previous studies have suggested that histamine, heparin and cannabinoids released by mast cells may be associated with cancer progression (Dimitriadou & Koutsilieris 1997;Masini, Fabbroni, Giannini, Vannacci, Messerini, Perna, Cortesini, & Cianchi 2005;Rudolph, Boza, Yefi, Luza, Andrews, Penissi, Garrido, & Rojas 2008). $TNF\alpha$ is a cytokine expressed by mast cells and there is conflicting evidence as to whether or not it is beneficial or harmful with respect to cancer development (Balkwill 2006;Mocellin, Rossi, Pilati, & Nitti 2005). The primary aims of the present study were therefore to define the phenotype of mast cells within NSCLC stroma and islets in terms of their protease content, to assess their state of activation defined by the extent of degranulation, and to evaluate the microlocalisation of mast cells expressing $TNF\alpha$.

Patients & Methods

Study Population

The tissue specimens evaluated were from 40 patients selected for the study based on their survival, without knowledge of their previous tumour cell counts. 20 patients had extended survival (ES) (mean ± SEM 81.8 ± 8.3 months), and 20 patients had poor survival (PS) (8.4 ± 0.8 months). Patient characteristics are summarised in Table 1.

Characteristic	Extended Survival	Poor Survival
No. of patients	20	20
Age – years	65.7±2.2	70.3±1.6
Male sex – no. (%)	14 (70)	14 (70)
Year of surgery – no. (%)		
1991	0 (0)	1 (5)
1992	2 (10)	1 (5)
1993	3 (15)	1 (5)
1994	5 (25)	2 (10)
1999	10 (50)	15 (75)
Tumour stage – no. (%)		
1	10 (50)	15(75)
2	8 (40)	5 (25)
3a	2 (10)	0 (0)
3b and 4	0 (0)	0 (0)
Histology – no. (%)		
Squamous	17 (85)	9 (45)
Adenocarcinoma	1 (5)	4 (20)
Large cell	0 (0)	4 (20)
Other	2 (10)	3 (15)
Tumour Grade – no. (%)		
Well	1 (5)	1 (5)
Moderate	11 (55)	3 (15)
Poor	7 (35)	16 (80)
Not recorded	1 (5)	0 (0)
Adjuvant Chemotherapy (%)	1 (5)	0 (0)
Radiotherapy (%)	2 (10)	2 (10)
Palliative Radiotherapy (%)	2 (10)	2 (10)
Survival – months	81.8±8.3	8.4±0.8

Plus-minus values are means ± SEM

Table 1. Patient Charactersitics

Immunohistology

Antibodies for phenotypic analysis were all mouse antihuman mAb as follows: tryptase (clone AA1; Dako Cytomation, Ely, Cambridgeshire, UK) as a specific marker for all mast cells, chymase (clone CCL1; Abcam, Cambridge, UK) as a specific marker for mast cells expressing chymase and tryptase, and TNFα (clone P/T2; Abcam, Cambridge, United Kingdom). Immunostaining was performed using the Envision double-stain kit (Dako Cytomation, Ely, Cambridgeshire, UK) according to the manufacturer's instructions and as described previously (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). Three slides were prepared for each patient – chymase versus tryptase, tryptase versus TNFα, and chymase versus TNFα. Peroxidase and DAB (brown reaction product), and alkaline phosphatase and fast red (red reaction product) were used to label cells expressing tryptase, chymase, and TNFα. Sections were then counterstained with haematoxylin and mounted in an aqueous mounting medium (BDH Chemicals Ltd, Poole, UK). Appropriate isotype controls were performed where the primary antibodies were replaced by irrelevant mouse mAb of the same isotype and at the same concentration as the specific primary mAb.

Analysis and Validation of Immunostaining

Analysis was repeated for 10 patients to assess repeatability and validity. To identify mast cell phenotype, cells positive for chymase were counted as MC_{TC} and all other mast cells were counted as MC_T.

A degranulation index score was established in order to assess the degree of degranulation by each individual mast cell as follows:

0	No degranulation
1	<33% degranulation
2	33-66% degranulation
3	>66% degranulation

Results

Patient Characteristics

Of the 40 patients studied, 33 had died at the time of analysis. Twenty-six tumours were squamous, 5 adenocarcinoma, 4 large cell, and 5 other. Twenty-five were stage I, 13 stage II, and 2 stage IIIa. One patient had additional chemotherapy and 4 had additional radiotherapy, all of whom had it for later palliation. The patient characteristics are summarized in Table 1.

Validation of Analysis

Clear and distinguishable staining was evident for tryptase, chymase and TNFα and double-stained cells were readily identifiable (Fig 1). Appropriate isotype controls were negative. Cells counts were repeated and an intraclass correlation coefficient was calculated as 0.797 ($p < 0.01$). This method of analysis has also been validated by our group previously (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). The degranulation index was validated by two separate observers with an intraclass correlation of 0.668 ($p<0.05$) (see Appendix A).
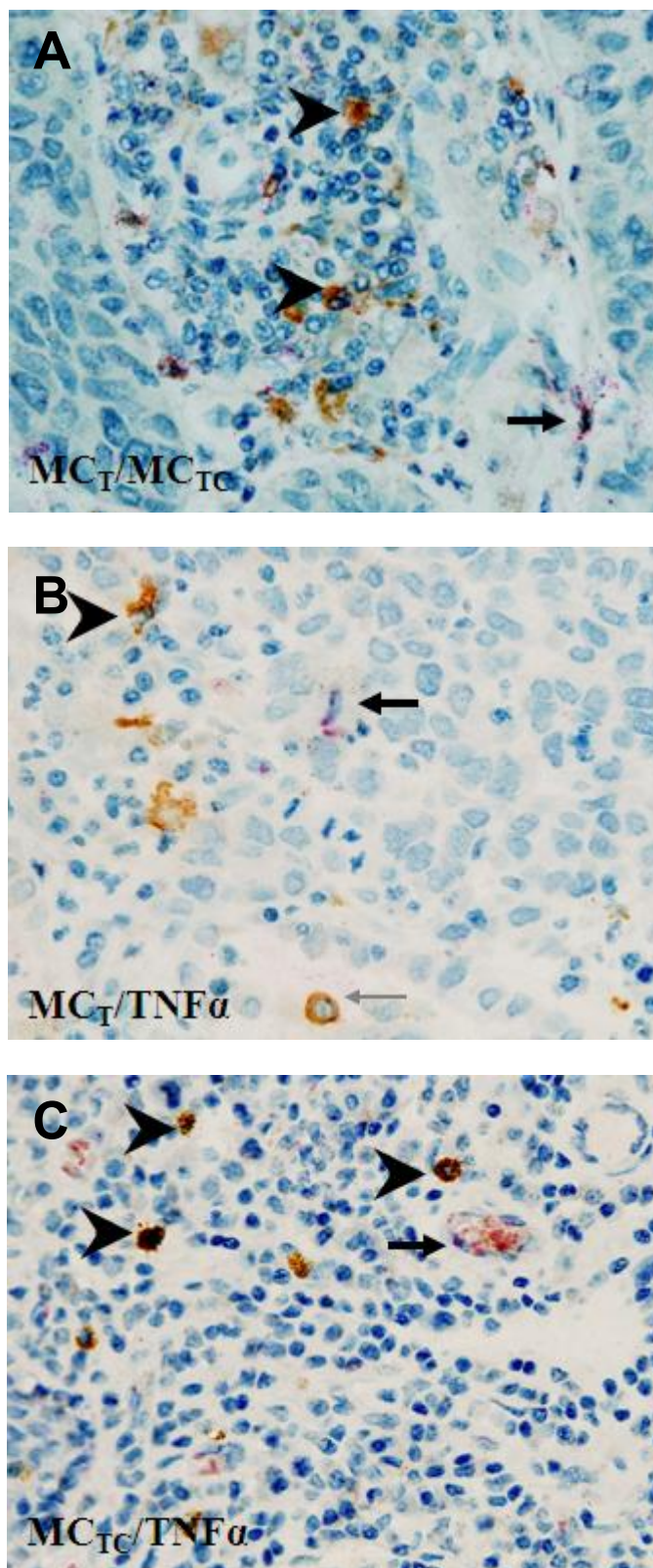
Fig 1. Immunohistochemical double-staining for A) chymase (brown) and tryptase (red) demonstrating the presence of MC_T (red) and MC_{TC} (reddish brown), B) tryptase (brown) and TNFα (red) demonstrating the presence of TNFα in tryptase+ mast cells (reddish brown), and C) chymase (brown) and TNFα (red) demonstrating the expression of TNFα in MC_{TC} mast cells (reddish brown). Arrowhead = double-stain cell. Black arrow = single-stain red cell. Grey arrow = single-stain brown cell. Magnification x400

Cellular Distribution

There were significantly more MC_T in the tumour islets of the ES group (median, 22.60 cells/mm^2 [range, 5.21 to 73.77]) compared to the PS group (median, 0.70 [range, 0 to 9.40], p<0.001) (Fig 2A). In contrast, in the stroma there was no significant difference between the MC_T densities in the ES group (median, 37.95 cells/mm^2 [range, 10.89 - 120.9]) and the PS group (median, 52.32 cells/mm^2 [range, 2.15 - 160.0], p=0.46) (Fig 2B).

There were also significantly more MC_{TC} in the tumour islets of the ES group (median, 4.63 cells/mm^2 [range, 0 - 82.91]) compared to the PS group (median, 0 [range, 0 - 2.21], p=0.003) (Fig 2C). In contrast, in the stroma there was no significant difference between the MC_{TC} densities in the ES group (median, 7.88 cells/mm^2 [range, 0 - 48.35]) and the PS group (median, 3.78 cells/mm^2 [range, 0 - 75.56], p=0.42) (Fig 2D). The percentage of mast cells which were MC_T or MC_{TC} in the tumour compartments are shown in Table 2.

The median density of MC_T and MC_{TC} expressing TNFα in the tumour islets of patients with ES was significantly greater (median 27.18 [range, 1.47 - 283.3] & median 9.32 cells/mm^2 [range, 0 - 27.48] respectively) than in patients with PS (median 0.79 [range, 0 - 19.8] & 0 cells/mm^2 [range, 0 - 1.69] respectively) (p<0.001 for MC_T & p=0.001 for MC_{TC}) (Figs 3A and C). The median density of MC_T and MC_{TC} expressing TNFα in the stroma of patients with ES was 16.81 (range, 0 - 67.21) & 10.86 (range, 0 - 31.25) cells/mm^2 respectively compared to 13.10 (range, 0 - 60.56) & 0 (range, 0 - 1.69) cells/mm^2 respectively in with patients with PS (p=0.52 for MC_T and p=0.02 for MC_{TC}) (Figs 3B and D). The median density of all cells expressing TNFα (mast cells and other cells) in tumour islets of patients with an above
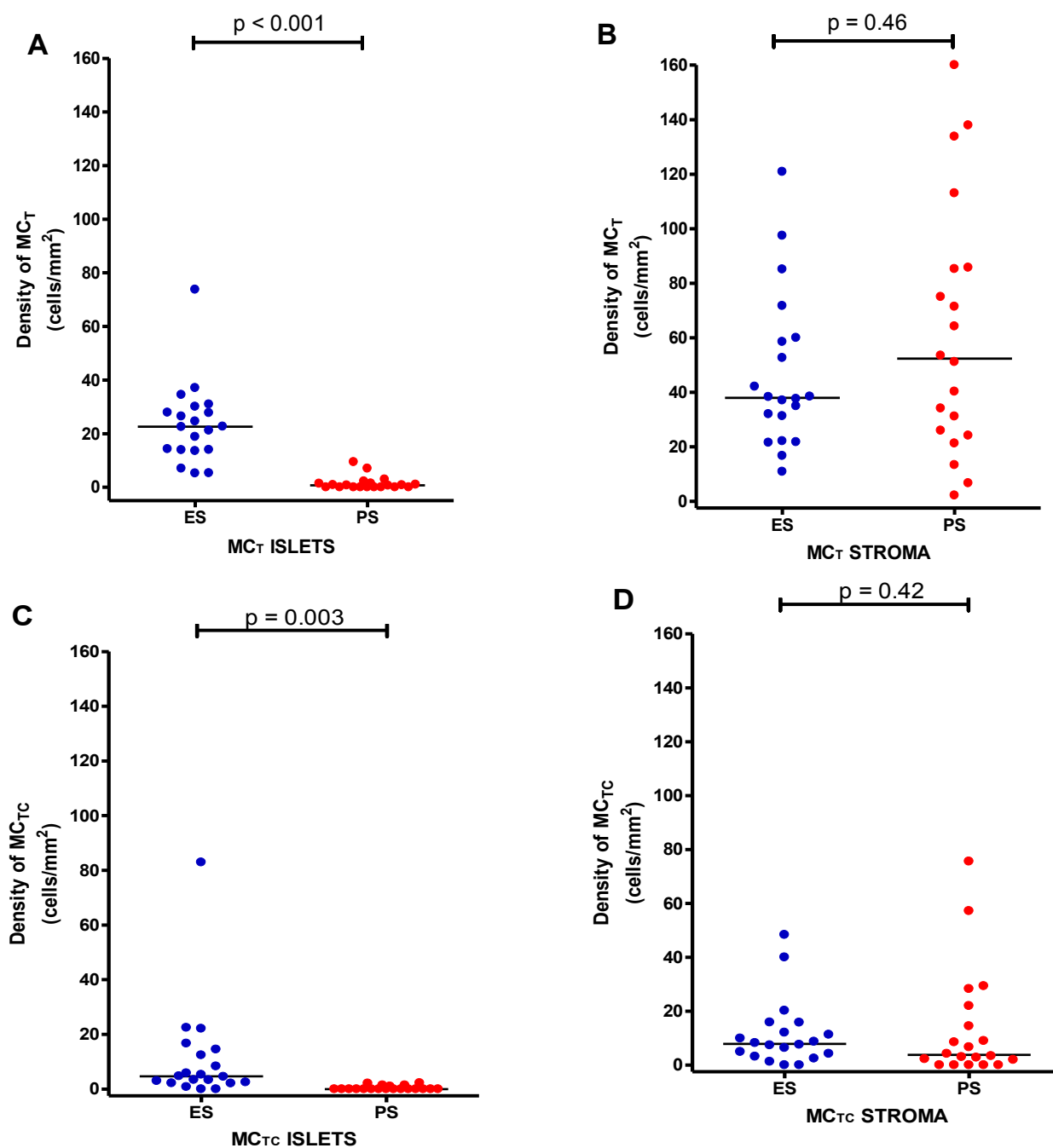
Fig 2. Mast cell densities for MC_T in the islets (A) and stroma (B) and for MC_{TC} in the islets (C) and stroma (D) in extended survival (ES) and poor survival (PS) patients.
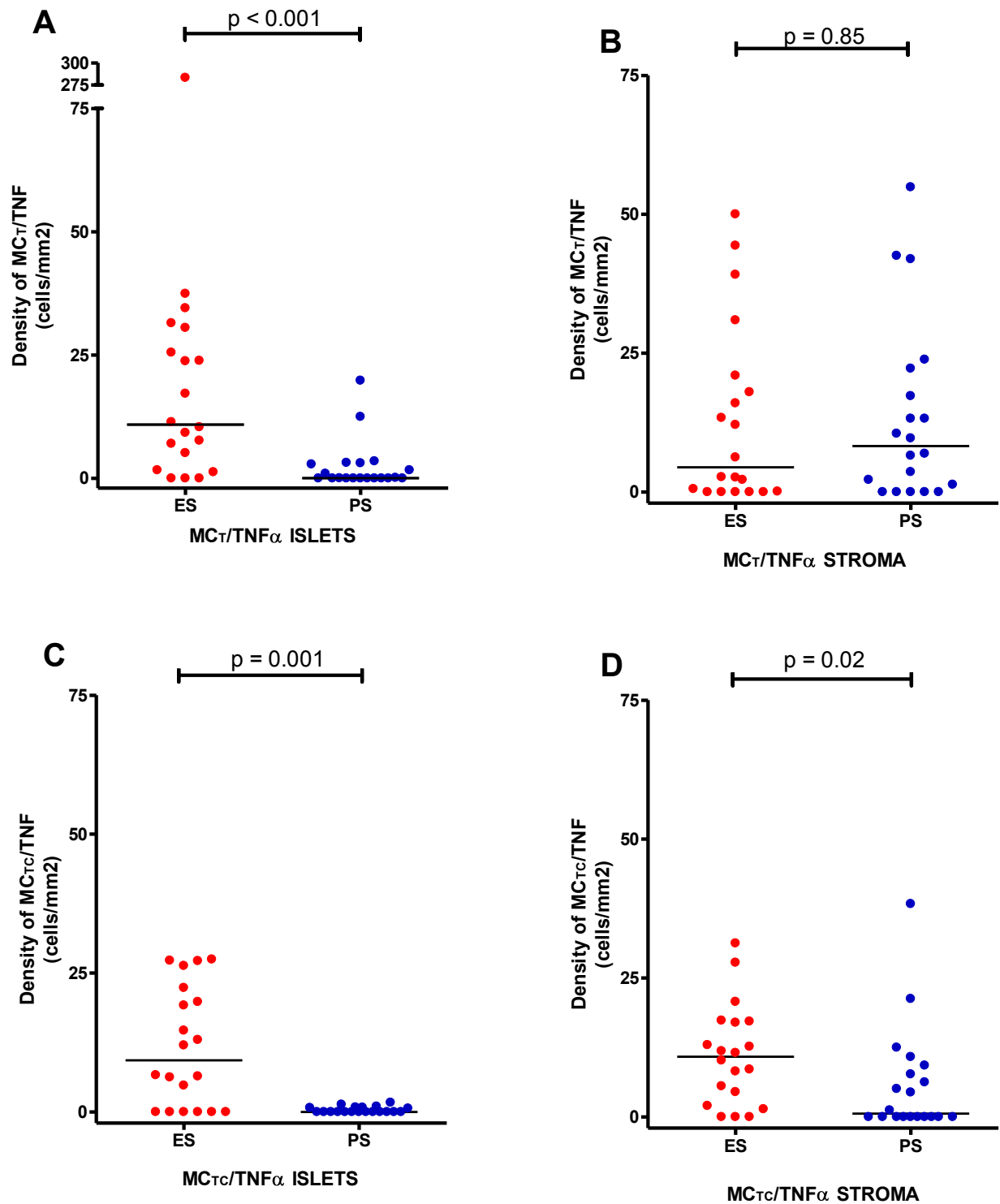
Fig 3. Double-stain densities of $MC_T/TNF\alpha$ in the islets (A), stroma (B), and $MC_{TC}/TNF\alpha$ in the islets (C) and stroma (D) in extended survival (ES) and poor survival (PS) patients. $MC_T/TNF\alpha$ densities were calculated by subtracting the chymase (MC_{TC}) count from the tryptase (total mast cells) count.

median survival was also noted to be significantly greater (47.83 cells/mm^2) than in patients with a below median survival (11.15 cells/mm^2) ($p<0.001$). The proportion of MC$_T$ and MC$_{TC}$ which expressed TNFα in the different tissue compartments are shown in Table 2.

Degranulation Index

In patients with extended survival, MC$_T$ in the stroma were degranulated to a greater degree than in those with poor survival (Fig 4B median degranulation index is 2.29 versus 1.89 respectively) ($p=0.007$). Fig 5 demonstrates a positive correlation between the stromal MC$_T$ degranulation index and survival. MC$_T$ in the ES stroma were also more degranulated than MC$_T$ in the ES islets (degranulation index 1.71, $p<0.001$). There was no significant difference between ES and PS mast cell degranulation index for MC$_T$ in the islets or MC$_{TC}$ in islets or stroma (Fig 4A, C and D).

Kaplan-Meier Survival Analysis

For further analysis, the data were divided into two groups above and below the median cell count values. Kaplan-Meier survival curves were plotted to investigate further the association of cell densities with survival. The log rank statistic was used to compare survival rates. In the tumour islets, patients with above median density of both MC$_T$ (Fig 6A) and MC$_{TC}$ (Fig 6C) had significantly greater predicted survival ($p<0.001$) but there was no correlation with stromal mast cell densities and survival (Figs 6B and D). There was a positive association between survival and tumour islet density of mast cells (MC$_T$ and MC$_{TC}$) expressing TNFα ($p<0.001$) (Figs 7A and B). In contrast there were no survival differences evident using the same analysis on

	ESI	PSI[1]	ESS	PSS
MC_{TC}	17.8 (0-81.5)	0 (0-100)	15.9 (0-65)	8.3 (0-68.4)
MC_T	82.2 (18.5-100)	100 (0-100)	84.1 (35-100)	91.7 (31.6-100)
$MC_{TC}/TNF\alpha$	36.8 (0-100)	20.1 (0-100)	50 (0-100)	48.3 (0-100)
$MC_T/TNF\alpha$	72.2 (17.4-100)	91.3 (0-100)	65.4 (0-100)	78.8 (0-100)

Table 2. Percentage of total mast cells positive for each phenotype (MC_{TC} or MC_T) and percentage of each phenotype expressing TNFα in the islets of extended survival patients (ESI) and poor survival patients (PSI) and stroma of extended survival patients (ESS) and poor survival patients (PSS). Median values are shown with (range). [1]Only 12 out of 20 poor survival patients had mast cells in their islets.

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[1] Only 12 out of 20 poor survival patients had mast cells in their islets
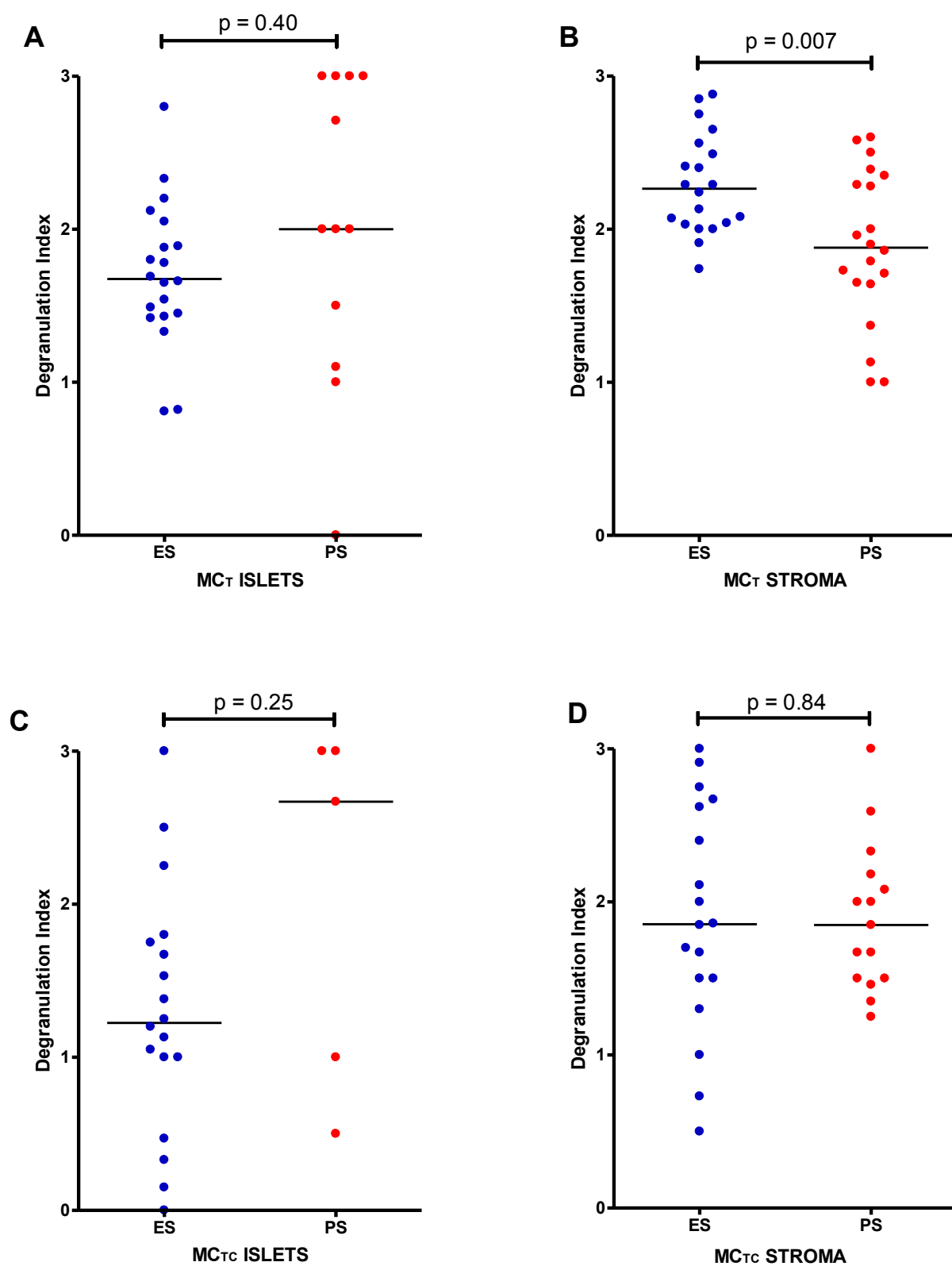
Fig 4. Degranulation Index of MC_T in the islets (A) and stroma (B), and MC_{TC} in the islets (C) and stroma (D)
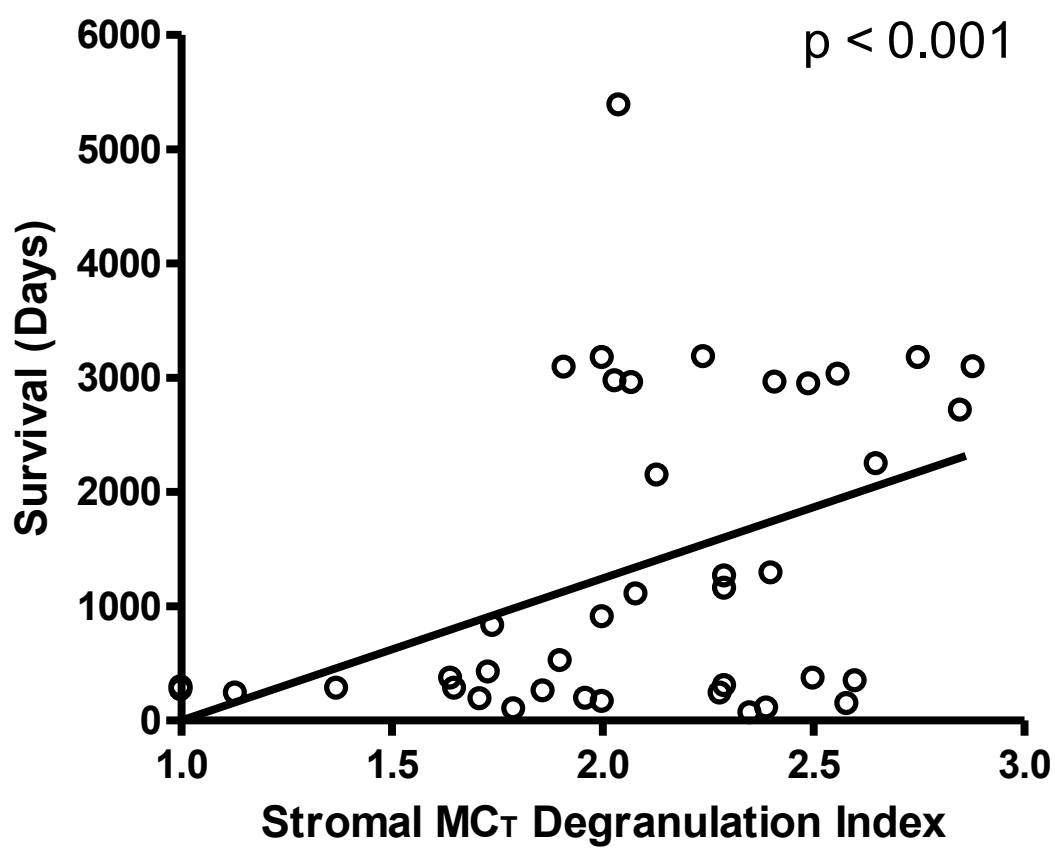
Fig 5. Linear regression showing positive correlation between stromal MC_T degranulation index and survival
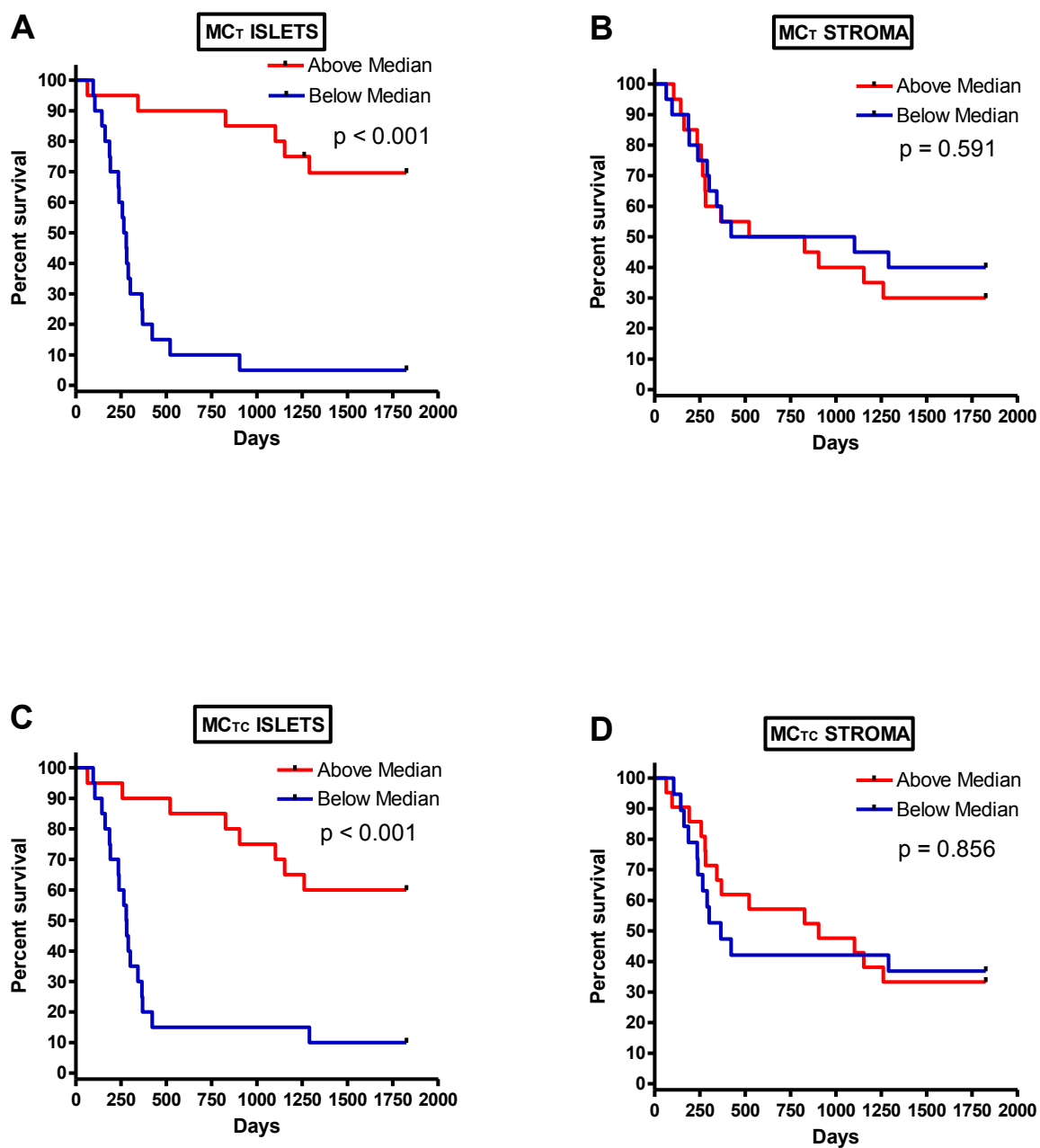
Fig 6. Kaplan-Meier five-year survival curves for MC_T in the islets (A) and stroma (B), and MC_{TC} in the islets (C) and stroma (D). Subjects were divided at the median value.
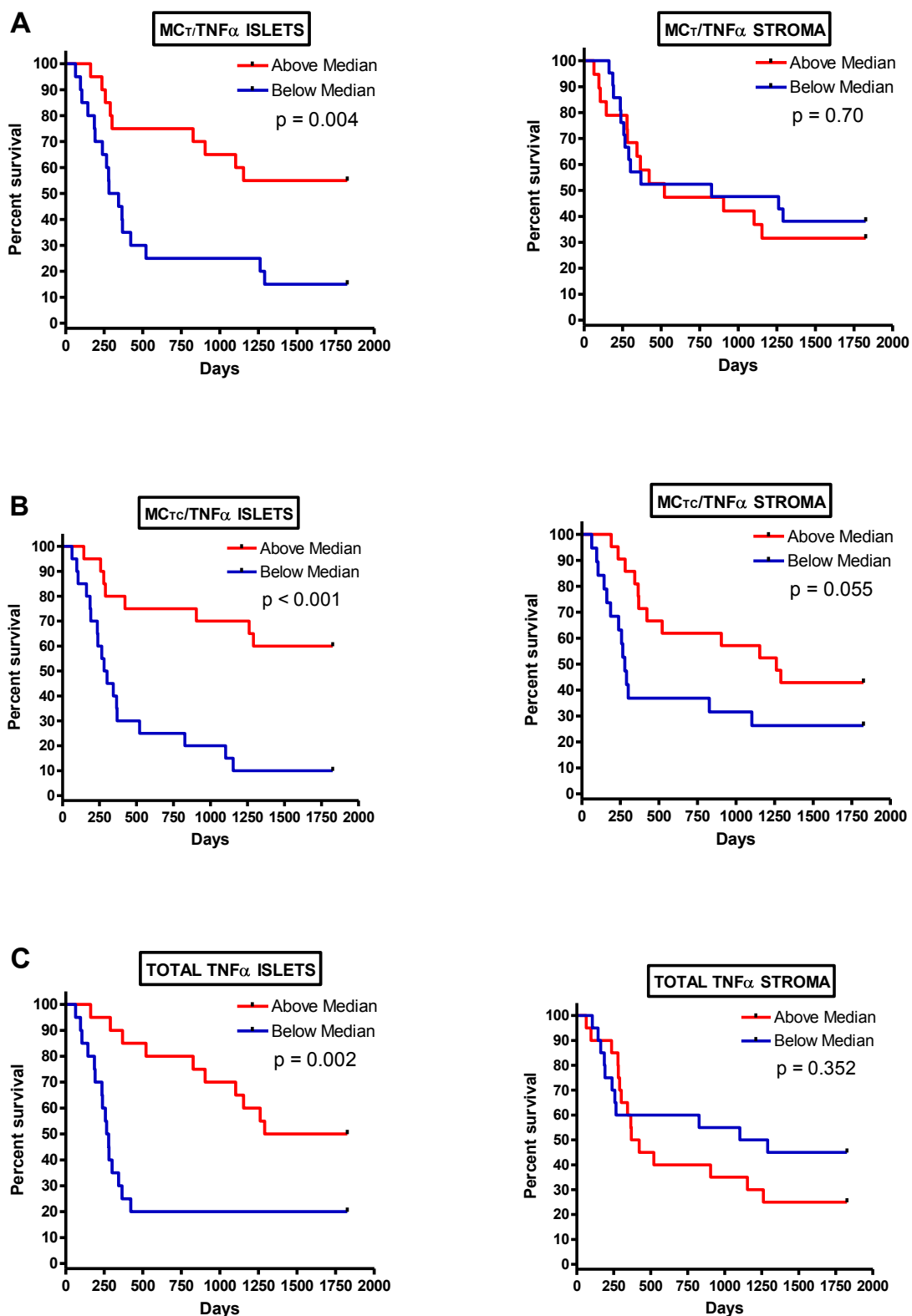
Fig 7. Kaplan-Meier five-year survival curves for MC_T/TNFα in the islets and stroma (A), and MC_{TC}/TNFα in the islets and stroma (B), and total TNFα+ cells in the islets and stroma (C).

stromal cell counts (Fig 7A and B). Above median total expression of TNFα in the islets was also associated with improved survival as opposed to stromal expression, which again had no significant relationship with survival (Fig 7C).

DISCUSSION

We have shown previously that mast cell infiltration of tumour islets in surgically resected NSCLC confers a marked survival advantage independently of tumour stage (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). This study extends those findings by delineating the compartmental microlocalisation of mast cell phenotypes within the tumour tissue.

In normal airways mast cell localisation is limited largely to the lamina propria, and the density of mast cells there and ratio of is similar to the density recorded here in the NSCLC tumour stroma. Furthermore, the normal airway lamina propria ratio of $MC_T:MC_{TC}$ is about 80:20 (Bradding, Okayama, Howarth, Church, & Holgate 1995;Irani & Schwartz 1989), which is similar to that in the NSCLC stroma. This suggests that the homeostatic mechanisms regulating mast cell density and phenotype is similar in normal bronchus and NSCLC stroma. Mast cells in airway epithelium of normal subjects are almost exclusively of the MC_T phenotype (Irani, Schechter, Craig, DeBlois, & Schwartz 1986). It is interesting therefore that in NSCLC epithelial islets, mast cells are also present in some patients, but not others, and that their presence correlates with extended survival. However, whereas the mast cell phenotype within the normal airway epithelium is almost exclusively MC_T, in the NSCLC epithelial islets of extended survival patients, approximately 20% of mast cells are of the MC_{TC} phenotype. This suggests that in patients with extended survival, the tumour epithelium expresses a specific repertoire of chemoattractants, growth

factors and cell-cell signals for the recruitment, differentiation and survival of the MC_{TC} phenotype.

The correlation of improved survival with mast cells of both the MC_T and MC_{TC} phenotype within the NSCLC tumour islets suggests that mast cells contribute to the anti-tumour immunological response, which also comprises of infiltration by CD68+ macrophages (Arenberg et al. 2000;Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005), natural killer and regulatory T cells (Esendagli et al. 2008). There are many mast cell products which may contribute to the recruitment and activation of these cells, but TNFα may be particularly important. This cytokine is stored pre-formed and secreted rapidly by mast cells in response to numerous diverse stimuli including IgE-dependent activation, TLR-dependent activation (Varadaradjalou et al. 2003) and cell-cell contact with T cells (Bhattacharyya et al. 1998). It is therefore likely to be important that mast cells within the tumour islets express this cytokine. The expression of TNFα by all cells in the tumour islets, not just mast cells, was also associated with better survival. These findings suggest that TNFα has a protective role to play for patients with NSCLC when present in the tumour islets, contributing to the limitation of tumour growth and dissemination. It has been shown in mouse models that mast cell-derived TNFα can significantly increase T cell proliferation and cytokine production (Nakae et al. 2005) and that IgE- and Ag-dependent mast cell enhancement of T cell activation requires TNFα (Nakae et al. 2006). TNFα also induces cytotoxic activity in macrophages, and so mast cell-derived TNFα may play a pivotal role in the cytokine pathways influencing cytotoxic T cells and macrophages within the

tumour islets, and thus have important consequences on cytotoxicity against tumour cells.

There is conflicting evidence as to whether or not TNFα plays a role in tumour progression or prevention. In a study investigating malignant melanoma, local administration of TNFα had a positive effect in treating metastatic disease (Lejeune, R³egg, & Liqnard 1998). In contrast, endogenous production of TNFα may act as a tumour promoter contributing to tissue remodelling and stromal development (Balkwill 2002). We found that while TNFα expression in the tumour islets was associated with improved survival, there was no correlation with survival in the tumour stroma. This raises the possibility of TNFα having different actions depending on its microlocalisation within the tumour. In addition, Shih investigated different genetic polymorphisms of TNFα in NSCLC and found that the -308 A allele has a promotive effect for lung cancer development and progression, whereas the -238 A allele has a protective function against lung cancers (Shih et al. 2006).

We found no correlation between the density of MC_{TC} and poor survival which conflicts with the results of Ibaraki and colleagues (Ibaraki, Muramatsu, Takai, Jin, Maruyama, Orino, Katsumata, & Miyazaki 2005) who reported a relationship between MC_{TC} density and microvessel density in NSCLC. This difference may be due to the fact that in that study no assessment of the microlocalisation of mast cells within the tumour was made. We also noted that patients with poor survival had low numbers of both phenotype of mast cell in their tumour islets (Fig 2A and C) which is in keeping with our previous findings (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005).

Our results also demonstrate that patients who have an increased degranulation of their MC_T in the stroma have a better outcome (Figs 4B and 5). No significant differences were noted for mast cell degranulation index in the tumour islets of the extended versus poor survival patients, but meaningful quantification in the poor survival group was hampered by the paucity of mast cell for analysis in the islets of these patients. It is possible that granular products such as heparin and proteases are able to disrupt the stroma after degranulation, thus inhibiting consequent tumour growth. Mast cell proteases are known to cause cell structural alterations and loss of the extracellular matrix integrity (Dimitriadou & Koutsilieris 1997). Interestingly, Rajput found that patients with infiltration of breast cancer tumour stroma by mast cells had an improved survival (Rajput et al. 2008). It is also possible that in the poor prognosis patients there is actually inhibition of degranulation by enhancement of pro-angiogenic cytokine production. For example, PGE_2, a product of epithelial cells, inhibits lung mast cell degranulation (Kay, Yeo, & Peachell 2006) but increases the synthesis and release of VEGF (Abdel-Majid & Marshall 2004).

It is recognised that mast cells exhibit heterogeneity with regards to their cytokine content. In the lung, the MC_T phenotype expresses predominantly IL-5 and IL-6, while the MC_{TC} phenotype expresses IL-4 and IL-13 (Bradding, Okayama, Howarth, Church, & Holgate 1995). This study demonstrates that in LC, mast cells of both the MC_T and MC_{TC} phenotype express $TNF\alpha$. The biological pathways producing the improved clinical outcome when these two mast cell phenotypes are present in NSCLC islets remain speculative and require further investigation.

In summary, we have shown that the density of the two mast cell phenotypes MC_T and MC_{TC} is increased in the tumour islets of patients with extended survival in NSCLC. The production of TNFα by these mast cells may be particularly important for their interaction with other immune cells and inhibition of tumour progression.

Chapter 6

Tumour necrosis factor-alpha microlocalisation and correlation with survival in non-small cell lung cancer

There is debate as to whether or not TNFα plays a role in NSCLC tumour cytotoxicity or conversely, tumour progression. Two small studies investigated previously the mRNA or protein expression of TNFα in NSCLC, and suggested overall that TNFα expression was either mildly beneficial but not an independent factor (Boldrini et al. 2000) or neutral (Tran, Kallakury, Ambros, & Ross 1998), respectively. However, the anatomical localisation of the TNFα expressed was not taken into account. We have shown previously that the site of inflammatory cell infiltration in NSCLC is critical in terms of prognosis. Patients with high expression of macrophages in the tumour islets have extended survival independently of tumour stage, and these macrophages demonstrate high expression of TNFα and other cytotoxic markers, suggesting they are of they are anti-tumorigenic cytotoxic M1 macrophage phenotype (Chapter 4;Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). We have also shown previously that mast cells within the NSCLC tumour islets express TNFα, and again this associated with prolonged survival (Chapter 5).

The studies in Chapters 4 and 5 investigating TNFα expression in macrophages and mast cells were aimed primarily at determining the phenotype of these cells rather than the prognostic significance of TNFα expression. Due to the nature of the work, relatively small numbers of patients were studied. The aim of this study was therefore to assess the prognostic significance of TNFα expression, irrespective of cell type, paying particular attention to its anatomical microlocalisation, in surgically resected NSCLC in our complete cohort of patients described previously (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005)

Patients & Methods

Study Population

133 patient samples were available for analysis in this study. Of the 133 patients studied, 88 were men and average age at surgery was 65.8 years (standard deviation, 9.8; range, 33 to 82 years). The median survival of all the patients (25.8 months) was used to subdivide the patients into two groups – above median survival (AMS) (mean ± SEM 84.0 ± 5.1 months) and below median survival (BMS) (mean ± SEM 10.8 ± 0.8 months). Macrophage and/or mast cell-associated TNFα expression has been described previously in 26 of these patients in Chapters 4 and 5. Patient characteristics are shown in Table 1.

Immunohistology

TNFα mouse antihuman antibody was used (clone P/T2; Abcam, Cambridge, United Kingdom. Immunostaining was performed and TNFα was developed with peroxidase and DAB (brown reaction product). Sections were then counterstained with haematoxylin and mounted in an aqueous mounting medium (BDH Chemicals Ltd, Poole, UK). Appropriate isotype controls were performed where the primary antibodies were replaced by irrelevant mouse mAb of the same isotype and at the same concentration as the specific primary mAb.

Analysis and Validation of Immunostaining

The number of nucleated cells with positive staining for both markers in each area were then counted manually and expressed as cells/mm^2 of stroma or

Characteristic	Extended Survival	Poor Survival
No. of patients	67	66
Age – years	66.3$\pm$1.2	65.3$\pm$1.2
Male sex – no. (%)	39 (58)	49 (74)
Year of surgery – no. (%)		
1991	0 (0)	5 (7)
1992	5 (7)	6 (9)
1993	6 (9)	4 (6)
1994	6 (9)	7 (11)
1999	50 (75)	44 (67)
Tumour stage – no. (%)		
1	40 (60)	20 (30)
2	18 (27)	19 (29)
3a	9 (13)	23 (35)
3b and 4	0 (0)	4 (6)
Histology – no. (%)		
Squamous	39 (58)	29 (44)
Adenocarcinoma	19 (28)	23 (35)
Large cell	4 (6)	8 (12)
Other	5 (7)	6 (9)
Tumour Grade – no. (%)		
Well	5 (7)	2 (3)
Moderate	31 (46)	17 (26)
Poor	30 (45)	46 (70)
Not recorded	1 (1)	1 (1)
Adjuvant Chemotherapy (%)	0 (0)	2 (3)
Radiotherapy (%)	8 (12)	12 (18)
Palliative Radiotherapy (%)	7 (10)	9 (14)
Survival – months	84.0 ± 5.1	10.8 ± 0.8

Plus-minus values are means $\pm$ SEM

Table 1. Patient Charactersitics

tumour islets. Analysis was repeated for 20 patients to assess repeatability and validity.

Results

Patient Characteristics

Patent characteristics are shown in Table 1. Of the 133 patients studied, 110 had died at the time of analysis. Thirty-day mortality for the cohort was 4% and patients who died within the first 60 days of surgery were not included in this analysis. Sixty-eight tumours were squamous, 42 adenocarcinoma, 12 large cell, and 11 other. Sixty were stage I, 37 stage II, 32 stage IIIa, and four stage IIIb or IV. Two patients had additional postoperative chemotherapy and 20 had additional radiotherapy, 16 of whom had it for later palliation. Neither had any effect on survival. The overall 5-year survival was 31.3%.

Validation of Analysis

Clear and distinguishable staining was evident for TNFα (Fig 1). Appropriate isotype controls were negative. In order to assess the validity of the method, area measurements and cell counts were repeated and intraclass correlation coefficients calculated. Good correlations were found for both: 0.997 (95% CI, 0.996 to 0.998, p<0.001) and 0.994 (95% CI, 0.992 to 0.996, p<0.001).

Cellular Distribution

There was increased expression of TNFα in the tumour islets of patients with AMS compared to those with BMS (median 27.2 versus 18.8 cells/mm^2
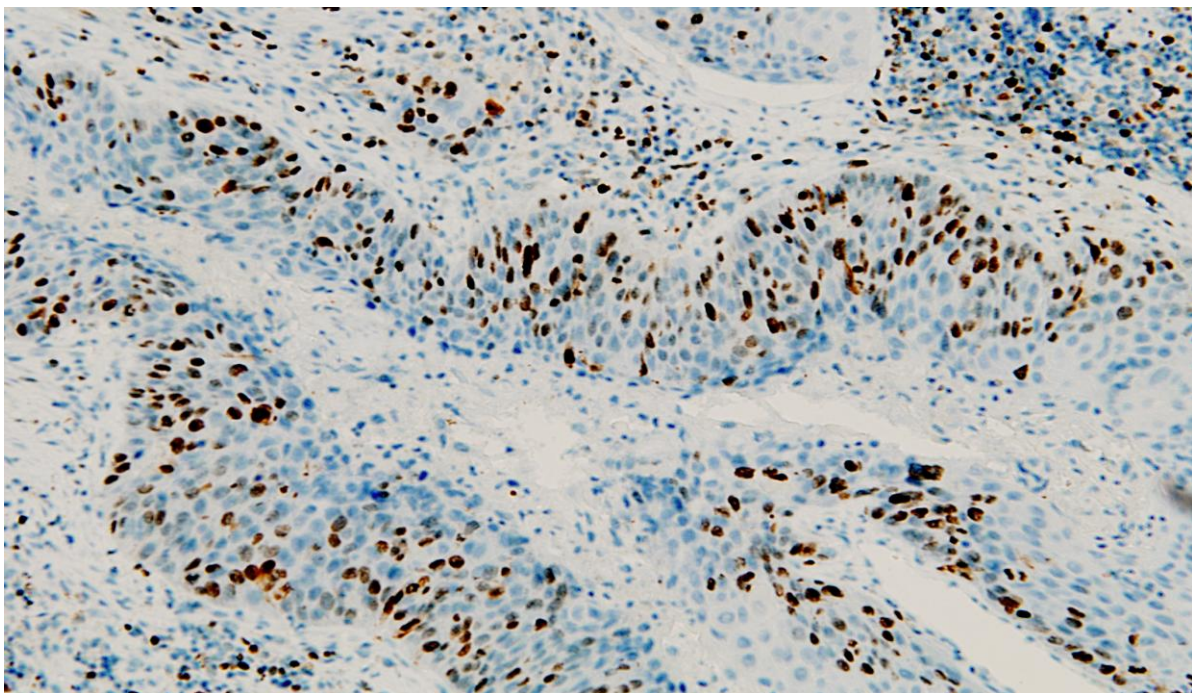
Fig 1. Immunohistology demonstrating positive TNFα expression

(brown).Magnification x100

respectively, p=0.006). There was no significant difference in the expression of stromal TNFα between the two groups (AMS 23.8 versus BMS 16.8 cells/mm^2, p=0.31) (Figs 2A and B).

Clinical Outcome

Scatter plots of the raw data of TNFα density versus survival are shown in Fig 3. Spearman's rank correlation coefficient was calculated to assess any potential relationship with survival. A direct relationship between tumour islet TNFα density and survival was noted (r_s = 0.213, p=0.01). No significant relationship was seen between survival and stromal TNFα density (rs = –0.01, p=0.90).

Kaplan-Meier Survival Analysis

For further analysis, the data were divided into two or three equal groups according to TNFα density. Kaplan-Meier survival curves were plotted and the log-rank statistic used to compare survival rates. When looking at two groups separated by the median, there was a non-significant trend for improved survival with above median TNFα expression in the tumour islets (p=0.15, Fig 4). When divided into tertiles, there was significantly improved survival in the top tertile of TNFα expression in the tumour islets compared to the middle and lower tertiles (Fig 5A). Thus patients with high islet TNFα expression (upper tertile) were noted to have a significantly higher 5-year predicted survival as opposed to patients with low TNFα expression (lower tertile) (43% versus 22%, p=0.01). There was no significant relationship between TNFα expression in the stroma and survival (Fig 5B). Similar
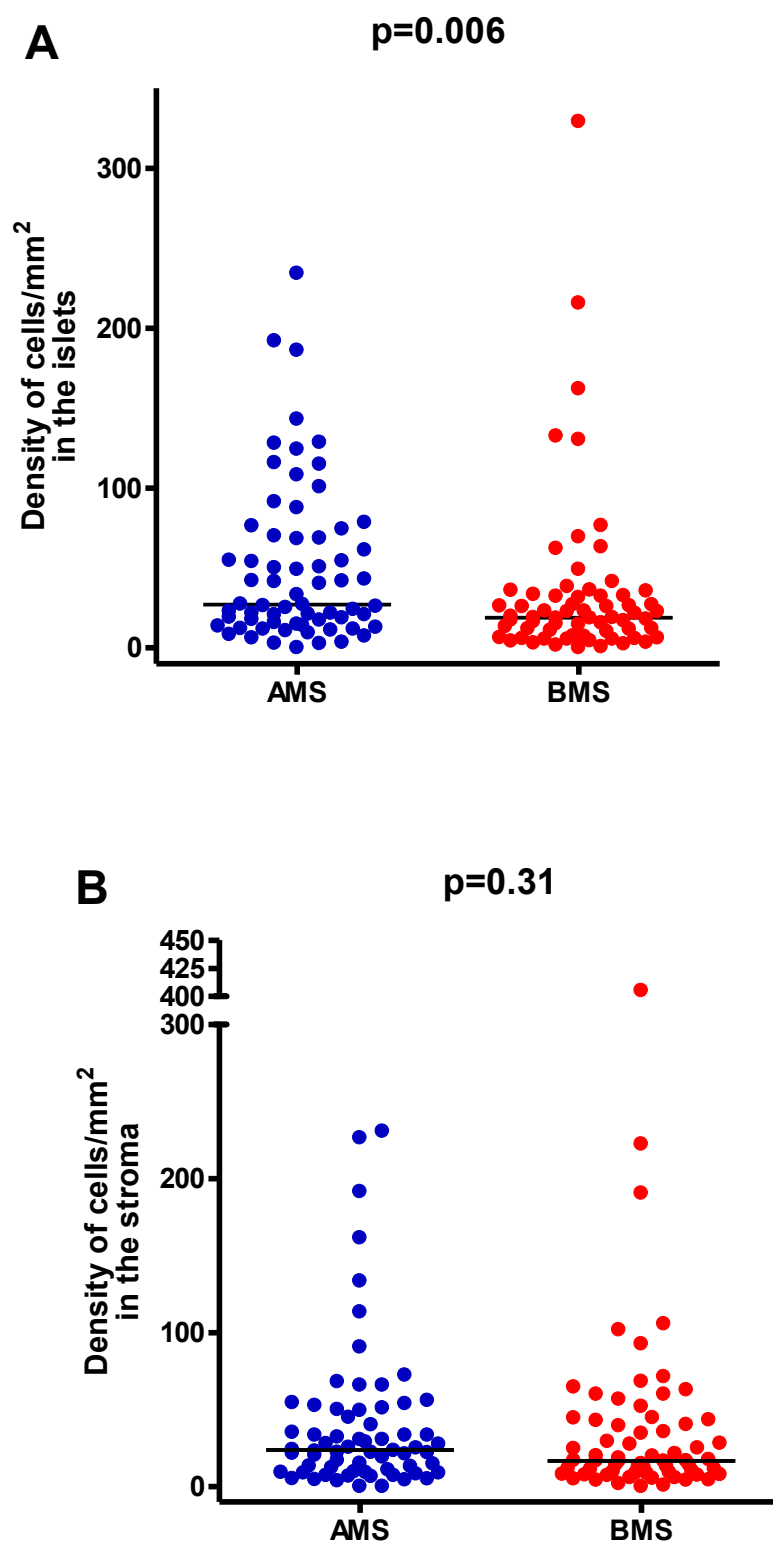
Fig 2. TNFα densities in Above Median Survival (AMS) and Below Median Survival (BMS) patients in the tumour islets (A) and stroma (B).
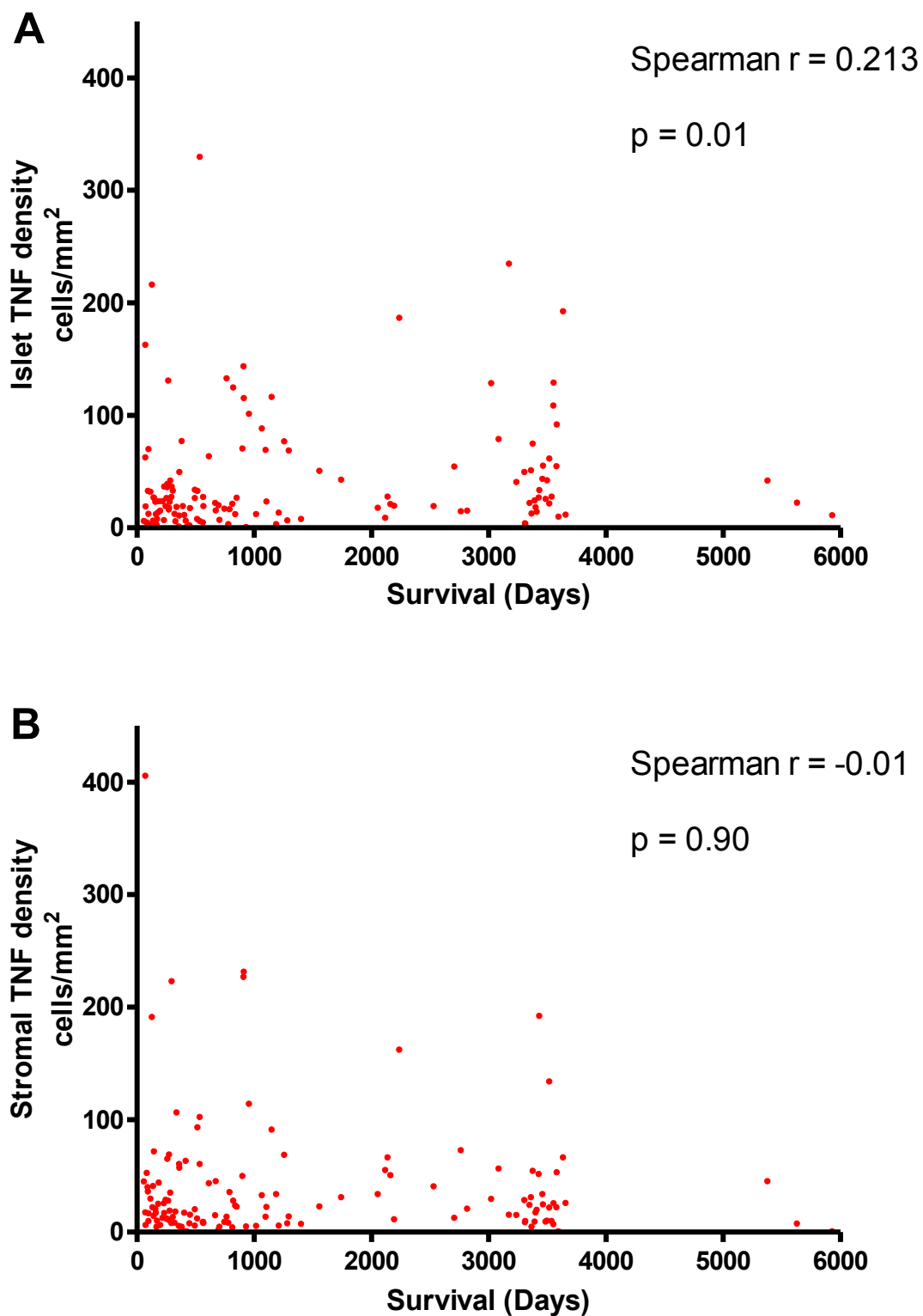
Fig 3. Raw data of cell counts expressing TNFα plotted against survival in days in the tumour islets (A) and stroma (B).
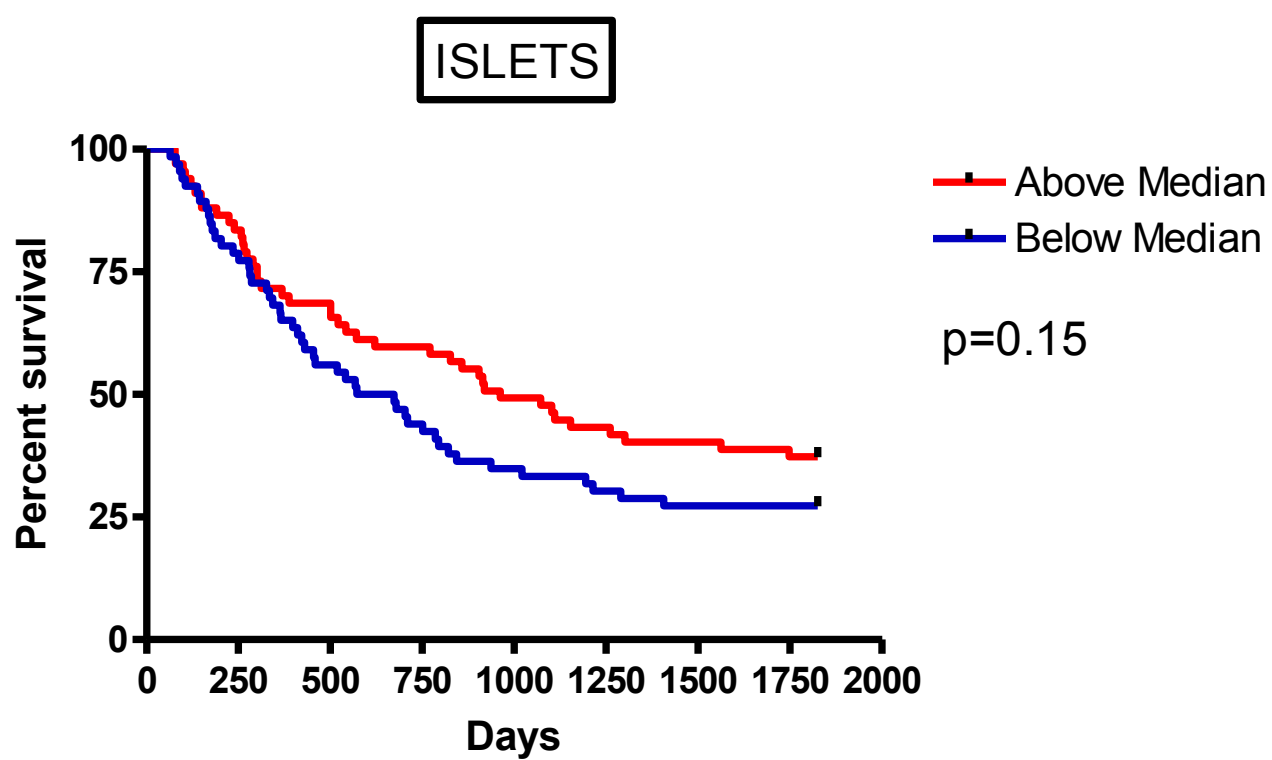
Fig 4. Kaplan-Meier five year survival curve for TNFα densities in the tumour
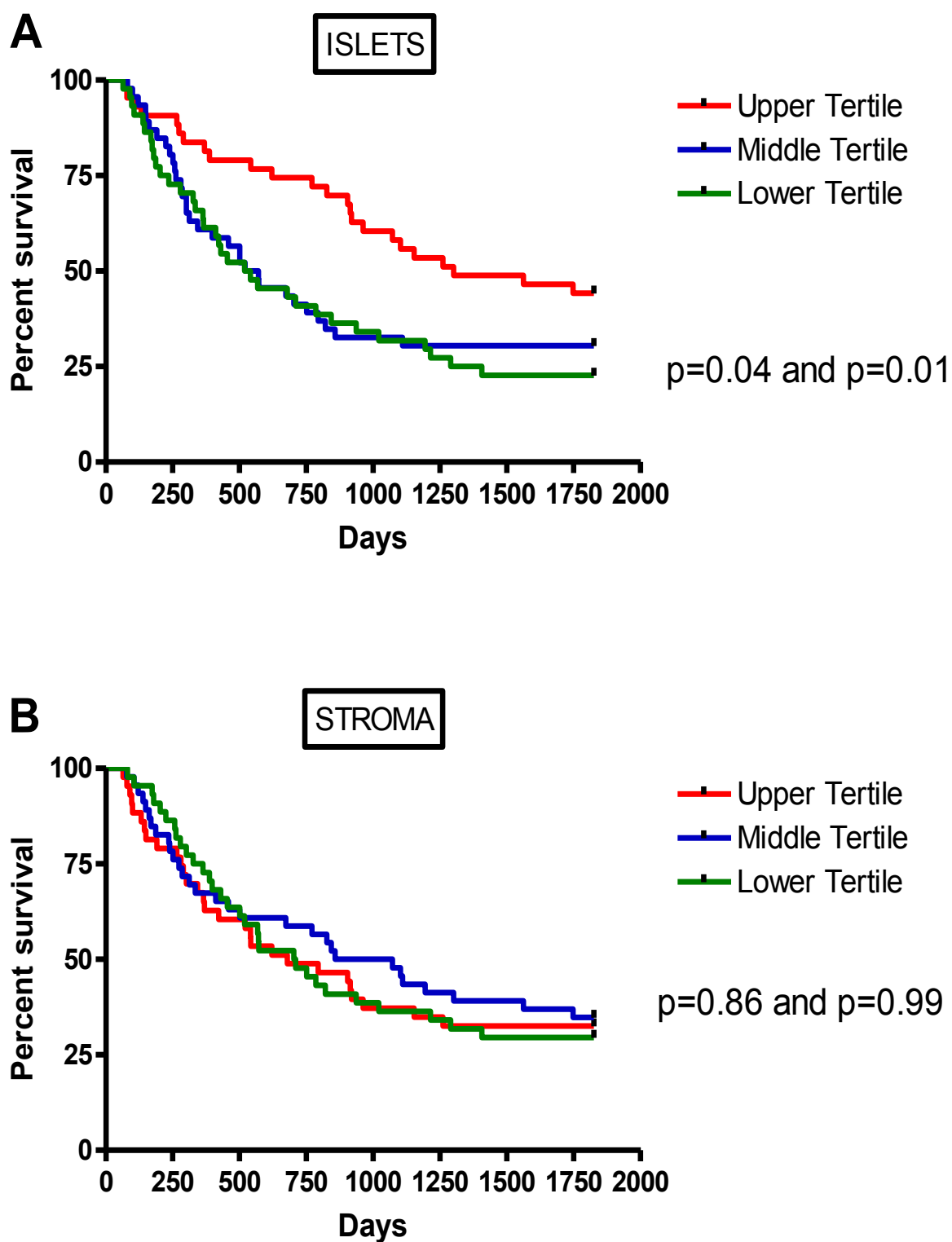
islets

Fig 5. Kaplan-Meier five year survival curve for TNFα densities in the tumour islets (A) and stroma (B) divided into high counts (upper tertile), midrange counts (middle tertile) and low counts (lower tertile).

differences in survival with respect to TNFα expression in the tumour islets were also evident within tumour stages (Fig 6). Interestingly, patients with stage IIIa disease in the top tertile of islet TNFα expression had a 5 year survival of 25% compared to 26% survival for those patients with stage I disease in the lower tertile of islet TNFα expression.

Multivariate Cox Proportional Hazards Analysis

Multivariate Cox Proportional Hazards Analysis was performed to assess whether TNFα islet or stromal counts were independently associated with survival. Only those variables that were associated with survival at a significance of $p<0.1$ were included in the multivariate analysis. Results of this analysis are shown in Table 2. Expression of TNFα in the tumour islets emerged as a significant independent predictor of survival (hazard ratio 0.994, 95% CI 0.989 to 1.000, p=0.048). Expression of TNFα in the tumour stroma emerged as a significant independent predictor of reduced survival (hazard ratio 1.007, 95% CI 1.002 to 1.011, p=0.007). There was no evidence of violation of the proportional hazards assumption.

Cellular localisation of TNFα

We have shown previously that in patients with extended survival, the expression of TNFα in mast cells and macrophages is markedly increased in the islets of patients with extended survival compared to poor survival. It is evident from Fig 2 that the difference in total TNFα expression in these 133 patients is not so marked. We have therefore analysed the cellular distribution of TNFα expressed by cells other than macrophages and mast cells in the

subset of samples previously stained as reported in Chapters 4 and 5.

Interestingly, while there is a marked difference in mast cell and macrophage-associated TNFα expression in above median survival versus below median survival patients, total islet TNFα expression is again not so marked (Fig 2).

When analyzing the cellular distribution, in the above survival patients 100% of islet TNFα is localized to mast cells or macrophages, while in below survival patients, only 28% is localized to these cells (Table 3). This is explained in part by the paucity of mast cells and macrophages in poor survival patients, but is interesting because it demonstrates that in poor survival patients there is TNFα expression by other cells, but which does not translate into extended survival.

These cells were predominantly mononuclear cells and rarely tumour epithelial cells. This suggests that TNFα is highly beneficial only when localized to macrophages and mast cells in tumour islets, and not when expressed by other cell types.

DISCUSSION

The purpose of this study was to investigate the relationship between the microanatomical expression of TNFα and survival in surgically resected NSCLC. The results demonstrate that expression of TNFα in the tumour islets is associated with a significant increase in 5-year survival, independently of other favorable prognostic factors including stage, and that TNFα expression in the stroma is an independent predictor of reduced survival.

The role of TNFα in tumour biology remains controversial with both pro-tumorigenic and anti-tumorigenic properties identified (Balkwill 2006;Balkwill 2002;Bongartz, Sutton, Sweeting, Buchan, Matteson, & Montori 2006;Lejeune &

	AMSI	AMSS	BMSI	BMSS
% of cells which were macrophages	61.8* (9.4-100)	76.9† (16.6-100)	22.2 (5-71.5)	39.7 (0-61.9)
% of cells which were mast cells	45.2# (20.3-58.4)	54.9¶ (0-79.1)	5.6 (0-100)	10.5 (0-55)
Estimated % of other cell types	0	0	72	50

Table 3. Assessment of the percentage of cell types expressing TNFα in patients with NSCLC in above median survival patients in the islets (AMSI) and stroma (AMSS) and below median survival patients in the islets (BMSI) and stroma (BMSS). *p<0.001 compared to BMSI; †p<0.001 compared to BMSS; #p<0.001 compared to BMSI; ¶p<0.001 compared to BMSS. Median values with (range)

Ruegg 2006;Mocellin, Rossi, Pilati, & Nitti 2005). Two small previous studies of patients with NSCLC suggested TNFα expression had little relationship to clinical outcome, but these did not distinguish between expression in tumour stroma and epithelial islets. The biology of these two tumour compartments demonstrates profound differences in matrix composition, cellular content and vascularity. Our previous studies (Chapters 4 and 5;Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005) have demonstrated the importance of anatomical microlocalisation in terms of potential sites for cytotoxicity against tumours. The key findings of these studies was that tumour epithelial islets are the likely site of host cytotoxic responses against tumour progression because patients with extended survival have infiltration of their tumour islets with mast cells and macrophages (Chapters 4 and 5;Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). Many cytokine-dependent effects are mediated through localized cell-cell contact, including the presentation of membrane-bound TNFα to TNFα-receptor+ cells (Chen & Goeddel 2002). The effects of cytokines including TNFα on tumour stroma versus islets are therefore likely to vary profoundly depending on the site of release, and the cell-cell interactions between TNFα-producing cells and the cells they interact with. We therefore analysed TNFα expression with close attention to expression in the stroma versus the tumour islets. In keeping with biological relevance of this concept, patients with AMS survival had greater immunoreactive TNFα expression in their tumour islets compared to those with BMS, and there was a positive correlation between increasing islet TNFα+ cell density and survival. In addition, when our patient cohort was divided into tertiles according to islet TNFα+ cell density we observed that patients with high expression (upper tertile) had

significantly improved 5-year survival. Thus increasing expression of TNFα in the tumour islets was associated with a significant increase in 5-year survival, independently of other favorable prognostic factors. Conversely, although the density of TNFα+ cells in the stroma was similar in AMS and BMS patients, TNFα expression in the stroma emerged as an independent predictor of reduced survival following Cox regression analysis. These results indicate that the micro-anatomical location of TNFα expression is potentially critical in determining its role in tumour biology.

Tumour stage is a key determinant of survival following surgery for NSCLC (Mountain 1997). We were able to show that the relationship between survival and islet TNFα expression was evident within tumour stages. Patients with stage I disease survive longer than patients with stage IIIa disease (Mountain 1997). However, when tumour islet TNFα expression was compared by stage with respect to survival, it was noted that patients with stage IIIa NSCLC and high TNFα expression had a 5-year survival of 25%, comparable to that of stage I NSCLC patients with low TNFα expression, who had a 5-year survival of 26% (Fig 6). Conversely, patients with stage I NSCLC and high TNFα expression have a 5-year survival of 46%. TNFα expression within tumour islets is therefore a key determinant of NSCLC survival within stage, even within in early stage disease.

A striking observation from this study was the cellular distribution of TNFα immunoreactivity in the tumour islets of patients with extended survival compared to those with poor survival. The macrophages which infiltrate the tumour islets in NSCLC are predominantly of the M1 cytotoxic phenotype (Chapter 4) which express TNFα. We have extended this work in the current

study and show that mast cells within the tumour islets also express TNFα. What is striking, however, is that in the subset of patients with extended survival, all TNFα islet immunoreactivity was accounted for by macrophages and mast cells. In the subset of patients with poor survival, TNFα expression in the islets was also evident, but was rarely present in mast cells or macrophages. Thus it is not only expression of TNFα in the tumour islets that is critical in determining survival, but perhaps more importantly, the type of cells which are expressing it.

The distinction of tumour islets versus stroma is important as there is evidence suggesting that TNFα located in the stroma contributes towards tumour proliferation via angiogenesis (Szlosarek, Charles, & Balkwill 2006). In support of this, although there was no difference in the stromal density of TNFα+ cells in AMS versus BMS patients, increasing stromal TNFα expression emerged as an independent predictor of worse survival. Whether this is due to the effects of TNFα on the stroma or a reflection of the inability of potentially beneficial TNFα-expressing macrophages and mast cells to infiltrate the islets is not known. The latter could be explained by an inappropriate chemokine repertoire released by the tumour stroma or even by a physical barrier, such as a thickened basement membrane, between the stroma and tumour islets.

The results of this study add to our previous work and have important clinical implications. We have shown that the density of TNFα+ cells in tumour islets is a predictor of extended survival in NSCLC following surgery. Its localisation to macrophages and mast cells in the tumour islets is the key factor relating to improved prognosis, and it seems unlikely that anti-TNFα strategies will be beneficial to such patients. In contrast, in poor prognosis patients whose

tumour islets contain relatively few TNFα+ macrophages and/or mast cells, anti-TNFα strategies may be worthy of further study as stromal expression is an independent predictor of poor survival. In view of the marked microanatomical and immunological heterogeneity within the tumour microenvironment in NSCLC, it is essential that attention is paid to this principle in future immunomodulatory trials in this disease. Targeting subphenotypes of disease with immunopathology predicted to respond to the intervention may then lead to the development of better anti-neoplastic therapeutic strategies.

Chapter 7

Non-macrophage expression

of markers associated with M1 and M2

macrophages in non-small cell lung cancer

In Chapter 4 we have demonstrated that macrophages within NSCLC tumour islets are predominantly of the cytotoxic M1 phenotype and are associated with extended survival. This supports the view that the survival advantage conferred by tumour islet macrophage infiltration is related to their cytotoxic potential. The panel of markers that were investigated in Chapter 4 are also known to be expressed by cells other than macrophages (see Table 1). CD163 is expressed by dendritic cells (Maniecki et al. 2006), HLA-DR by NK cells (Carrega, Morandi, Costa, Frumento, Forte, Altavilla, Ratto, Mingari, Moretta, & Ferlazzo 2008), and MRP 8/14 by neutrophils (Robinson & Hogg 2000). TNFα is expressed by lymphocytes (Scheurich et al. 1987), mast cells (Bradding, Walls, & Holgate 2006), endothelial cells (Lamas et al. 1991), adipose tissue (Fantuzzi 2005), fibroblasts (Saika et al. 2006). From the findings of the studies presented in Chapters 5 and 6, the majority of non-macrophage expression of TNFα in the tumour islets is by mast cells. However, this does not appear to be the case in the stroma. iNOS (Wink et al. 1998) and VEGF (Ferrara & Kerbel 2005) are expressed by tumour cells.

Thus, it was hypothesised that the cell type expressing a particular marker, along with its microlocalisation, influences the role of that particular marker. Therefore, in this study it was our aim was to investigate the non-macrophage expression of these markers with respect to microlocalisation and survival in NSCLC.

Marker	Non-macrophage expression
CD163	dendritic cells
HLA-DR	B cells and activated T cells
iNOS	tumour cells
MRP 8/14	neutrophils
TNFα	lymphocytes, mast cells, endothelial cells, adipose tissue and fibroblasts
VEGF	tumour cells

Table 1. Non-macrophage cell types expressing markers associated with M1 and M2 macrophages

Patients & Methods

Study Population

The tissue specimens evaluated were from 40 patients selected for the study based on their survival, without knowledge of their previous tumour cell counts. 20 patients had ES (mean 92.7 months, S.E.M.7.2), and 20 patients had PS (mean 7.7 months, S.E.M. 0.7). Patient characteristics are shown in Table 2.

Immunohistology

Mouse antihuman macrophage CD68 mAb (clone PGM1; Dako Cytomation, Ely, Cambridgeshire, United Kingdom) was used as a specific marker for macrophages. Antibodies for phenotypic analysis were all mouse antihuman mAb as follows: i) CD163 mAb (clone 10D6, Novocastra, United Kingdom), ii) HLA-DR mAb (clone TAL.1B5; Hycult biotechnology, the Netherlands), iii) iNOS mAb (clone 2D2-B2; R&D systems, Abingdon, United Kingdom), iv) MRP 8/14 mAb (clone 27E10; Bachem Distribution Services, Germany), v) TNFα mAb (clone P/T2; Abcam, Cambridge, United Kingdom) and vi) VEGF mAb (clone 14-124; Abcam, Cambridge, United Kingdom). Immunostaining for CD68 and each individual phenotype marker was performed using the Envision double-stain kit (Dako Cytomation) according to the manufacturer's instructions and as described previously (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). Thus, six slides were prepared for each patient – CD68 versus CD163, CD68 versus HLA-DR, CD68 versus iNOS, CD68 versus MRP 8/14, CD68 versus TNFα, and CD68 versus VEGF. CD68 was developed with peroxidase and DAB (brown reaction product), and each

Characteristic	Extended Survival	Poor Survival
No. of patients	20	20
Age – years	69.1$\pm$1.8	69.6$\pm$1.6
Male sex – no. (%)	16 (80)	11 (55)
Year of surgery – no. (%)		
1991	0 (0)	1 (5)
1992	3 (15)	1 (5)
1993	2 (10)	1 (5)
1994	1 (5)	1 (5)
1999	14 (70)	16 (80)
Tumour stage – no. (%)		
1	13 (65)	13 (65)
2	5 (25)	3 (15)
3a	2 (10)	3 (15)
3b and 4	0 (0)	1 (5)
Histology – no. (%)		
Squamous	14 (70)	10 (50)
Adenocarcinoma	4 (20)	5 (25)
Large cell	0 (0)	3 (15)
Other	2 (10)	2 (10)
Tumour Grade – no. (%)		
Well	2 (10)	0 (0)
Moderate	8 (40)	1 (5)
Poor	11 (55)	18 (90)
Not recorded	1 (5)	1 (5)
Adjuvant Chemotherapy (%)	0 (0)	0 (0)
Radiotherapy (%)	3 (15)	1 (5)
Palliative Radiotherapy (%)	2 (10)	1 (5)
Survival – months	92.7$\pm$7.2	7.7$\pm$0.7

Plus-minus values are means $\pm$ SEM

Table 2. Patient Characteristics

phenotype marker with alkaline phosphatase and fast red (red reaction product).

Sections were then counterstained with haematoxylin and mounted in an

aqueous mounting medium (BDH Chemicals Ltd, Poole, United Kingdom).

Appropriate isotype controls were performed where the primary antibodies were

replaced by irrelevant mouse mAb of the same isotype and at the same

concentration as the specific primary mAb.

Results

Patient Characteristics

These are shown in Table 2.

Validation of Analysis

Clear and distinguishable staining was evident for both CD68 and each

phenotype marker and double-stained cells were readily identifiable (Chapter 4

Fig 1B-F). Appropriate isotype controls were negative. Cell counts were

repeated and an intraclass correlation coefficient was calculated as 0.998 (p <

0.001).

Cellular Distribution

There was less non-macrophage expression of CD163 in the tumour islets of

ES patients compared with PS patients (3.9 versus 5.2 cells/mm^2) and also of

VEGF (2.1 versus 11.4 cells/mm^2) (p=0.39 and p<0.001, respectively) but more

non-macrophage expression in the islets of ES compared to PS patients of

HLA-DR (111.7 versus 15.9 cells/mm^2), iNOS (19.7 versus 9.6 cells/mm^2), MRP

8/14 (6.3 versus 2.2 cells/mm^2) and TNFα (25.8 versus 14.6 cells/mm^2) (p <0.001, p=0.02, p=0.02 and p=0.25 respectively) (Figs 1A and B). There was significantly less non-macrophage expression of CD163 in the islets of ES patients compared with non-macrophage expression of HLA-DR, iNOS and TNFα (p<0.001, p<0.001 and p=0.003, respectively) (Fig 1A). There was also significantly less non-macrophage expression of VEGF in the islets of ES patients compared to HLA-DR, iNOS, MRP 8/14 and TNFα (p<0.001, p<0.001, p=0.04 and p<0.001, respectively).

There was more non-macrophage expression of CD163 in the stroma of ES patients compared with PS patients (45.9 versus 22.2 cells/mm^2) (p=0.04) and less non-macrophage expression in the stroma of ES compared to PS patients of VEGF (18.6 versus 20.1 cells/mm^2), HLA-DR (22.6 versus 66.6 cells/mm^2), iNOS (6.4 versus 17.7 cells/mm^2) and MRP 8/14 (3.9 versus 9.8 cells/mm^2) and TNFα (10.7 versus 22.9 cells/mm^2) (p=0.25, p=0.16, p=0.002, p=0.01 and p=0.01 respectively) (Figs 1C and D). There was significantly more non-macrophage expression of CD163 in the stroma of ES patients compared with non-macrophage expression of iNOS, MRP 8/14 and TNFα (p<0.001) (Fig 1C). There was also significantly more non-macrophage expression of VEGF in the stroma of ES patients compared to iNOS, MRP 8/14 and TNFα (p=0.003, p<0.001 and p=0.03, respectively).

In the ES group there was more non-macrophage expression of CD163 and VEGF (p<0.001) in the stroma compared to the islets and conversely more non-macrophage expression of HLA-DR, iNOS, MRP 8/14 and TNFα in the islets compared to the stroma (p=0.002, p<0.001, p=0.08 and p=0.05) (Figs 1A and C).
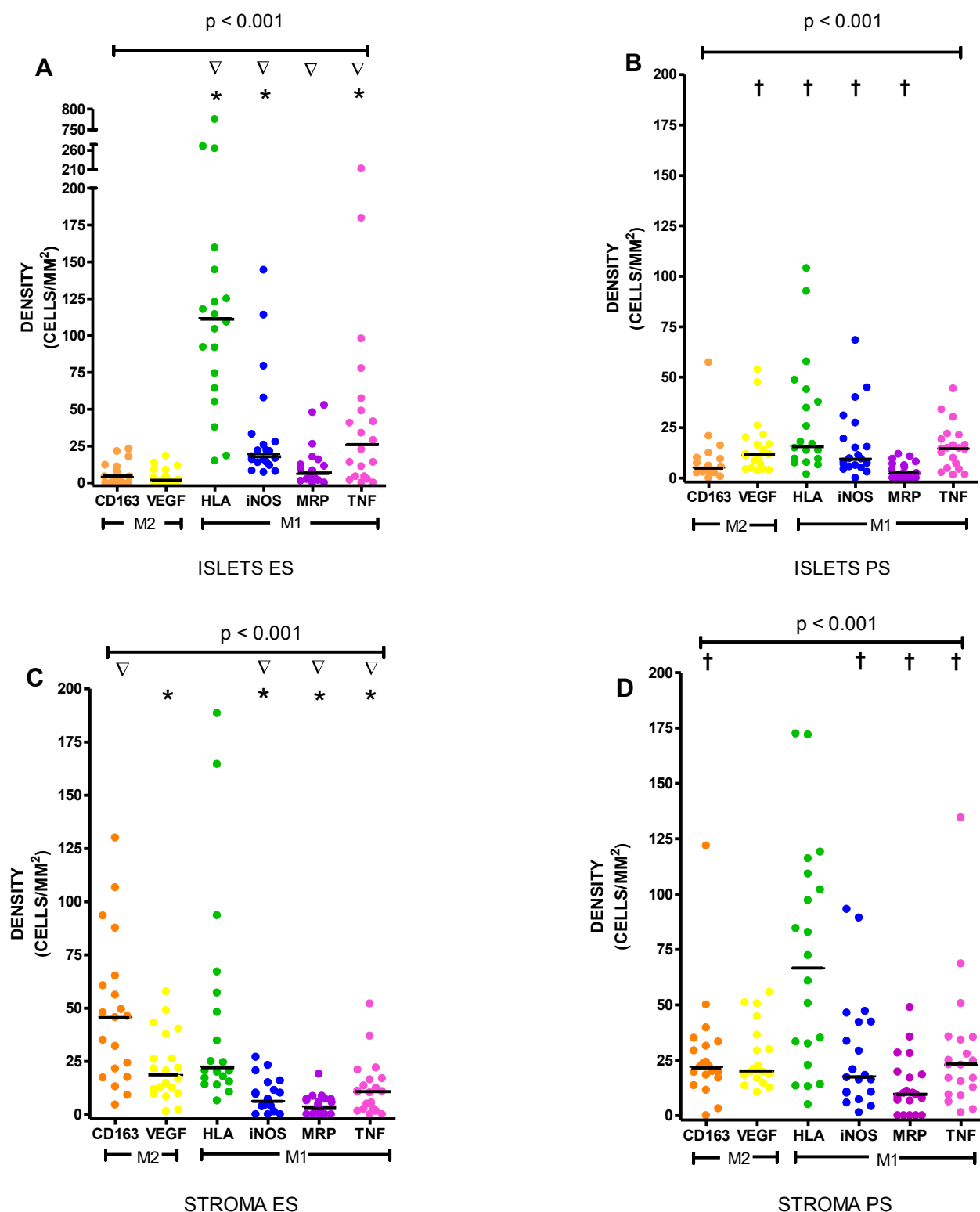
Fig 1. Non-macrophage double-stain densities in the islets in (A) extended survival (ES) and (B) poor survival (PS) and in stroma in (C) ES and (D) PS patients. * represents a significant difference (p<0.05) in expression compared with CD163. ∇ represents a significant difference (p<0.05) in expression compared with VEGF. † represents a significant difference (p<0.05) between the corresponding macrophage marker in the ES group.

Kaplan-Meier Survival Analysis

For further analysis, the data were divided into two groups above and below the median cell-count values and Kaplan-Meier survival curves were plotted. Looking at non-macrophage-associated staining for each marker, there was an inverse relationship between tumour islet density and survival for CD163 and VEGF (p=0.41 and p<0.001, respectively), and significant associations for HLA-DR (p<0.001), iNOS (p=0.003) and MRP 8/14 (p=0.04) but not TNFα (p=0.45) (Fig 2). There was an association between survival and tumour stroma non-macrophage CD163 density (p=0.005), but an inverse relationship for VEGF, HLA-DR, iNOS, MRP 8/14 and TNFα (p=0.90, p=0.31, p=0.11, p=0.02 and p=0.04, respectively) (Fig 3).

DISCUSSION

Our group has shown previously that macrophage infiltration of tumour islets in surgically resected NSCLC confers a marked survival advantage independently of tumour stage, while increasing numbers of macrophages in the tumour stroma are associated with a worse prognosis (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). We have shown in Chapter 4 that there are two distinct macrophage phenotypes in NSCLC tissue: M1 macrophages that express HLA-DR, iNOS, MRP 8/14 and TNFα, and M2 macrophages that express CD163 and VEGF (14). We also found that macrophages within NSCLC tumour islets are predominantly of the cytotoxic M1 phenotype and are associated with extended survival. This study aimed to examine non-macrophage expression of markers associated with M1 and M2 macrophages in NSCLC.
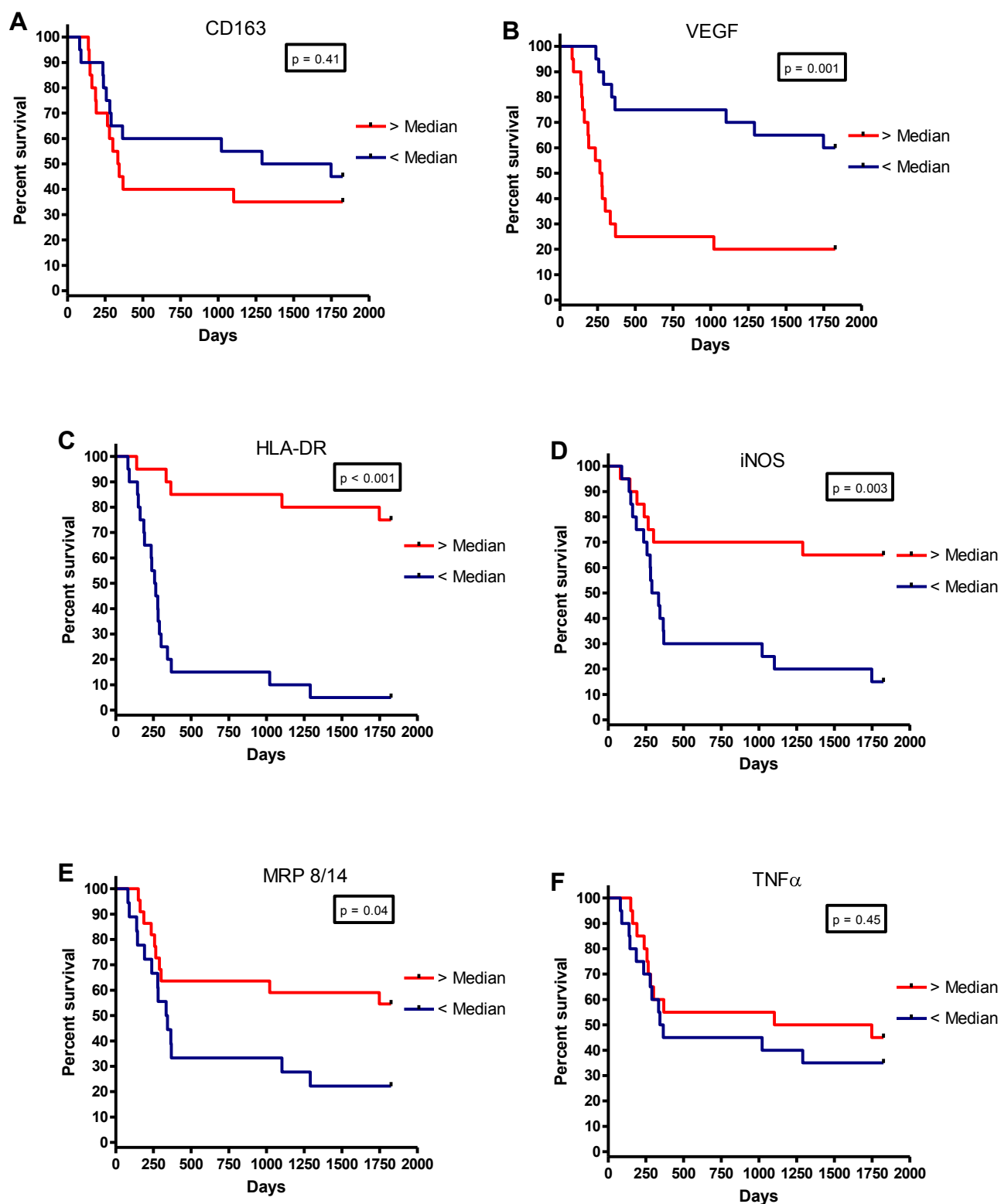
Fig 2. Kaplan-Meier five year survival curves for non-macrophage densities in the tumour islets for (A) CD163, (B) VEGF, (C) HLA-DR, (D) iNOS, (E) MRP 8/14 and (F) TNF.
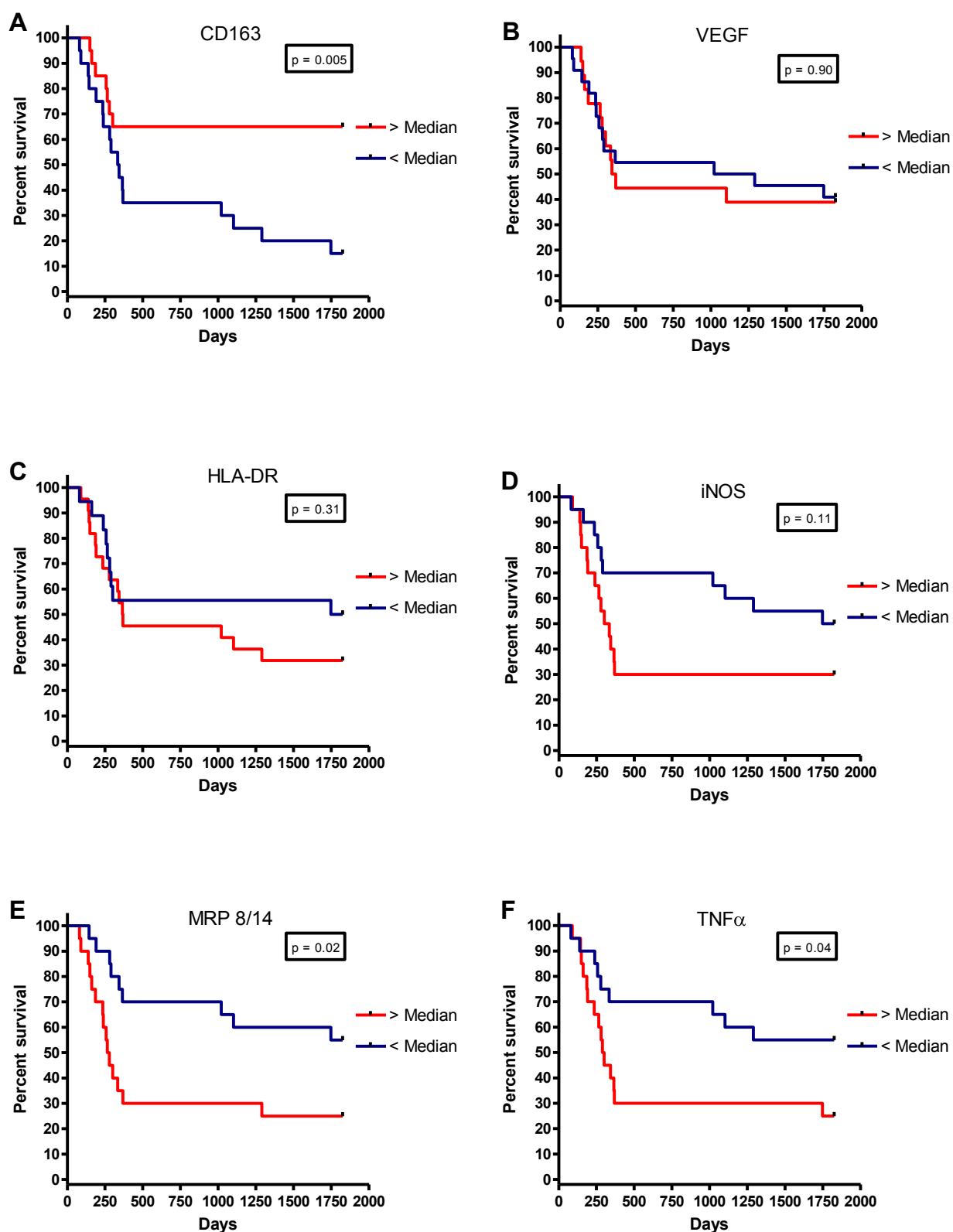
Fig 3. Kaplan-Meier five year survival curves for non-macrophage densities in the tumour stroma for (A) CD163, (B) VEGF, (C) HLA-DR, (D) iNOS, (E) MRP 8/14 and (F) TNF.

We noted that cells other than macrophages express CD163, HLA-DR, iNOS, MRP 8/14, TNFα and VEGF in both tumour islets and stroma in NSCLC. The majority of these markers demonstrated increased cellular expression in the islets of extended survival patients compared to poor survival patients suggesting a more extensive inflammatory response in patients with better outcome.

When macrophages were excluded, there was increased expression of markers associated with cellular cytotoxicity (M1= HLA-DR, iNOS, MRP 8/14 and TNFα) in the tumour islets of ES compared with PS patients. Conversely, there was increased expression of pro-tumorigenic VEGF (M2) in the islets of patients with PS compared to ES. PS patients also had more M1 markers in their stroma compared to their islets. This reinforces the idea that M1 markers must be in the islets to fulfil their cytotoxic potential. Therefore, we can postulate that in PS patients cytotoxic M1 markers are either unable to get into the tumour islets from the stroma or they are drawn into the stroma by a pro-tumorigenic mechanism.

Another interesting observation was that TNFα expression by non-macrophages did not mirror macrophage expression in the islets of extended survival patients (Chapter 4). Some studies previously have suggested that TNFα is in fact a marker of poor prognosis and tumour progression. Our findings in Chapters 4 and 6 add weight to the argument that macrophages play a key role in cytotoxicity against tumours, given that when TNFα expression occurs by macrophages in the tumour islets as opposed to other cells, there is a more favorable prognosis. We have assessed mast cell expression of TNFα (Chapter 5) and from that study can state that the majority of non-macrophage

expression of TNFα in NSCLC in extended survival patients is from mast cells (Chapter 6 Table 3).

We noted increased non-macrophage expression of CD163 in the stroma compared to islets and ES particularly had low expression in their islets. Dendritic cells are known to express CD163 (Maniecki, Moller, Moestrup, & Moller 2006). Bergeron has suggested that the function of dendritic cells is inhibited in NSCLC (Bergeron et al. 2006) suggesting their importance in the immune response to cancer. We also noted comparatively high non-macrophage expression of HLA-DR in the islets of ES patients. It is known that HLA-DR is expressed by NK cells and it is therefore possible that the survival advantage conferred by high non-macrophage expression of HLA-DR relates to the presence of NK cells in the tumour islets. Again this is compatible with our previous findings suggesting that when cytotoxic cells, for example macrophages (Chapter 4), are present in the tumour islets, patients have improved survival.

With respect to VEGF, it is recognised to be a marker of angiogenesis, expressed by tumour cells. Our results demonstrate that patients with high expression of VEGF in their tumour islets have poor survival. This suggests that patients predisposed to factors which lead to angiogenesis in their tumour islets are unable to amount a sufficient cytotoxic response leading to cancer progression and ultimately, a poor prognosis. It is also possible that patients with increased numbers of tumour cells in their islets have a worse prognosis as these cells express VEGF.

In summary, we have shown that cells other than macrophages express markers associated with M1 and M2 macrophages, and that they may make an important contribution to cytotoxic and pro-tumorigenic responses in NSCLC.

Crucially, cytotoxic markers appear to need to be present in the tumour islets in order to fulfil their cytotoxic potential.

Chapter 8

Chemokine receptors CXCR1-5 & CCR1

In non-small cell lung cancer

We have shown previously that the microlocalisation of immune cells is important with respect to cytotoxicity against tumour cells (Chapters 4-7) (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). In particular, when macrophages and mast cells are located within tumour islets a significant improvement in patient survival is noted. It is not clear why some patients have high levels of potentially cytotoxic cells in their tumour islets and others do not, therefore an improved understanding of chemoattractant pathways may lead to the development of novel chemotherapeutic agents.

IL-8 (CXCL8) is a mediator of angiogenesis in LC (Smith et al. 1994) and correlates with angiogenesis, tumour progression and poor survival in NSCLC (Masuya et al. 2001;Yuan et al. 2000). IL-8 has two chemokine receptors, CXCR1 and CXCR2 (Zhu et al. 2004). CXCR4 may be involved in metastasis regulation in NSCLC (Phillips et al. 2003). With respect to CXCR5, its ligand, CXCL13, has been shown to be over expressed in breast cancer (Panse et al. 2008).

It has been shown that CXCR3, along with IFN-γ, may play an important role with respect to NK cell infiltration of tumours (Wendel et al. 2008). The chemokine ligand for CCR1 is CCL5. CCL5 expression by tumour cells in patients with stage I lung adenocarcinoma has been associated with improved survival (Moran et al. 2002).

We hypothesised in this study that patients with improved survival would have high tumour islet expression of chemokine receptors known to be associated with favourable prognosis in cancer (CXCR3 and CCR1) and low islet expression of chemokines receptors associated with poor prognosis in cancer (CXCR1,2,4 and 5). The ligands for these chemokine receptors as well

as the cell types which express them are shown in Table 1 (Mahalingam & Karupiah 1999).

Patients & Methods

Study Population

The tissue specimens evaluated were from 20 patients. Of the 20 patients studied, 14 were men and average age at surgery was 72.3 years (standard deviation, 6.53; range, 60.2 to 82.4 years). Patients were divided into two groups, ES (mean ± SEM 90.8 ± 11.8 months) and PS (mean ± SEM 7.9 ± 0.8 months). Patient characteristics are shown in Table 2.

Immunohistology

Mouse antihuman antibodies were used (all R & D Systems Europe, Abingdon, United Kingdom) as follows: CXCR1 (clone 42705), CXCR2 (clone 48311), CXCR3 (clone 49801), CXCR4 (clone 44716), CXCR5 (clone 51505) and CCR1 (clone 53504). Immunostaining was performed and the chemokine receptors were developed with peroxidase and DAB (brown reaction product). Sections were then counterstained with haematoxylin and mounted in an aqueous mounting medium (BDH Chemicals Ltd, Poole, United Kingdom). Appropriate isotype controls were performed where the primary antibodies were replaced by irrelevant mouse mAb of the same isotype and at the same concentration as the specific primary mAb.

Chemokine Receptor	Chemokine Ligand	Cells expressing Chemokine Receptor
CXCR1	IL-8 = CXCL8	Neutrophils, monocytes
CXCR2	IL-8 = CXCL8	neutrophils, monocytes, eosinophils
CXCR3	CXCL4,9,10,11	activated T cells, B cells, NK cells
CXCR4	CXCL12	T cells
CXCR5	CXCL13	B cells
CCR1	CCL3, 4, 5 and 7	T cells, B cells, monocytes, eosinophils

Table 1. Chemokine receptors and their ligands, and cells which express them

Characteristic	Extended Survival	Poor Survival
No. of patients	10	10
Age – years	72.0±2.5	72.6±1.6
Male sex – no. (%)	8 (80)	6 (60)
Year of surgery – no. (%)		
1992	1 (10)	0 (0)
1994	0 (0)	1 (10)
1999	9 (90)	9 (90)
Tumour stage – no. (%)		
1	7 (70)	7 (70)
2	3 (30)	3 (30)
Histology – no. (%)		
Squamous	8 (80)	6 (60)
Adenocarcinoma	2 (20)	1 (10)
Large cell	0 (0)	1 (10)
Other	0 (0)	2 (20)
Tumour Grade – no. (%)		
Moderate	4 (40)	0 (0)
Poor	6 (60)	10 (100)
Adjuvant Chemotherapy (%)	0 (0)	0 (0)
Radiotherapy (%)	1 (10)	1 (10)
Palliative Radiotherapy (%)	1 (10)	1 (10)
Survival – months	90.8 ± 11.8	7.9 ± 0.8

Plus-minus values are means ± SEM

Table 2. Patient Characteristics

Results

Patient Characteristics

Patent characteristics are shown in Table 2. Of the 20 patients studied, 15 had died at the time of analysis. Fourteen tumours were squamous, 3 adenocarcinoma, 2 large cell, and 1 other. Fourteen were stage I and 6 stage II. No patients had additional postoperative chemotherapy and 2 had additional radiotherapy for later palliation.

Validation of Analysis

Clear and distinguishable staining was evident for the chemokine receptors (Fig 1). The number of nucleated cells with positive staining for both markers in each area were then counted manually and expressed as cells/mm^2 of stroma or tumour islets. Appropriate isotype controls were negative. In order to assess the validity of the method, area measurements and cell counts were repeated and intraclass correlation coefficients calculated. Good correlations were found for both: 0.997 (95% CI, 0.996 to 0.998, p<0.001) and 0.995 (95% CI, 0.993 to 0.997, p<0.001).

Cellular Distribution

There was more expression of CXCR2, CXCR3, CXCR4, CXCR5 and CCR1 in the tumour islets of ES compared with PS patients (median 6.5 versus 2.6, (p=0.007), 12.7 versus 3.2, (p=0.01), 6.5 versus 3.6, (p=0.32), 3.6 versus 0.4, (p=0.25) and 24.3 versus 2.4 cells/mm^2, (p=0.002), respectively) and less expression of CXCR1 in the tumour islets of ES compared with PS patients (median 3.3 versus 5.2 cells/ mm^2, (p=0.80)) (Figs 2A and B). There
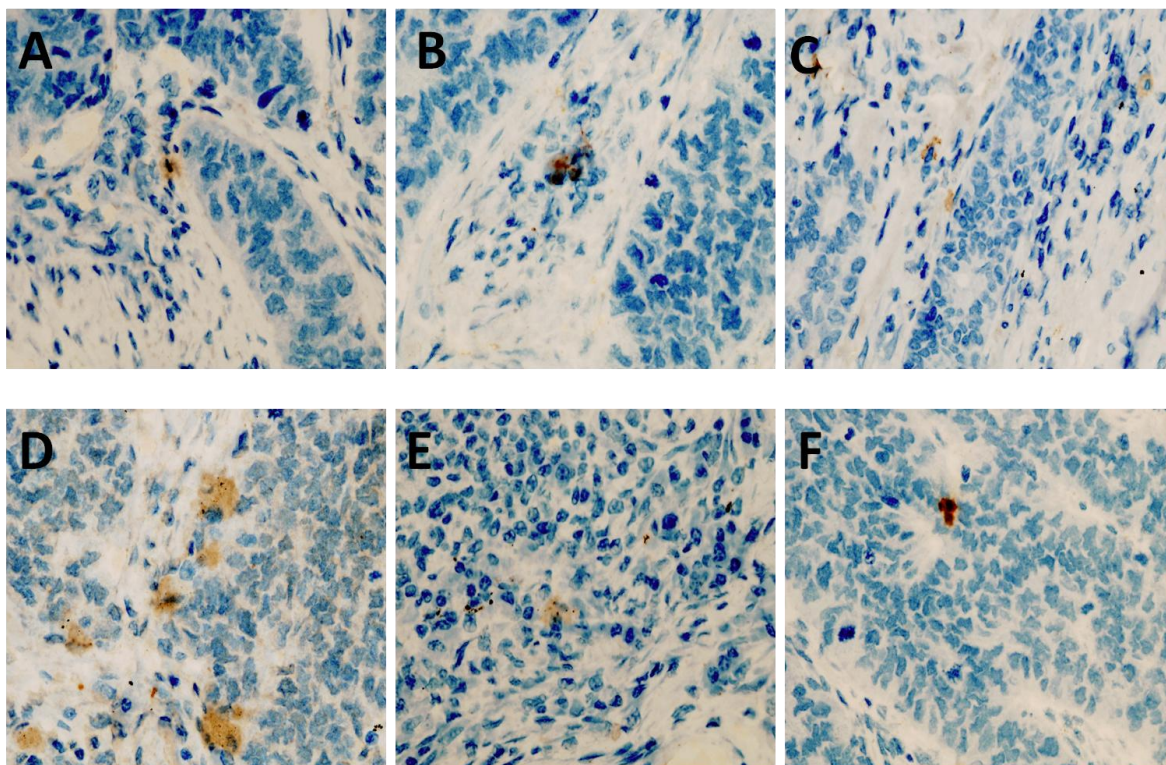
Fig 1. Immunohistology demonstrating positive chemokines receptor expression (brown) for CXCR1 (A), CXCR2 (B), CXCR3 (C), CXCR4 (D), CXCR5 (E) and CCR1 (F). Magnification x400
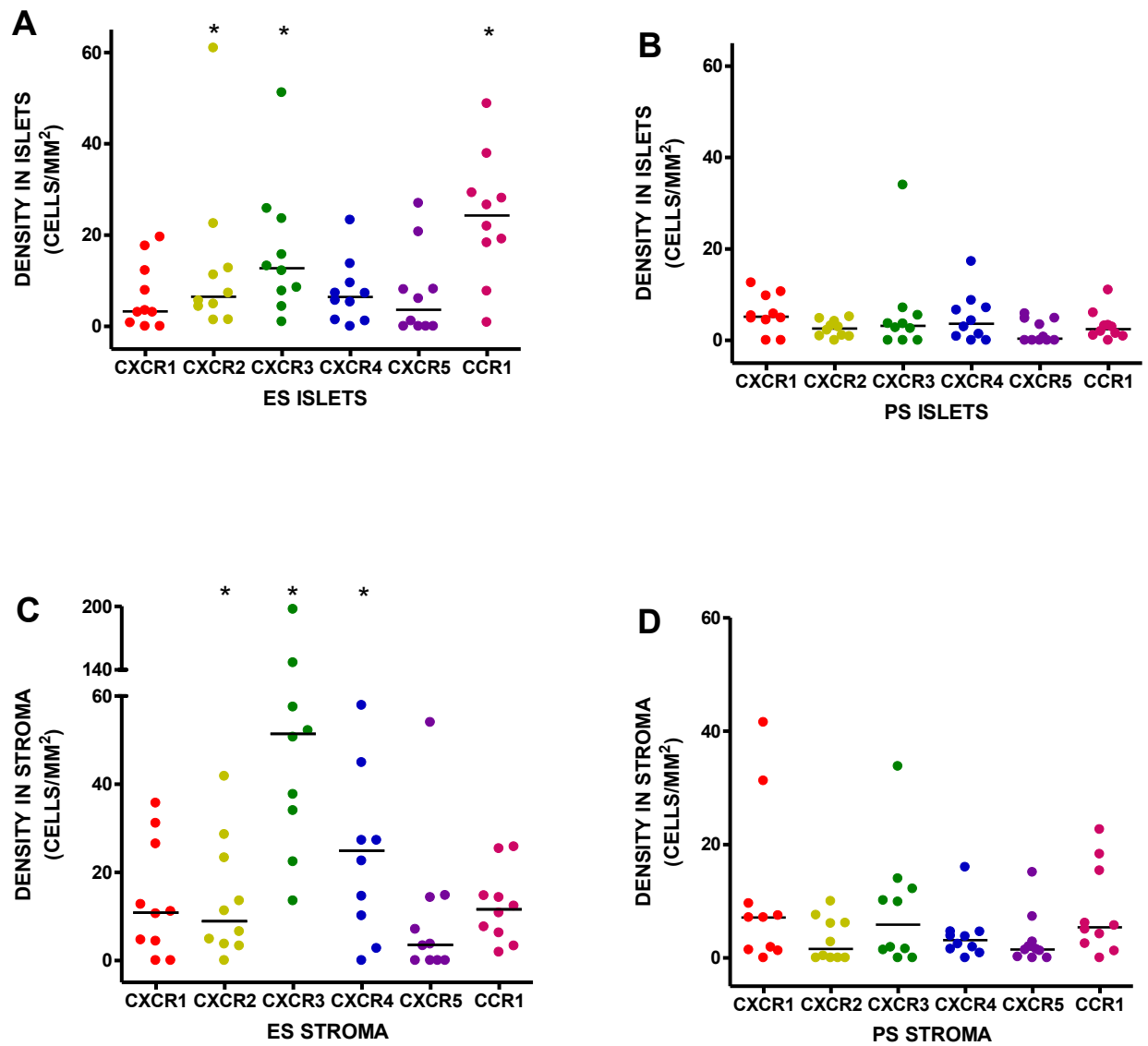
Fig 2. Chemokine receptor densities in the tumour islets for extended survival (ES) patients (A) and poor survival (PS) patients (B) and in the stroma for ES patients (C) and PS patients (D).

was more expression of all of the receptors in the stroma of ES compared with PS patients (10.9 versus 7.1, (p=0.58), 8.9 versus 1.6, (p=0.04), 51.4 versus 5.9, (p<0.001), 24.9 versus 3.1, (p=0.004), 3.5 versus 1.5, (p=0.68) and 11.6 versus 5.4 cells/mm^2, (p=0.22) for CXCR1, CXCR2, CXCR3, CXCR4, CXCR5 and CCR1, respectively) (Figs 2C and D).

Kaplan-Meier Survival Analysis

For further analysis, the data were divided into two groups above and below the median cell-count values and Kaplan-Meier survival curves were plotted. These are shown for expression of the chemokine receptors in the tumour islets (Fig 3) and stroma (Fig 4).

Correlation with macrophage counts

Chemokine receptor expressing cell counts were correlated against islet and stromal macrophage counts (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). It was noted that there were positive correlations for CXCR3 (r_s = 0.520, p=0.02) and CCR1 (r_s = 0.432, p=0.06) in the islets (Fig 5). With respect to stromal counts there were negative correlations for CXCR2 (r_s = -0.455, p=0.04), CXCR3 (r_s = -0.844, p<0.001) and CXCR4 (r_s = -0.606, p = 0.005) (Fig 6).

DISCUSSION

The purpose of this study was to investigate the expression of chemokine receptors and their microlocalisation in surgically resected NSCLC. The results demonstrate that patients with extended survival have significantly increased expression of CXCR2, CXCR3 and CCR1 in both their tumour islets compared
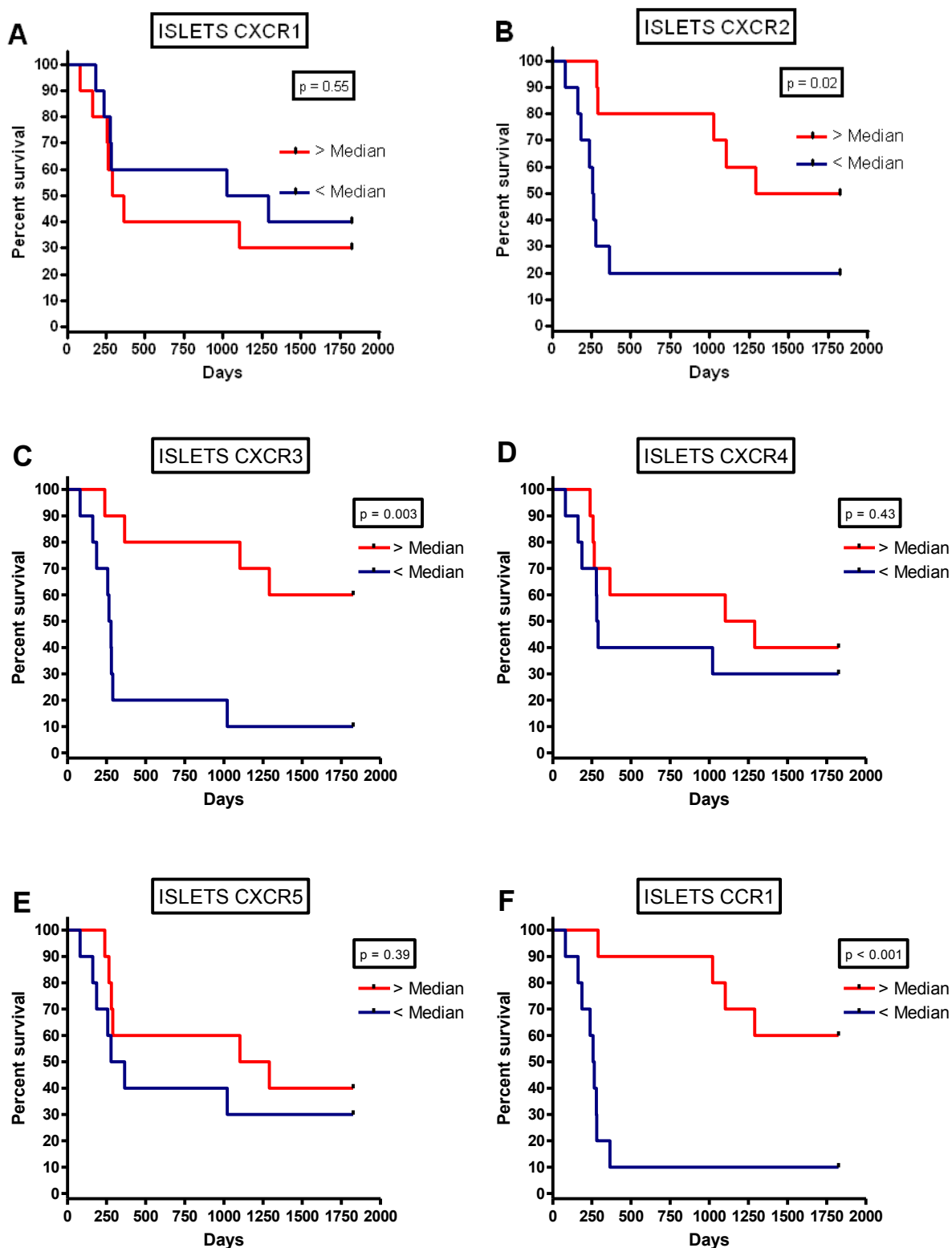
Fig 3. Kaplan-Meier five year survival curve for chemokine receptor densities in the tumour islets for CXCR1(A), CXCR2 (B), CXCR3 (C), CXCR4 (D), CXCR5 (E) and CCR1 (F)
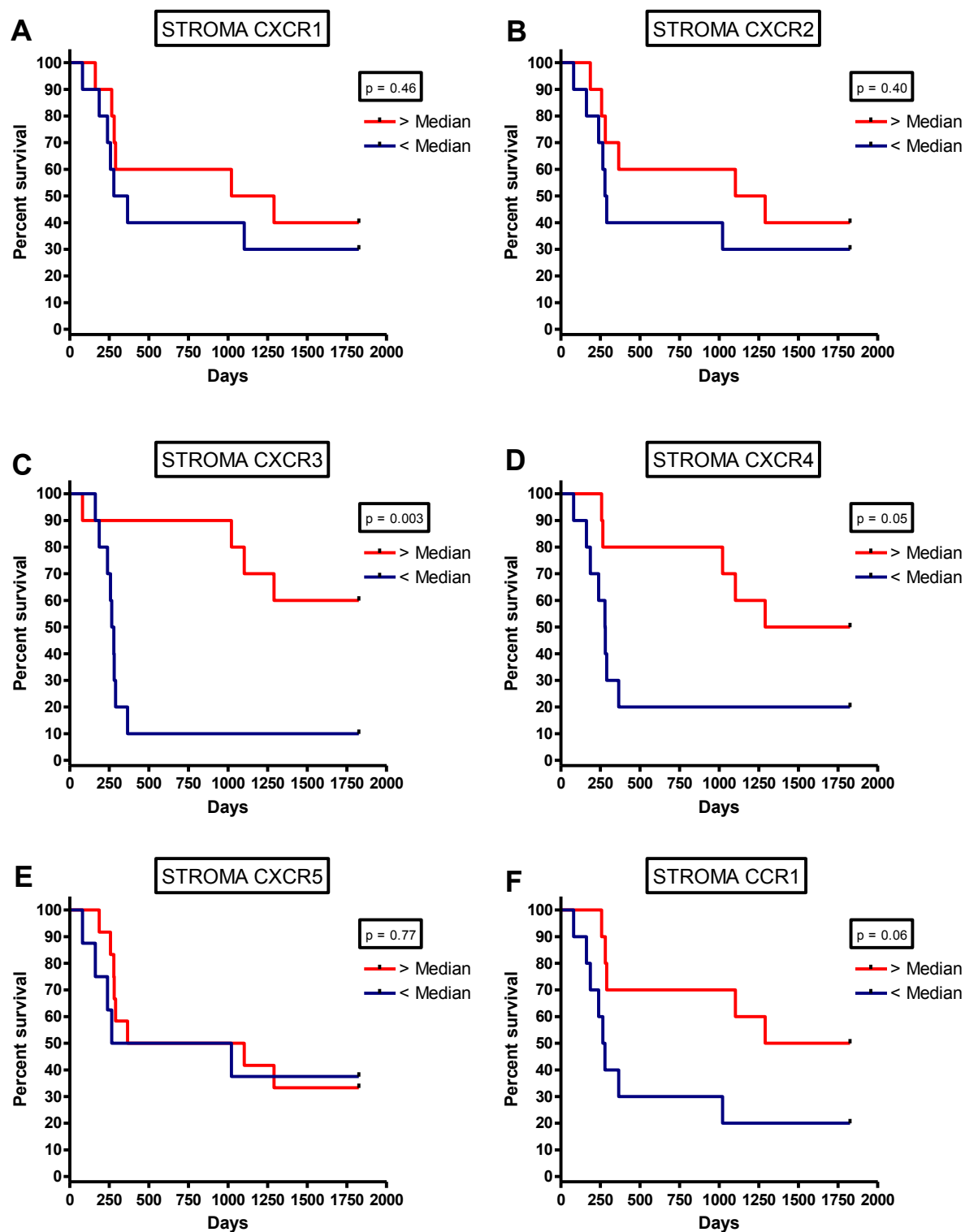
Fig 4. Kaplan-Meier five year survival curve for chemokine receptor densities in the tumour stroma for CXCR1(A), CXCR2 (B), CXCR3 (C), CXCR4 (D), CXCR5 (E) and CCR1 (F)
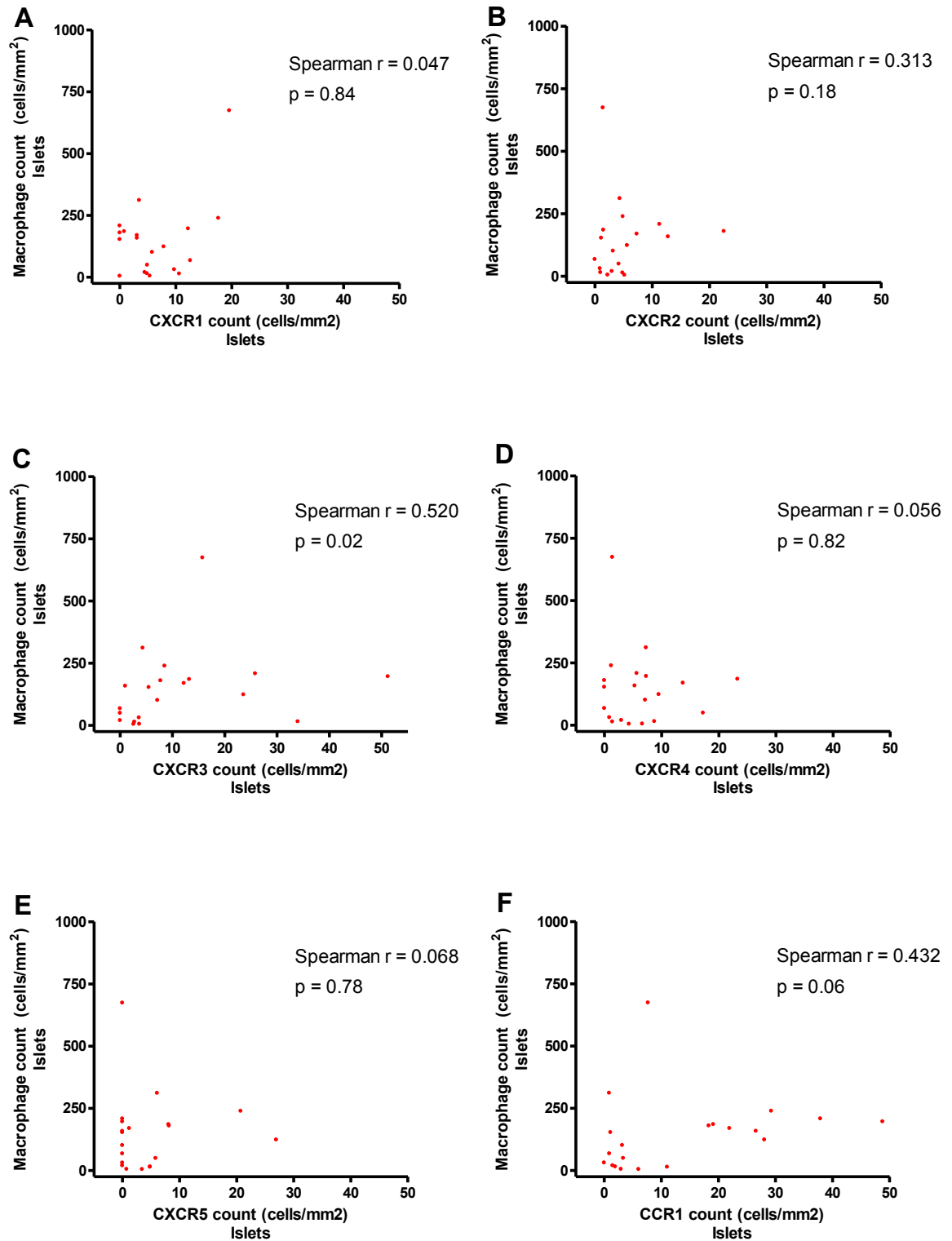
Fig 5. Correlations between chemokine receptors and macrophage cell counts in the tumour islets for CXCR1(A), CXCR2 (B), CXCR3 (C), CXCR4 (D), CXCR5 (E) and CCR1 (F)
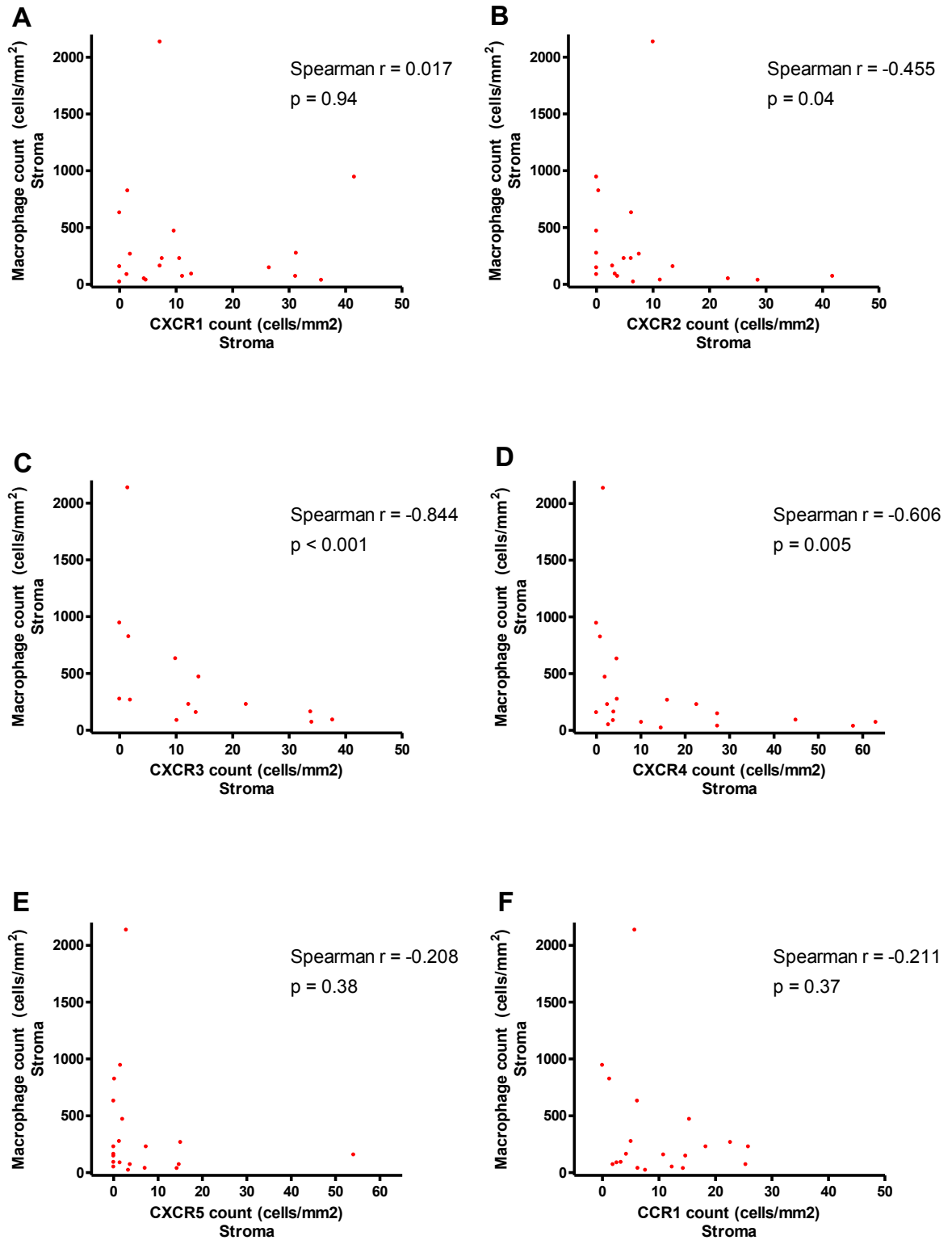
Fig 6.

Correlations between chemokine receptors and macrophage cell counts in the

stroma for CXCR1(A), CXCR2 (B), CXCR3 (C), CXCR4 (D), CXCR5 (E) and

CCR1 (F)

to patients with poor survival, as well as significantly increased expression of CXCR2, CXCR3 and CXCR4 in their stroma (Fig 2). In addition, we observed that patients with above median expression of CXCR2, CXCR3 and CCR1 in their tumour islets (Fig 3) and patients with above median expression of CXCR3 in their stroma (Fig 4) had significantly improved 5-year survival. Interestingly, when chemokine receptor expression was compared with macrophage density in the tumour islets (Fig 5), it was noted that there was a positive correlation with increasing islet macrophage count and expression of CXCR3 and CCR1.

Our group (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005) has previously demonstrated the importance of microlocalisation in terms of potential sites for cytotoxicity against tumours. In particular, tumour islets are the site of host cytotoxic responses against tumour development (Chapters 4-7). We have seen previously that patients with extended survival have predominantly M1 cytotoxic macrophages in their tumour islets (Chapter 4). Our current results provide evidence that the chemokine receptors CXCR3 and CCR1 may be involved in the chemotactic pathway for attracting M1 macrophages into the islets where they can achieve their cytotoxic potential. One of the ligands for CCR1 is CCL3 which is thought to stimulate the production and release of TNFα, a cytokine that we have demonstrated has cytotoxic potential in the tumour islets (Chapter 6). CXCR3 and CCR1 are both expressed by B and T cells suggesting that the adaptive immune system also plays a key role in cytotoxicity in the tumour islets. CXCR3 is also expressed by NK cells and interestingly in Chapter 7 it was noted that above median non-macrophage expression of HLA-DR (also expressed by NK cells) in the tumour islets is associated with a five-year survival of 74% (Chapter 7 Fig 2C),

irrespective of tumour stage implying that NK cells also play a critical role in the immune response to NSCLC.

The negative correlations that we have shown between CXCR2, CXCR3 and CXCR4 expression and macrophage counts in the stroma (Fig 6) may relate to the fact that patients with improved prognosis have lower expression of macrophages in their stroma (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). CXCR2 is expressed by neutrophils, CXCR3 by activated T and CXCR4 by T cells (Mahalingam & Karupiah 1999). Therefore less expression of these three receptors in the stroma may result in less infiltration by neutrophils and T cells. This suggests that patients with improved prognosis have lower expression of not only macrophages, but also potentially neutrophils and T lymphocytes, in their stroma. Using principals that we have demonstrated previously (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005) we can hypothesise that patients with improved survival have increased expression of neutrophils and T lymphocytes in their tumour islets where they can achieve maximum cytotoxic potential.

In summary, above median expression of the chemokine receptors CXCR2, CXCR3 and CCR1 in the tumour islets is associated with increased 5-year survival. Increased expression of CXCR3 and CCR1 correlates with increased macrophage expression in the tumour islets and therefore these receptors may be involved in the pathway that attracts cytotoxic M1 macrophages, B cells, T calls and NK cells into the tumour islets. Therefore, if a therapeutic agent had the effect of increasing CXCR3 and CCR1 expression in the islets, this may lead to an improved immune response to NSCLC and better survival.

Chapter 9

Cleaved cytokeratin 18 (a marker of apoptosis)

and Ki-67 (a marker of cellular proliferation)

expression in

non-small cell lung cancer

It has been demonstrated that the microlocalisation of immune cells, as wells as cytokines and chemokines (Chapters 4-8), influences survival for patients with resected NSCLC. In particular, the tumour islets seem to be a critical site for cytotoxic pathways. Conversely, the tumour stroma may be a site of chronic inflammation, cellular proliferation and angiogenesis. A monoclonal antibody, named M30 antibody, specifically recognises CK 18 exposed after caspase cleavage. The M30 antibody detects only the caspase-cleaved fragment of CK 18 (also called M30 antigen) to enable the detection of apoptotic cells but not necrotic or non-apoptotic cells. Ki-67 is a protein and marker of cellular proliferation (Scholzen & Gerdes 2000). The role of Ki-67 as a diagnostic marker for NSCLC remains unclear.

In this study, the microlocalisation of markers of apoptosis and cellular proliferation were assessed. The correlation between microlocalisation of these markers to the tumour islets with macrophage expression was also investigated. Given that apoptosis may relate to cytotoxicity we hypothesised in this study that patients with increased expression of a marker of apoptosis (M30 CytoDeath) in their tumour islets have a better survival and conversely, that patients with increased expression of a marker of cellular proliferation (Ki-67) in their stroma have a worse prognosis. We also hypothesised that M30 expression (apoptosis) would correlate with macrophage expression (cytotoxicity) in the tumour islets.

Patients & Methods

Study Population

The tissue specimens evaluated were from 50 patients. Of the 50 patients studied, 34 were men and average age at surgery was 68.5 years (standard deviation, 8.34; range, 45.5 to 82.4 years). Patients were divided into two groups, ES (mean ± SEM 96.1 ± 8.3 months) and PS (mean ± SEM 8.0 ± 0.7 months). Patient characteristics are shown in Table 1.

Immunohistology

Mouse antihuman antibodies were used as follows: M30 CytoDeath (clone M30, Roche Diagnostics Ltd, West Sussex, UK) as a marker of apoptosis and Ki-67 (clone MIB-1, Dako Cytomation, Ely, Cambridgeshire, UK) as a marker of cellular proliferation). Immunostaining was performed and the chemokine receptors were developed with peroxidase and DAB (brown reaction product). Sections were then counterstained with haematoxylin and mounted in an aqueous mounting medium (BDH Chemicals Ltd, Poole, United Kingdom). Appropriate isotype controls were performed where the primary antibodies were replaced by irrelevant mouse mAb of the same isotype and at the same concentration as the specific primary mAb.

Analysis and Validation of Immunostaining

The number of nucleated cells with positive staining for both markers in each area were then counted manually and expressed as cells/mm^2 of stroma or tumour islets. Analysis was repeated for 10 patients to assess repeatability and validity.

Characteristic	Extended Survival	Poor Survival
No. of patients	25	25
Age – years	67.6$\pm$1.8	69.4$\pm$1.5
Male sex – no. (%)	18 (72)	16 (64)
Year of surgery – no. (%)		
1991	0 (0)	0 (0)
1992	4 (16)	0 (0)
1993	3 (12)	3 (12)
1994	1 (4)	3 (12)
1999	17 (68)	19 (76)
Tumour stage – no. (%)		
1	16 (64)	9 (36)
2	7 (28)	6 (24)
3a	2 (8)	8 (32)
3b and 4	0 (0)	2 (8)
Histology – no. (%)		
Squamous	15 (60)	11 (44)
Adenocarcinoma	7 (28)	7 (28)
Large cell	2 (8)	2 (8)
Other	1 (4)	5 (20)
Tumour Grade – no. (%)		
Well	1 (4)	2 (8)
Moderate	7 (28)	6 (24)
Poor	16 (64)	16 (64)
Not recorded	1 (4)	1 (4)
Adjuvant Chemotherapy (%)	1 (4)	0 (0)
Radiotherapy (%)	3 (12)	3 (12)
Palliative Radiotherapy (%)	2 (8)	3 (12)
Survival – months	96.1$\pm$8.3	8.0$\pm$0.7

Plus-minus values are means $\pm$ SEM

Table 1. Patient Characteristics

Results

Patient Characteristics

Patent characteristics are shown in Table 1. Of the 50 patients studied, 39 had died at the time of analysis. Twenty-six tumours were squamous, 14 adenocarcinoma, 4 large cell, and 6 other. Twenty-five were stage I, 13 stage II, 10 stage IIIa and 2 stage IIIb/IV. One patient had additional postoperative chemotherapy and 6 had additional radiotherapy, 5 of whom had it for palliation.

Immunostaining and Validation of Analysis

Clear and distinguishable staining was evident for both Ki-67 and M30 (Fig 1). Appropriate isotype controls were negative. In order to assess the validity of the method, area measurements and cell counts were repeated and intraclass correlation coefficients calculated. Good correlations were found for both: 0.998 (95% CI, 0.997 to 0.999, $p<0.001$) and 0.995 (95% CI, 0.993 to 0.996, $p<0.001$).

Cellular Distribution

There was more expression of Ki-67 in the tumour islets of ES compared with PS patients but this was not statistically significant (median 9.6 versus 2.2 cells/mm^2, $p=0.23$) (Fig 2A). There was more expression of Ki-67 in the stroma of ES compared with PS patients (4.9 versus 0 cells/mm^2, $p=0.05$) (Figs 2B). There was no difference in expression of M30 in the tumour islets of ES compared with PS patients (median 7.8 versus 8.7 cells/mm^2, $p=0.29$).
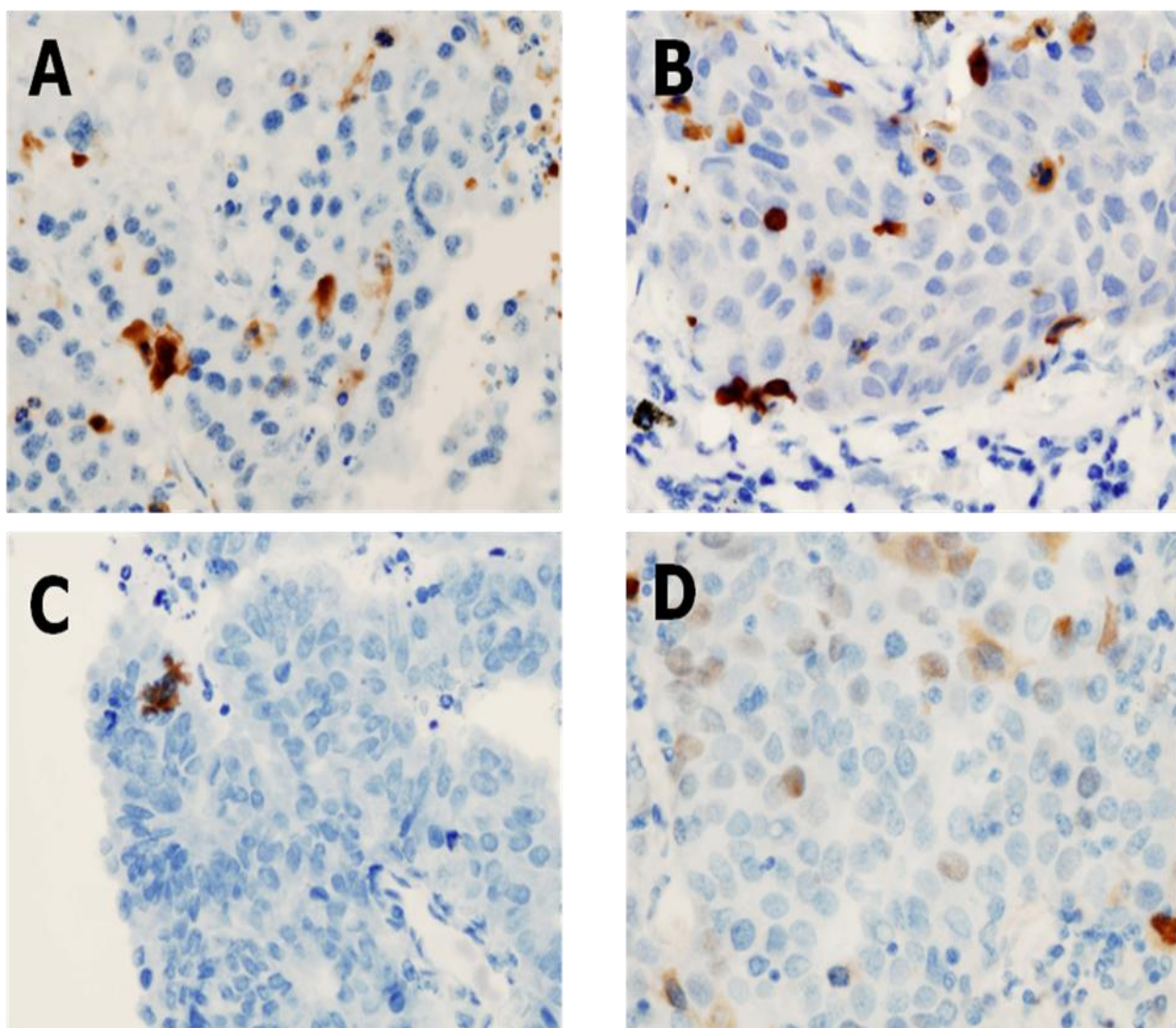
Fig 1. Immunohistology of M30 (apoptosis) in extended survival (A) and poor survival (B) and Ki-67 (cellular proliferation) in extended survival (C) and poor survival (D). Magnification x400
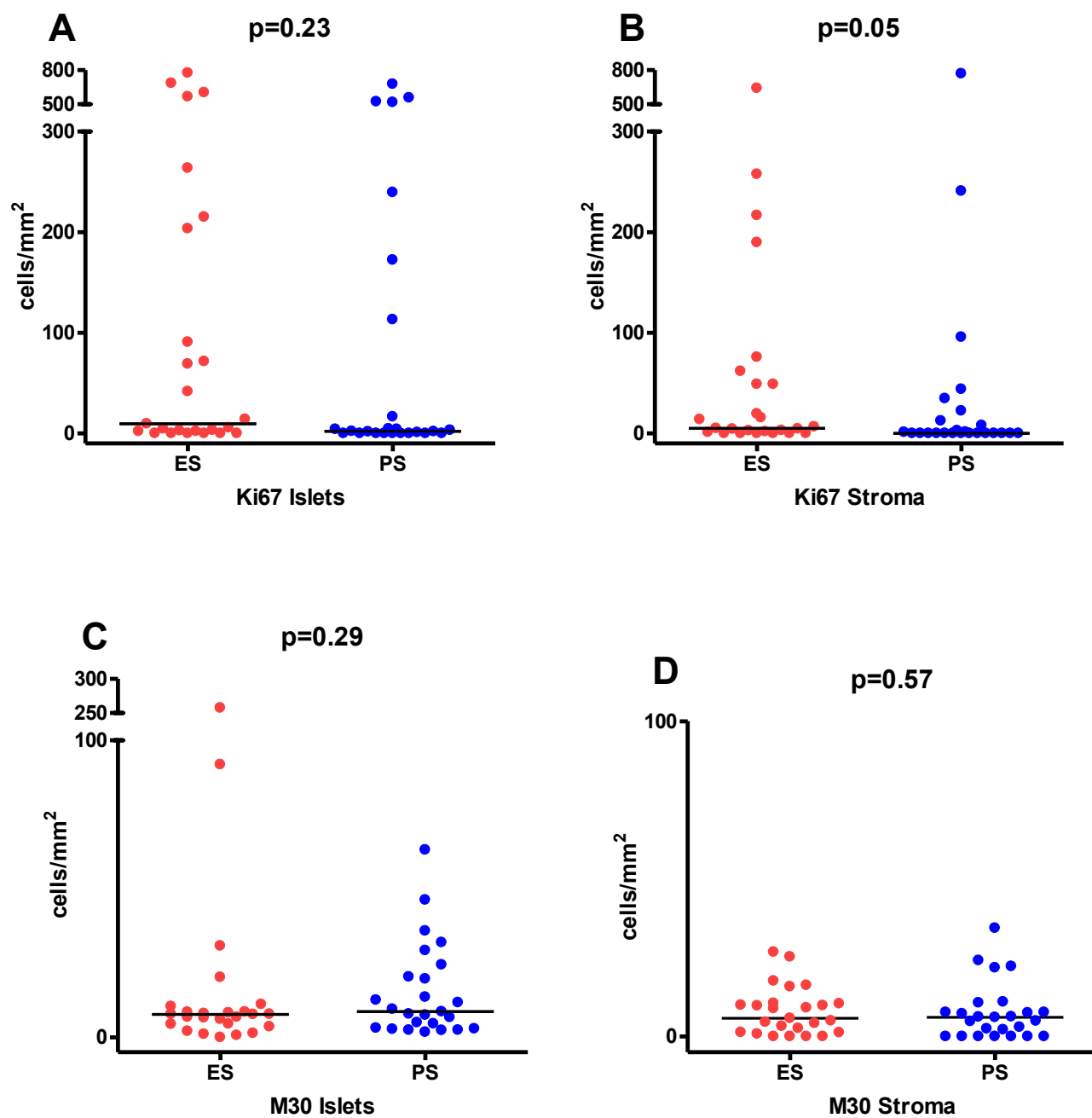
Fig 2. Ki-67 density in the tumour islets (A) and stroma (B) and M30 density in the islets (C) and stroma (D).

(Fig 2C) and also no difference in the expression of M30 in the stroma of ES compared with PS patients (5.8 versus 6.1 cells/mm^2, p=0.57) (Fig 2D).

Kaplan-Meier Survival Analysis

For further analysis, the data were divided into two groups above and below the median cell-count values and Kaplan-Meier survival curves were plotted. Looking at the expression of Ki-67, there was no association between islet density and survival but there was a positive association with stromal density (p=0.07 and p=0.03, respectively) (Figs 3A and B). There was no association between survival and tumour islet or stromal M30 density (p=0.53 and p=0.82, respectively) (Figs 3C and D).

Correlation with macrophage counts

Ki-67 and M30 expressing cell counts were correlated against islet and stromal macrophage counts (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005). It was noted that there were no significant correlations (Fig 4).

DISCUSSION

The aim of this study was to investigate the microlocalisation of apoptosis and cellular proliferation in resected samples of NSCLC, as well as correlate these processes with macrophage densities. The initial hypotheses were that patients with extended survival would have increased expression of M30 (apoptosis) in their tumour islets, patients with poor survival would have increased expression of Ki-67 in their islets, and that there would be a correlation between M30 expression and macrophage density in the islets
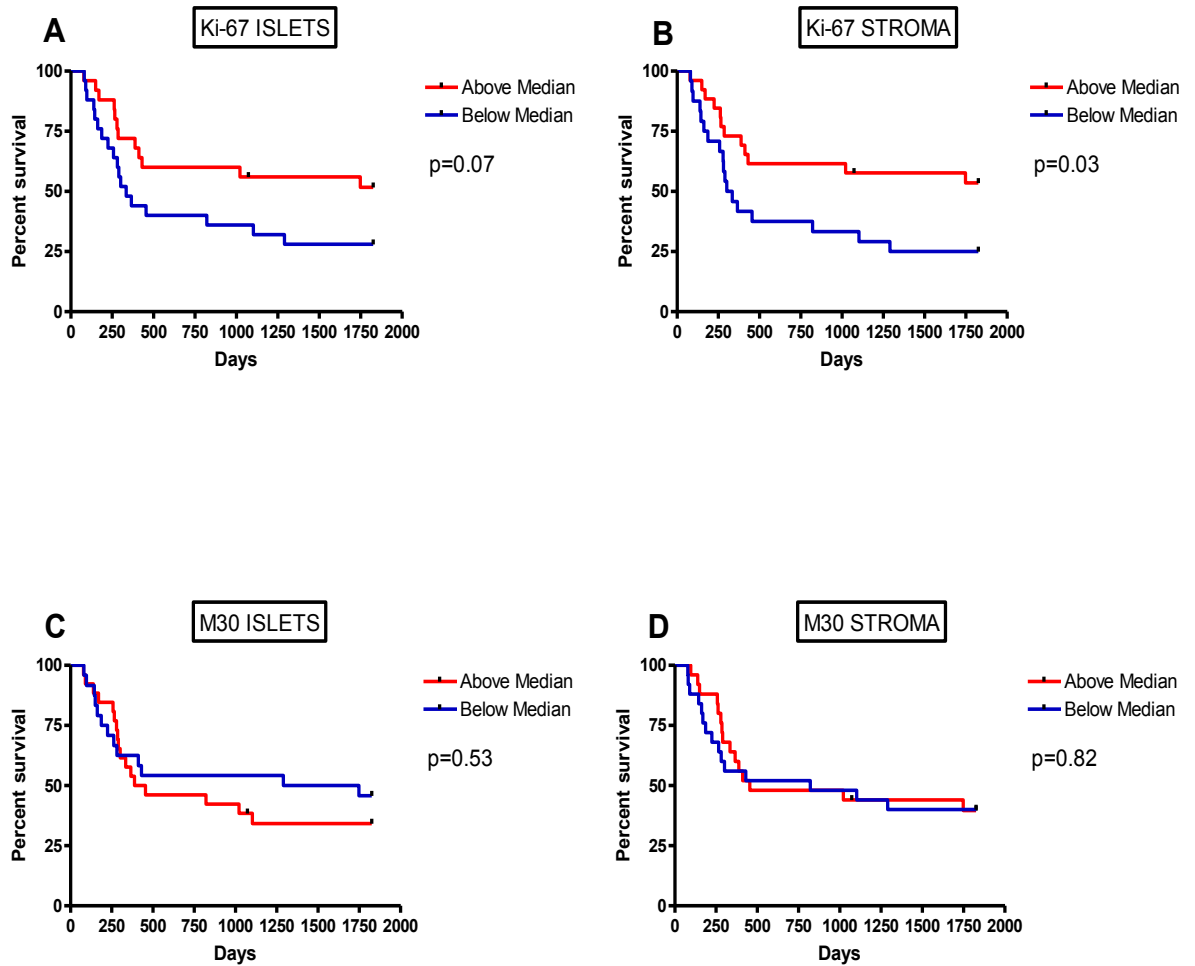
Fig 3. Kaplan-Meier five year survival curve for Ki-67 densities in the tumour islets (A) and stroma (B) and M30 densities in the islets (C) and stroma (D).
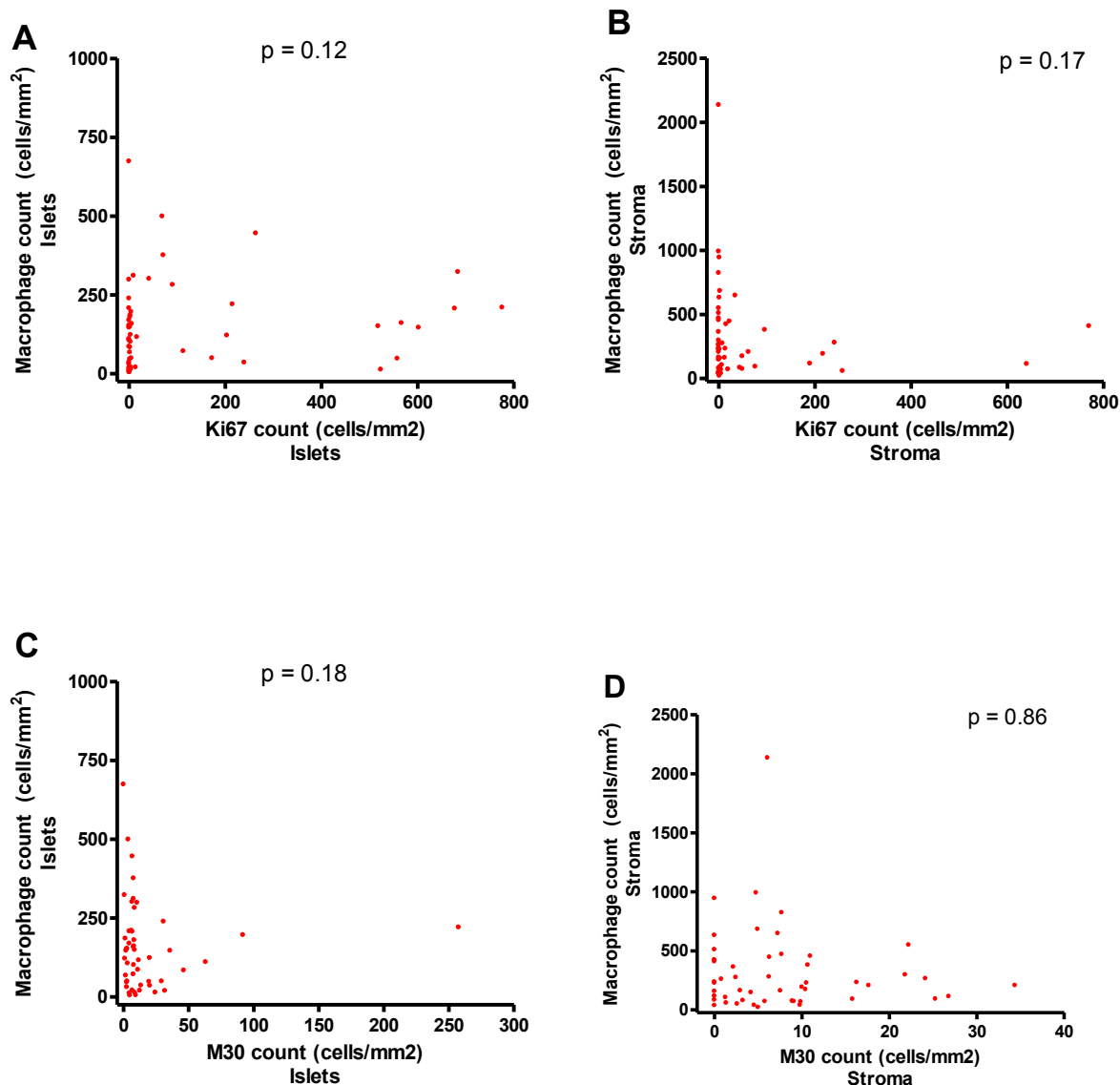
Fig 4. Correlations between macrophage cell counts and Ki-67 in the tumour islets (A) and stroma (B), and M30 in the tumour islets (C) and stroma (D).

It was found that there was no association between survival and the density of cells expressing the marker of apoptosis (M30) or cellular proliferation (Ki-67), in either tumour islets or stroma. This may be explained by the possibility of both processes of apoptosis and cellular proliferation taking place in NSCLC patients regardless of prognosis and also irrespective of microlocalisation within the tumour. Apoptosis and cellular prolferation may simply be a reflection of an individual cell's activity in relation to the cell cycle and not bear any influence on whether or not the tumour is progressing or being suppressed by cytotoxic responses. There is no strong evidence from previous studies to support a role for M30 or Ki-67 markers in NSCLC with respect to prognosis.

We also correlated the expression of M30 and Ki-67 with macrophage densities in both tumour islets and stroma. Given that our group has shown previously that increased macrophage counts in the islets relates to survival (Welsh, Green, Richardson, Waller, O'Byrne, & Bradding 2005) we hypothesised that patients with increased macrophage islet counts would also have increased M30 expression in their islets. In this study, we found no correlation between M30 expression and macrophage density in the islets. With respect to apoptosis, a previous study observed increased expression of the M30 marker in serum of patients with lung cancer 48 hours post chemotherapy (Ulukaya, Yilmaztepe, Akgoz, Linder, & Karadag 2007), however, no further data with respect to survival was presented. Our results also demonstrate that there is no relationship between cellular proliferation (Ki-67 expression) in either the islets or stroma and poor prognosis.

There are several possible explanations for the fact that our results did not match our hypotheses including: 1) the processes of apoptosis and cellular

proliferation may not be easily measurable using immunohistochemistry and may be better measured in serum or alternative methods, and 2) apoptosis and cellular proliferation may not have any relationship with tumour cytotoxicity or tumour progression, and therefore no association with prognosis. Apoptosis and cellular proliferation may be a reflection of an individual cell's position in the cell cycle and in no way reflect the state of the tumour as a whole.

In summary, we have shown that there is no association between the microlocalisation of markers of apoptosis or cellular proliferation with survival or cytotoxicity reflected by macrophage counts.

Chapter 10

General Discussion

Macrophage expression in NSCLC

Our findings demonstrate the importance of microlocalisation with respect to macrophage expression in NSCLC. Critically, we have noted that patients with increased expression of cytotoxic M1 macrophages in their tumour islets have a significantly improved predicted survival. It still remains unclear as to how and why M1 macrophages enter tumour islets – the cytokine / chemokine pathway responsible for this needs to be evaluated in the future in order to attempt to exploit this potential novel therapeutic target. However, the study presented in Chapter 8 suggests that patients with increased CXCR3 and CCR1 expression in their islets have better prognosis and these chemokine receptors may be responsible for attracting cytotoxic macrophages into the tumour islets.

With respect to macrophages located within the stroma, it is possible that they contribute towards tumour growth. Lingen proposed three possible mechanisms by which macrophages may contribute to cancer growth (Lingen 2001). Taken in conjunction with the findings of this thesis these mechanisms can be applied to stromal macrophages:

- they are activated by hypoxia in tumour tissue and subsequently secrete factors which lead to neovascularisation
- they are capable of secreting factors that may degrade connective tissue matrix
- they may secrete factors that stimulate other cell types, such as endothelial cells, fibroblasts, or keratinocytes, to secrete additional or higher levels of angiogenic factors

It is unclear how macrophages become activated in tumours. It is possible that they recognise tumour specific antigens expressed on tumour cells leading to an immune response and influx of T cells but it is also possible that T cell derived IFN-y is required to attract macrophages towards the tumour site.

In summary, we have shown that there are 2 distinct macrophage phenotypes, M1 and M2, in NSCLC tissue. The cytotoxic M1 phenotype accounts for the majority of macrophages present within the tumour islets in patients with extended survival. This supports the view that immune responses in the tumour islets play a crucial role in preventing NSCLC progression. The key questions now are how are these cytotoxic M1 macrophages generated and how are they recruited into the tumour islets? If this scenario can be mimicked by clinical intervention, it may lead to the development of new anti-cancer agents.

Mast cell expression in NSCLC

We have demonstrated that two major phenotypes of mast cell, MC_T and MC_{TC}, exist in both tumour islets and stroma in NSCLC. Increased expression in the tumour islets of either phenotype is associated with extended survival. This suggests that mast cells, irrespective of phenotype, may have a significant role to play in cytotoxic responses when present in tumour islets. This cytotoxic activity may relate to cytokines that they express, such as $TNF\alpha$. We have shown that patients with increased mast cell expression of $TNF\alpha$ in their islets have better survival. This provides further recognition of the importance of the microlocalisation of immune components with respect to NSCLC.

The granular contents of mast cells may also influence cytotoxic responses in NSCLC. Our findings suggest that increased degranulation of MC_T in the stroma relates to extended survival. The reasons for this remain unclear, however, it is possible that these granular contents, when shed into stroma, limit progression of stromal formation, thus limiting tumour proliferation. Future studies to assess the granular contents of MC_T in NSCLC stroma would be

useful. We also acknowledge that the airway inflammatory status of patients with high numbers of mast cells in this study was not known and that in future studies this would be a useful parameter to assess.

Thus, regardless of mast cell phenotype, increased expression in the tumour islets is associated with extended survival. Furthermore, expression of TNFα by mast cells in the tumour islets is also associated with extended survival. Taken in conjunction with our findings regarding macrophage expression, it is estimated that all of the expression of TNFα in patients with above median survival is exclusively from macrophages and mast cells. However, in below median survival patients, the majority of TNFα expression is from cells which are not macrophages and mast cells. This highlights that the cell type expressing TNFα may influence the action of this cytokine. Finally, we noted that increased degranulation of MC_T in the stroma is also associated with extended survival.

TNFα expression in NSCLC

Several studies have investigated the role of TNFα in NSCLC, as well as cancer in general, and the results have been conflicting as to what the specific role of TNFα actually is. Our results suggest the TNFα expression by macrophages in NSCLC relates to M1 macrophages and the presence of these in high numbers in tumour islets relates to improved prognosis. Similarly, when TNFα is expressed by mast cells located in the tumour islets, survival also appears to be improved. When we examined the expression of TNFα by all cells we noted that again increased expression in the islets is associated with improved survival but also that increased expression in the stroma is a predictor of poor prognosis.

This may explain why previous evidence as to the role of TNFα has been so conflicting. Our results once again emphasise the critical role that microlocalisation plays in NSCLC and open up the possibility of the existence of two different phenotypes of TNFα depending on whether it is located in the tumour islets or stroma - one phenotype, located predominantly in tumour islets may be cytotoxic and the other phenotype may have an effect on tumour progression. It is also possible that the phenotype of TNFα is influenced by the cell type which expresses it. In support of this, although there was no difference in the overall staining of AMS versus BMS patients, increasing stromal TNFα expression emerged as an independent predictor of worse survival. Whether this is due to the effects of TNFα on the stroma or a reflection of the inability of potentially beneficial TNFα-expressing macrophages and mast cells to infiltrate the islets is not known. The latter could be explained by an inappropriate chemokine repertoire released by the tumour stroma or even by a physical barrier, such as a thickened basement membrane, between the stroma and tumour islets.

The results of this study suggest that TNFα expression in NSCLC could be used to stratify patients, both in terms of prognosis and also to determine which patients may benefit from novel therapeutic strategies such as TNFα. In view of the marked microanatomical and immunological heterogeneity within the tumour microenvironment in NSCLC, it is essential that attention is paid to this principal in future immunomodulatory trials in this disease. This may then lead to the development of better anti-neoplastic therapeutic strategies.

Non-macrophage expression of M1 and M2 markers in NSCLC

The results presented in Chapter 7 demonstrate that markers associated with M1 and M2 macrophages are expressed by cells other than macrophages in both tumour islets and stroma in patients with NSCLC. Interestingly, HLA-DR, iNOS, MRP 8/14 and TNFα (markers associated with M1 macrophages) were found to be expressed more in the tumour islets of patients with ES compared to PS. This again reinforces the concept that these markers are potentially associated with cytotoxic responses when expressed in the tumour islets. However, the cell type expressing the marker appears to be important – the predicted 5-year survival was better when HLA-DR, iNOS, MRP 8/14 and TNFα were expressed by macrophages as opposed to non-macrophages in the tumour islets. Conversely, patients with ES had less expression of CD163 and VEGF (markers associated with M2 macrophages) in their islets compared to PS patients. The importance of microlocalisation is also strengthened by the finding that ES patients had more expression of M1 markers in their islets compared to stroma and less expression of M2 markers in their islets compared to stroma.

The 5-year survival for patients with above median tumour islet non-macrophage expression of HLA-DR was noted to be 74% irrespective of tumour stage. HLA-DR is known to be expressed by NK cells and therefore the cytotoxic result of high HLA-DR expression in the islets may indicate the important role that NK cells play combating tumour cells in the tumour islets.

This study further emphasises the importance of microlocalisation of immune cells with respect to cytotoxic response against NSCLC and also that the cell type expressing a cytokine is critical to the function of that particular cytokine.

Chemokine receptor expression in NSCLC

Our results highlight potential differences in chemokine receptor expression according to survival and microlocalisation although the numbers of patients assessed in this study was small. We found that patients with above median tumour islet expression of CXCR2, CXCR3 and CCR1 had improved survival.

It was also seen that increased expression of CXCR3 and CCR1 correlates with increased macrophage expression in the tumour islets. This suggests a role for these receptors in the pathway that attracts cytotoxic M1 macrophages into the tumour islets. Also, one of the ligands for CCR1 is CCL3 which is thought to stimulate the production and release of TNFα, a cytokine that we have demonstrated has cytotoxic potential in the tumour islets. Therefore, the fact that extended survival patients have increased expression of CCR1 in their islets may represent increased attraction of macrophages expressing TNFα into the islets with resulting enhanced cytotoxic responses.

CXCR3 and CCR1 are expressed by B cells and T cells and CXCR3 is also expressed by NK cells. Therefore, the correlation between increased expression of these two chemokine receptors in the tumour islets with increased survival reinforces the potential role that B cells, T cells and NK cells may play in the tumour islets with respect to cytotoxic responses.

Given that a relatively small patient sample was used in this study, it would be important to look at these receptors in more detail in the future as well as the chemokine ligands associated with them. A complete understanding of the chemokine pathways involved in attracting immune cells into either tumour islets or stroma may lead to the development of novel therapies for NSCLC.

Apoptosis and cellular proliferation in NSCLC

We found no association between the microlocalisation of markers of apoptosis or cellular proliferation and survival. These negative findings may reflect the possibility that there is actually no association between an individual cell's status in terms of apoptosis or cellular proliferation with the overall progression or modulation of a tumour. Additionally, there was also no correlation between the expression of these markers and macrophage expression in tumour islets and stroma. This suggests that there is no association between cytotoxicity and the processes of apoptosis and cellular proliferation. Therefore, it seems that M30 as a marker of apoptosis and Ki-67 as a marker of cellular proliferation do not have a useful role to play in assessing the immune response to NSCLC.

Future studies

There are a number of questions which require addressing following on from the studies presented in this thesis. Firstly, it would be important to validate our findings in a separate cohort of patients in order to determine whether or not the methods used would be of significant benefit in terms of patient management.

It would be fascinating to explore the microlocalisation of T cells and NK cells in NSCLC, as well as their correlation with the expression of macrophages, mast cells, cytokines, chemokines and chemokine receptors. This may lead to an enhanced understanding of how cytotoxic responses against NSCLC are mounted in patients with extended survival.

A better understanding of what particular granular components have an effect on tumour stroma would be helpful as well as investigating any correlation between mast cell expression in NSCLC with airways disease status.

Clinical trials examining the role of TNFα as a therapeutic agent according to TNFα expression in tumour islets and stroma may be worthy of future consideration. Its localisation to macrophages and mast cells in the tumour islets is the key factor relating to improved prognosis, and it seems unlikely that anti-TNFα strategies will be beneficial to such patients. In contrast, in poor prognosis patients whose tumour islets contain relatively few TNFα+ macrophages and/or mast cells, anti-TNFα strategies may be worthy of further study as stromal expression is an independent predictor of poor survival.

With respect to underlying mechanisms in NSCLC further work is needed looking into chemokine ligand and chemokine receptor pathways in the tumour islets and stoma. A better understanding of these pathways may help to uncover novel therapeutic targets for patients with NSCLC.

Summary

The work presented in this thesis can be summarised in Fig 1. It demonstrates the two microanatomical areas of tumour – the islets and stroma. There is a physical barrier between them (dashed line) – this concept has been introduced previously (Hagenaars, Ensink, Basse, Hokland, Nannmark, Eggermont, van de Velde, Fleuren, & Kuppen 2000). It is possible that this barrier may prevent the migration of potentially cytotoxic immune cells into the tumour islets in some patients. Fig 1 demonstrates that if cytotoxic M1 macrophages are able to pass through this barrier this may be to chemotraction towards CXCR3 and CCR1.
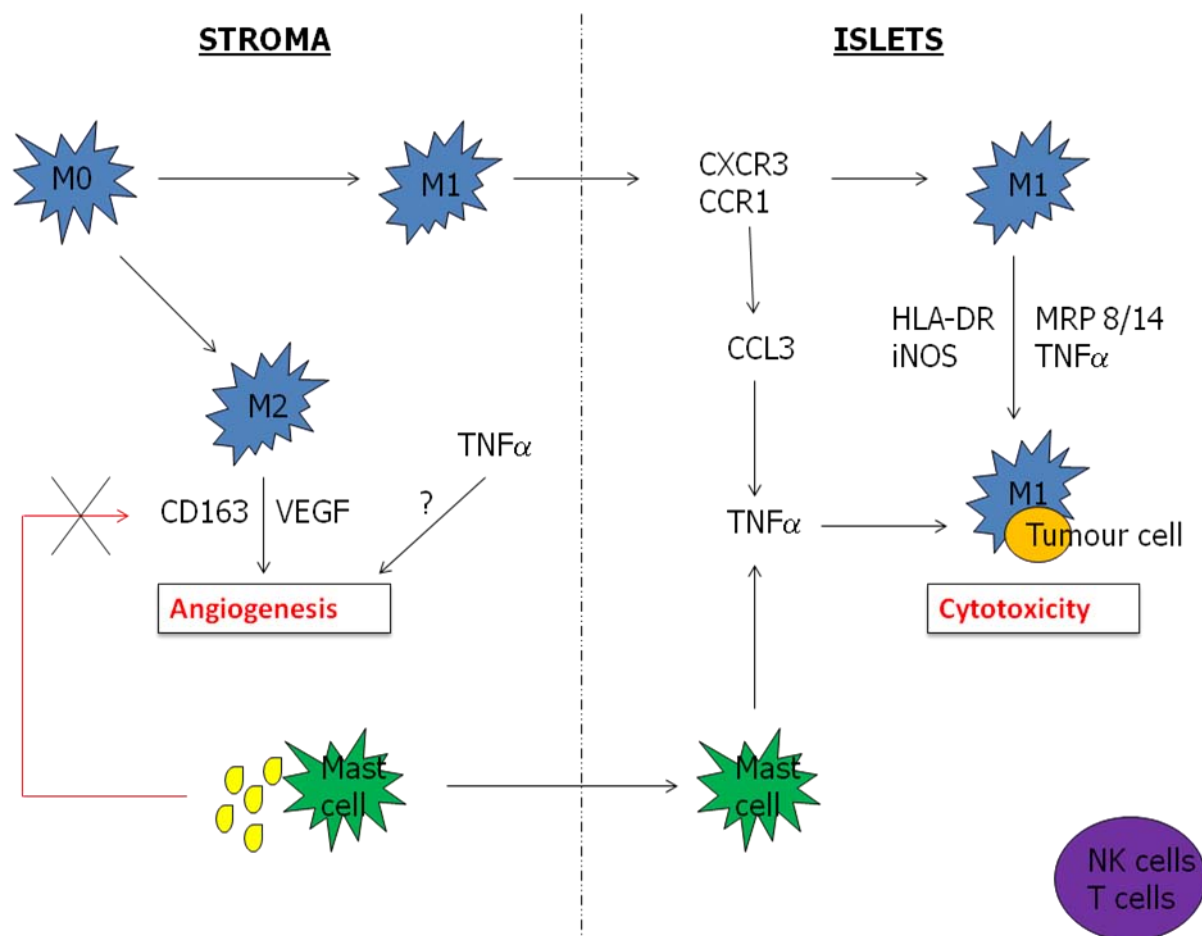
Fig 1. Summary of thesis findings demonstrating possible pathways involved in the immune response to non-small cell lung cancer

Once in the tumour islets, M1 macrophages are then capable of killing tumour cells, a response enhanced by local TNFα expression. One of the ligands for CCR1 is CCL3 which is thought to stimulate the production and release of TNFα. Therefore, TNFα appears to fulfill its cytotoxic potential when expressed in the islets by M1 macrophages, mast cells and CCL3. Fig 1 also shows that M2 macrophages situated in the stroma may be associated with angiogenesis, a process which may be further enhanced by the presence of TNFα in the stroma. Our results also suggest that mast cell degranulation in the stroma may inhibit the angiogenic pathway.

In conclusion, the microlocalisation of immune cells and cytokines, within NSCLC tumours, is critical to their phenotype and subsequently their function. Also, the cell type expressing a cytokine appears to be important in determining the function of that particular cytokine. If the mechanisms for attracting cytotoxic cells into the tumour islets can be completely understood there is potential to develop very powerful immunotherapies which could treat and possibly cure NSCLC.

APPENDIX A

Intra- and inter-observer variation of cell counts in NSCLC sections

Degranulation Index

Observer 1		Observer 2
Count 1	Count 2	Count 1
1	1	2
1	1	1
1	1	1
1	1	1
2	1	0
3	3	2
2	2	3
2	1	2
1	1	1
3	3	3
2	2	2
1	1	2
3	3	3
3	3	3
2	2	2
2	2	2
1	2	2
3	3	2
2	2	0
3	3	3
3	3	2
2	2	3
2	1	2
2	2	2
1	1	1
2	2	3
1	1	2
2	2	2
3	3	3
3	3	3

Intra-observer intraclass correlation 0.898

Inter-observer intraclass correlation 0.668

APPENDIX B

Possible avenues of future research

1. <u>Macrophage phenotypes</u>

   What influences the differentiation of macrophages into either the M1 or M2 phenotype?

2. <u>Mast cell phenotypes</u>

a) What are the roles of the granular contents released by mast cells in relation to cancer progression in particular when shed in tumour stroma?

b) Is there an association between mast cell expression in NSCLC and airways disease status?

3. <u>TNFα</u>

a) Is there are a role for anti-TNFα drugs for patients with low TNFα expression in their tumour islets?

b) What is the pathway responsible for influencing TNFα attraction into the tumour islets?

4. <u>Non-macrophage expression of markers associated with M1 and M2 macrophages</u>

   What exactly are the cell types, other than macrophages, expressing markers associated with M1 and M2 macrophages?

5. <u>Chemokine Receptors</u>

   What are the chemokine ligands and receptors responsible for the microlocalisation of cytotoxic cells?

6. <u>Markers of apoptosis and cellular proliferation</u>

   Do these markers correlate with survival when present in serum?

7. <u>Other possible studies</u>

a) How does the microlocalisation of T cells and NK cells correlate with survival?

b) Is there a relationship between the microlocalisation of T cells and NK cells with expression of macrophages, mast cells, cytokines, chemokine ligands and chemokine receptors?

c) What is the chemokine ligand/receptor pathway responsible for attracting T cells and NK cells into either the tumour islets or stroma?

d) Is there a physical barrier between the tumour islets and stroma and could it be therapeutically altered to allow for migration of cytotoxic cells into the islets?

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