Phenotypic and functional characterisation of regulatory T cells in head and neck squamous cell carcinoma

Samantha Drennan

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The University of Hull and The University of York

Hull York Medical School

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Abstract

Head and neck cancer is the sixth most common cancer worldwide. Despite advances in therapy, the five year survival rate remains poor. The presence of regulatory T cells (Tregs) may be a mechanism by which head and neck squamous cell carcinoma (HNSCC) evades the immune system. Using flow cytometry to identify distinct Treg populations on the basis of phenotype and a CFSE proliferation assay, the frequency and suppressive activity of Treg populations from the peripheral circulation and tumour microenvironment of newly-presenting HNSCC patients was assessed. No difference in the circulating Treg prevalence was observed between HNSCC patients (n=39) and healthy controls (n=14), or between patients with HNSCC from different subsites. However, patients with advanced stage tumours and those with nodal involvement had significantly elevated levels of peripheral $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs compared with patients who had early stage tumours (p=0.03) and those without nodal involvement (p=0.03) respectively. Circulating $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs from the entire HNSCC patient cohort and patients whose tumours had metastasised to the lymph nodes suppressed the proliferation of effector T cells significantly more compared with those from healthy controls (p=0.04) or patients with no nodal involvement (p=0.04). Additionally, peripheral $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs consistently induced greater suppressive activity than $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs. The tumour microenvironment had an elevated frequency of Tregs ($p \leq 0.002$) and a lower frequency of effector T cells ($p \leq 0.03$) compared with the patient's peripheral circulation (n=15). No difference was observed in the level of suppression between tumour and peripheral Tregs. Using immunohistochemistry, patients with oropharyngeal tumours showed a significantly greater infiltration of $Foxp3^{+}$ cells in the tumour and stroma compared with laryngeal tumours. Furthermore, as determined by ELISA, the dispersed tumour specimens secreted detectable levels of TGF-β and IL-10, but secretions from HNSCC (dissociated tumour samples and cell lines) did not influence the suppressive activity of Tregs. Clarifying the role of $CD127^{low/-}$ Tregs in HNSCC and the influence the tumour may have on the regulatory population will provide the opportunity, through future work, to establish whether Tregs can be used as a prognostic determinant or manipulated by immunotherapy.

Thesis associated manuscripts

Drennan S., Stafford N. D., Greenman J., Green V.L. Increased frequency and suppressive activity of $CD127^{low/-}$ Tregs in the peripheral circulation of patients with head and neck squamous cell carcinoma are associated with advanced stage and nodal involvement Immunology, manuscript submitted February 2013

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Thesis associated presentations and published abstracts

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Green V.L., Michno A., Murray P., **Drennan S**., Stafford N.D., Greenman J. (2011) What effects does head and neck squamous cell carcinoma have on the systemic and intra-tumoral cytokine environment and Treg cell number? (Poster) Yorkshire Cancer Research Annual Conference

Michno A., **Drennan S**., Stafford N., Greenman J., Green V., (2010) T regulatory cell phenotype in HNSCC pre- and post- treatment. (Oral) Otolaryngology - Head and Neck Surgery. *143*, 176

Murray P., Green V.L., **Drennan S**., Stafford N.D., Greenman J. (2010) Investigating the Treg/Th17 cytokine axis within head and neck squamous cell carcinoma (Poster) Yorkshire Cancer Research Annual Conference

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Abbreviations

AD	Active Disease
APC	Allophycocyanin
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CFDASE	Carboxyfluorescein Diacetate Succinimidyl Ester
CFSE	Carboxyfluorescein Succinimidyl Ester
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetra Acetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ENT	Ear, Nose and Throat
ERCC1	Excision Repair Cross-Complementation group 1 enzyme
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
Foxp3	Forkheadbox Transcription Factor p3
FSC	Forward Scatter
GITR	Glucocorticoid-Induced TNF Receptor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HCl	Hydrochloric Acid
HEPES	4-(2-Hydroxyethyl) Piperazine-1-Ethanesulfonic Acid
HNSCC	Head and Neck Squamous Cell Carcinoma
HRP	Horseradish Peroxidase
HPV	Human Papillomavirus
H_2SO_4	Sulphuric Acid
ICOS	Inducible Co-Stimulator
IDO	Indoleamine 2,3-Dioxygenase
IFN-γ	Interferon-gamma
IL	Interleukin
INT	Iodonitrote-trazolium
IPEX	Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked Syndrome
iTreg	Induced T Regulatory Cells
LSM	Lymphocyte Separation Medium
MACS	Magnetic Activated Cell Sorting
MDSC	Myeloid Derived Suppressor Cells
MHC	Major Histocompatibility Complex
miRNA	Micro Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
N^+	Nodal Involvement

N0	No Nodal Involvement
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
NaOH	Sodium Hydroxide
NED	No Evidence of Disease
NK	Natural Killer
NPC	Nasopharyngeal Carcinoma
NSCLC	Non Small Cell Lung Cancer
nTreg	Naturally Occurring T Regulatory Cells
OVC	Ovarian Cancer
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein Complex
PGE2	Prostaglandin E2
PI	Proliferation Index
pRb	Retinoblastoma Protein
R and D	Research and Development
RNA	Ribonucleic Acid
SCC	Squamous Cell Carcinoma
SSC	Side Scatter
TBS	Tris-Buffered Saline
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-Beta
Th1	T Helper-1
Th2	T Helper-2
Th3	T Helper-3
Th17	T Helper-17
TIL	Tumour Infiltrating Lymphocytes
TMB	Tetramethylbenzidine
TNF	Tumour Necrosis Factor
TNM	Tumour, Node, Metastases
Tr1	T Regulatory Type 1
Treg	Regulatory T cells
VEGF	Vascular Endothelial Growth Factor

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Author's declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

Chapter 1.

Introduction

1.1 Head and neck cancer

Head and neck cancer is a term used to group together epithelial malignancies that develop from anatomically defined locations within the upper aerodigestive tract; including the larynx, nasopharynx, oropharynx, hypopharynx, oral cavity and nasal cavity (Figure 1.1; Choong and Vokes, 2008). Approximately 90% of head and neck cancer cases are squamous cell carcinomas (SCC) arising from organs lined by squamous epithelium (Curado and Hashibe, 2009). It is widely recognised that patients with head and neck squamous cell carcinoma (HNSCC) have a suppressed immune system and that the tumour employs numerous mechanisms to evade immune recognition, inhibit anti-tumour responses and promote an immunosuppressive environment (Duray *et al.*, 2010; Young, 2006).

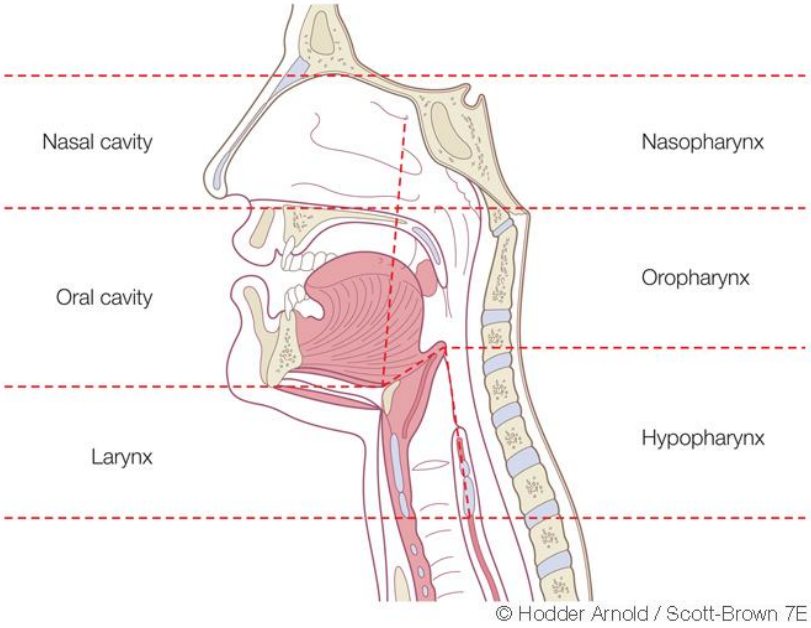


Figure 1.1 Anatomical regions of the head and neck

Taken from Scott-Brown *et al.*, 2008

The disease burden and treatment of HNSCC can cause severe loss of function in the area where the tumour has developed, effecting eating, drinking, speech, swallowing, breathing, social interactions and work capabilities. Despite advances in surgical and

chemoradiotherapy regimes, the overall survival of HNSCC patients has not significantly improved in the past 30 years. In addition, surgical resection of the tumour in such a compact and anatomically complex region can have a negative aesthetic impact (Wrangle and Khuri, 2007). These aspects can greatly reduce a patient's quality of life emphasising the need for continued research to understand further the cellular pathways and interactions involved, in the hope of both providing better prognostic information and the development of new treatment strategies.

1.2 Anatomy and clinical presentation of HNSCC

Although HNSCC are united by general location and histology, the different primary sites vary in presentation, pathogenesis and survival. The studies performed in this thesis have assessed the HNSCC cohort as a whole, as is the case in many research publications (Schaefer *et al.*, 2005; Schott *et al.*, 2010; Strauss *et al.*, 2007a), but have also analysed individual subsites and examined the differences that may occur between the primary sites. The conducted studies have predominately focused on HNSCC developing from the larynx and oropharynx along with their associated tumour infiltrated lymph nodes; these primary subsites and associated regional nodes will be discussed briefly in turn.

1.2.1 The larynx

The larynx, located in the anterior neck, acts to provide voice and protect the respiratory airways from the digestive tract; it is commonly referred to as the voice box. The larynx consists of a framework of cartilage, connected by ligaments and muscles which are lined by a mucous membrane, comprising of pseudostratified and squamous epithelium. Anatomically, the larynx can be divided into three regions: supraglottis (the area above the vocal cords), glottis (the area containing the vocal cords) and subglottis (the area below the vocal cords); SCC can develop in any of these locations (American Cancer Society, 2011a; Scott-Brown *et al.*, 2008;).

The clinical presentation of laryngeal cancer varies depending on the region involved. For early stage glottic lesions and advanced supraglottic tumours, hoarseness of

the voice is one of the most common symptoms. Consequently, glottic cancer is diagnosed early with a greater survival rate, in contrast to supraglottic tumours which commonly present in the advanced stage and usually have nodal involvement with a low probability of cure (Licitra *et al.*, 2003; Marur and Forastiere, 2008). In addition, a sore throat, shortness of breath, ear pain, a mass in the neck or a feeling of a "lump in the throat" are further symptoms observed with cancer of the larynx, in particular with advanced staged cancers of the supraglottis or subglottis (American Cancer Society, 2011a; Licitra *et al.*, 2003).

1.2.2 The oropharynx

The pharynx is divided in to three parts and the oropharynx is one of these regions (Figure 1.1). At the crossroad between the respiratory and digestive tract the oropharynx is located directly behind the oral cavity and includes the base of the tongue (the back third of the tongue), the soft palate (the back area of the roof of the mouth), the tonsillar region and the posterior and lateral pharyngeal wall (American Cancer Society, 2011b; Scott-Brown *et al.*, 2008). SCC can arise at any of these sites however, the commonest site of oropharyngeal cancer is the tonsillar region (Cohan *et al.*, 2009; Scott-Brown *et al.*, 2008). Cancer of the oropharynx has a high frequency of lymph node involvement with approximately 60 to 70% of patients presenting with nodal disease (Jose *et al.*, 2002).

Oropharyngeal cancer can be late to present due to small tumours in this region not always producing symptoms or being easy to detect, consequently the patient's tumour will usually be of the advanced stage at the time of diagnosis. Symptoms can include a lump or swelling in the upper neck, a foreign body sensation in the throat, a sore throat, altered voice or ear ache. If the tumour advances and infiltrates the tongue muscles, speech and swallowing are also affected (Scott-Brown *et al.*, 2008).

1.2.3 The lymph nodes

Due to the rich lymphatic network present in the head and neck region, when HNSCC cells break away from the primary tumour mass they often infiltrate the lymph nodes and establish SCC growth. Lymphatic drainage varies depending on the anatomical

subsite, therefore the primary site of a HNSCC has a great impact on the risk of nodal involvement (Mukherji *et al.,* 2001). For example, the base of the tongue has a significantly higher metastatic risk compared with tumours arising on the oral tongue, because of the close locality of the regional lymph nodes. Similarly, there is a lower incidence of metastases for primary cancers of the glottic larynx due to the sparse lymphatic drainage around the vocal cords (Genden *et al.,* 2003; Marur and Forastiere, 2008; Scott-Brown *et al.,* 2008).

Nodal involvement has a significant impact on prognosis; HNSCC patients who develop nodal metastatic disease are associated with a higher incidence of distant metastasis, consequently their 5 year survival rate is reduced by approximately 50% (Scott-Brown *et al.,* 2008; Wenzel *et al.,* 2004).

1.3 Progression of HNSCC

The accumulation of numerous genetic and molecular alterations in the cells of the upper aerodigestive tract leads to the development of HNSCC. The proposed stepwise progression model for HNSCC is illustrated by Figure 1.2 as the normal mucosa develops towards invasive carcinoma. Many of the genetic disruptions occur in chromosomal regions encoding tumour suppressor genes, thus disrupting the expression of proteins involved in cell cycle regulation and progression which leads to the loss of cell cycle control, continued cell proliferation and the accumulation of genetic mutations (Section 1.5.4; Kim and Califano, 2004; Mao *et al.,* 2004).

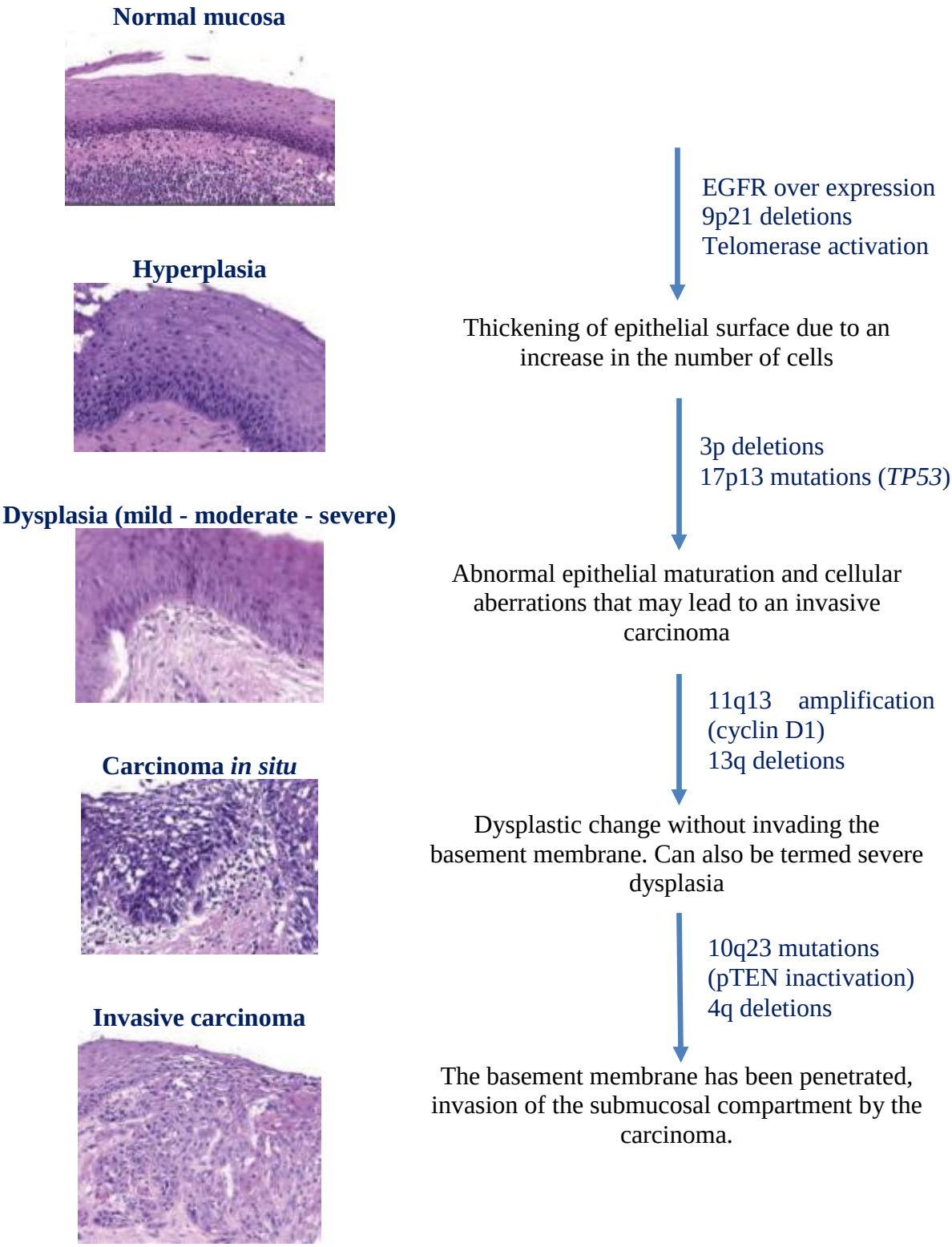


Figure 1.2 Presentation of the stepped progression model for HNSCC

Images taken from Argiris *et al.*, 2008 and definitions adapted from Wenig, 2002.

1.4 Epidemiology of head and neck cancer

Head and neck cancer is the sixth most common type of cancer with the incidence estimated to be 650,000 new cases per year and accounting for approximately 350,000 deaths worldwide (Parkin *et al.,* 2005). However, the rates of incidence and mortality can varying greatly depending upon geographical location, HNSCC subsite and gender.

In England and Wales approximately 6,700 new cases of HNSCC are diagnosed each year (National Head and Neck Cancer Audit, 2008). Between October 2007 and November 2008, 29% of cases were cancer of the larynx and 25% were oropharyngeal cancer (National Head and Neck Cancer Audit, 2008).

Worldwide, an estimated 130,000 males were diagnosed with laryngeal cancer in 2008 compared with 21,000 cases in women (Ferlay *et al.,* 2010). In the UK, cancer of the larynx is almost five times more common in men than women with around 1,900 males and 400 females diagnosed in 2010 (Cancer Research UK, 2013a). In Europe, depending upon the country, cancer of the larynx was 4 (Scotland) to 49 (Spain) times more common in men than women (Karim-Kos *et al.,* 2008). For both males and females, due to the reduction in smoking habits, the incidence rate for laryngeal cancer has declined in Europe over the past decade. In contrast, the UK incidence rates in women have remained stable over the last 40 years whilst for men the rates steadily fell in the early 2000's before reaching a stable rate of approximately 5.4 per 100,000 (Figure 1.3; data shown for the last 33 years). It has been projected that between 2007 and 2030 the incidence rates for laryngeal cancer will fall by more than 1% per year in the UK (Mistry *et al.,* 2011). However, the five year survival rates for cancer of the larynx have not considerably improved over the last 30 years with an increase of approximately 3%, reaching a five year survival rate of 66.8% (Cancer Research UK, Laryngeal cancer statistics).

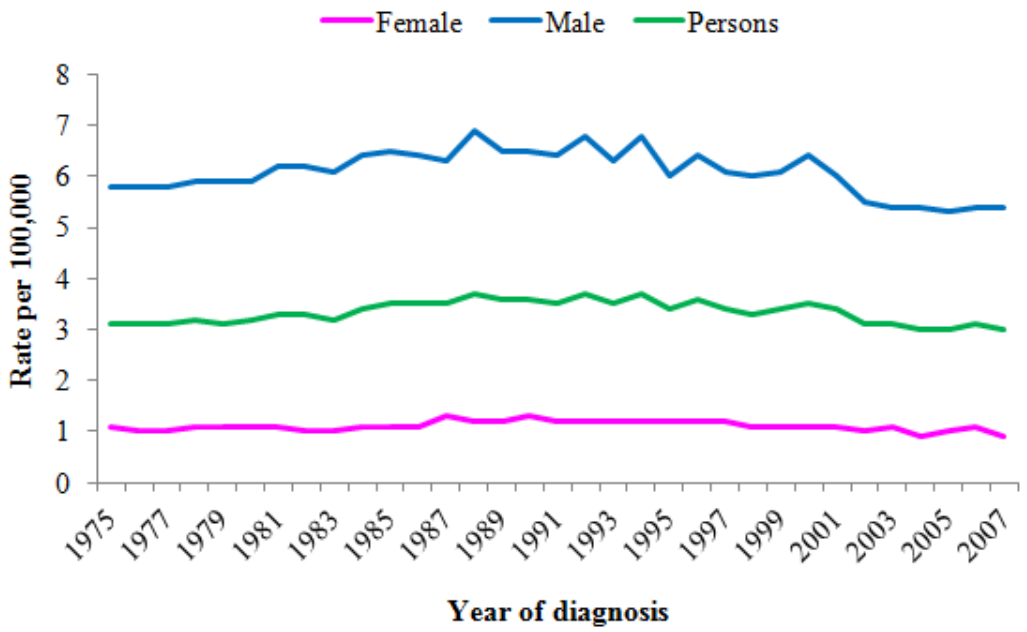


Figure 1.3 Incident rates for laryngeal cancer by sex
Age standardised incident rates, per 100,000, for laryngeal cancer in the UK by sex from 1975-2008. Adapted from statistics reported by Cancer Research UK, Laryngeal cancer statistics

Globally, cancer of the oral cavity is the most common HNSCC and accounted for an estimated 274,000 cases in 2002, of which approximately two thirds were male (Parkin *et al.*, 2005). In Europe, the incidence of oral cavity and pharyngeal cancer combined varies considerably, amongst males it is between 5.9 (Finland) and 32 (France) per 100,000 and for women between 1.9 (Finland) and 10.3 (Lithuania) per 100,000 (Karim-Kos *et al.*, 2008). The statistics presented by Cancer Research UK group cancer of the oropharynx with several other head and neck cancers (lip, tongue, mouth, hypopharynx) to form a group termed oral cancer. The charity report that in the UK in 2010 approximately 4,300 men and 2,200 women were diagnosed with oral cancer, a male:female ratio of around 2:1 (Cancer Research UK, 2013b). Over the last decade (1999-2009) oral cancer incidence has increased by 25% for males and 33% for females (Figure 1.4) and it has been projected that between 2007-2030 the incidence of oral cancer will increase by more than 1% per year in the UK (Mistry *et al.*, 2011). In England the incidence rate for oropharyngeal cancer has more than doubled from 1990-2006, a rise greater than any other head and neck cancer site; this has been attributed to infection with the human papillomavirus (HPV; Profiles of Head and Neck Cancers in England, 2010; Section 1.5.2). The five year survival rate for cancer

of the oropharynx is approximately 50% (Profiles of Head and Neck Cancers in England, 2010) however, positive HPV status has been associated with an improved overall survival (Section 1.5.2).

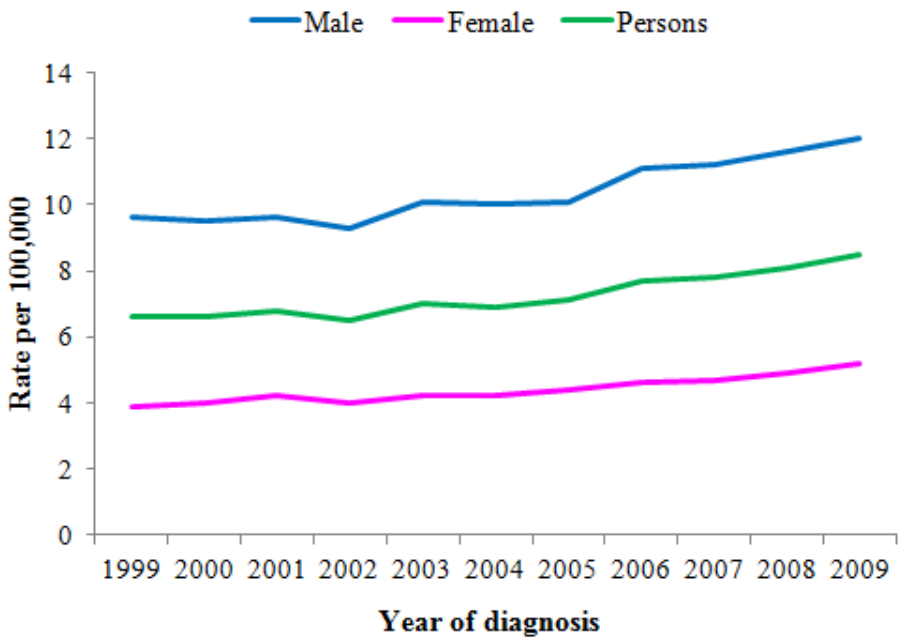


Figure 1.4 Incident rates for oral cancer by sex
Age standardised incident rates, per 100,000, for oral cancer in the UK, by sex, from 1999-2009. Adapted from statistics reported by Cancer Research UK, Oral cancer statistics

1.5 Aetiology of head and neck cancer

1.5.1 Alcohol and tobacco

Smoking and alcohol, either independently or synergistically, are historically the most common aetiological factors for the development of HNSCC. In the UK in 2010, approximately 79% of laryngeal cancers were attributable to smoking and 25% were linked to alcohol consumption (Parkin, 2011a; Parkin, 2011b).

The risk of developing head and neck cancer has been shown to be proportional to smoking frequency and duration, with a strong association for the development of laryngeal cancer (Hashibe *et al.,* 2007; Lewin *et al.,* 1998). One study demonstrated this dose response relationship clearly with the odds ratio of laryngeal cancer being 5.7 for smokers

of up to ten cigarettes per day increasing to 18.4 when consumption was thirty-forty cigarettes per day (Hashibe *et al.*, 2007). Individuals who cease smoking considerably reduce their risk of head and neck cancer in comparison with current smokers; however, the risk is still approximately twice that of never smokers for individuals who have ceased smoking for less than twenty years (Bosetti *et al.*, 2008; Lewin *et al.*, 1998).

Alcohol, or more accurately ethanol, is not classed as a carcinogen *per se* but has been shown to independently increase the risk of developing head and neck cancer in association with high drinking frequency (Hashibe *et al.*, 2007; Lewin *et al.*, 1998). In particular, the risk of cancer of the head and neck is approximately twice for individuals who consume three to four alcoholic drinks per day in comparison with never drinkers. The elevated risk appears to be strongest for cancer of the pharynx (Hashibe *et al.*, 2007; Lewin *et al.*, 1998).

The effect of tobacco and alcohol has been estimated to account for 72% of the head and neck cancer burden, of which 33% was due to smoking alone, 4% explained by alcohol alone and approximately 35% by the combined effect of tobacco and alcohol consumption (Hashibe *et al.*, 2009). The elevated risk of developing head and neck cancer due to the use of tobacco and alcohol together has been reported by several groups (Anantharaman *et al.*, 2011; Franceschi *et al.*, 1990; Hashibe *et al.*, 2009; Lewin *et al.*, 1998) and the risk is highest for laryngeal cancer (Table 1.1).

Table 1.1 Risk of developing head and neck cancer at various subsites for the highest alcohol and tobacco frequencies

Cancer subsite	Alcohol	Tobacco	Risk
Head and neck	Never	Never	1
	≥ 3 drinks / day	≥ 20 cigarettes / day	14.23
Oral cavity	≥ 3 drinks / day	≥ 20 cigarettes / day	15.49
Pharynx	≥ 3 drinks / day	≥ 20 cigarettes / day	14.29
Larynx	≥ 3 drinks / day	≥ 20 cigarettes / day	36.87

Adapted from Hashibe *et al*., 2009

1.5.2 Human papillomavirus (HPV)

HPV, perhaps more commonly associated with cancer of the cervix, has recently been associated with HNSCC, particularly cancer of the oropharynx. In the western world the incidence of HNSCC at specific sites arising as a result of alcohol and tobacco use has seen a slow decline or stabilisation over the past decade, however, the incidence of oropharyngeal cancer has risen steadily, and this increase has been attributed to infection with HPV and changes in sexual behaviour (Dufour *et al.,* 2012; Profiles of Head and Neck Cancers in England, 2010).

Approximately 25% of HNSCC cases are positive for HPV DNA, with the prevalence of infection higher in cancer of the oropharynx compared with oral cavity and laryngeal cancer (Dayyani *et al.,* 2010; Gillison *et al.,* 2000). The majority of these cases are related to the high risk subtype HPV-16. In a review of sixty studies Kreimer *et al.* (2005) reported that of the HPV positive HNSCC cases HPV-16 accounted for 86.7% of oropharyngeal, 68.2% of oral and 69.2% of laryngeal cancers. HPV-18 was the next most common oncogenic HPV type detected but with a different subsite profile, being more prevalent in oral cavity cases (34.1%) compared with cancer of the oropharynx (2.8%) and larynx (17%).

HPV-16 mediates carcinogenic effects through the viral oncoproteins E6 and E7 which bind and degrade p53 and retinoblastoma protein (pRb), tumour suppressor proteins, respectively. Consequently the virus causes the cell to lose cell cycle regulation and the ability to induce apoptosis thereby promoting cell proliferation and survival (Gonzalez *et al.,* 2001; Scheffner *et al.,* 1992).

It appears that HPV positive tumours create a distinct group within cancers of the head and neck. As summarised in Table 1.2 differences between individuals with either HPV positive or HPV negative HNSCC have been highlighted in relation to age, risk factors and molecular characteristics. In particular, although the prevalence of HPV positive tumours is on the increase, patients with these tumours have been associated with improved overall survival and progression free survival with approximately a 60% reduction in risk of death compared with HPV negative patients (Ang *et al.,* 2010; Dayyani *et al.,* 2010; Gillison *et al.,* 2000).

Table 1.2 Different features of HNSCC dependent on HPV status

Feature	HPV negative HNSCC	HPV positive HNSCC
Incidence	Decreasing	Increasing
Age	Older (above 55 years)	Younger (below 55 years)
Risk factors	Smoking, alcohol	Sexual behaviour
Tumour	Well/moderately differentiated	Poorly differentiated
TP53 mutations	Frequent	Infrequent
Prognosis	Poor	Favourable

Correlated from Ang *et al.*, 2010; Dayyani *et al.*, 2010; D'Souza *et al.*, 2007; Gillison *et al.*, 2000.

1.5.3 Epstein Barr Virus

The association between the Epstein Barr Virus (EBV) and nasopharyngeal carcinoma (NPC) is well established. However, distinctive geographical variation indicates that the viral infection is not the sole causative factor, but in conjunction with other risk factors, such as genetic predisposition or environmental events, may contribute to NPC development (Parkin *et al.*, 2005; Spano *et al.*, 2003).

The expression of latent genes by the virus (including EBV nuclear antigens and latent membrane proteins) is important for the maintenance and replication of the EBV genome and maintaining the latent infection within the cells. In addition, latent membrane proteins have been shown to influence the expression of genes involved in cell cycle induction and inhibition of apoptosis, thereby enabling the continued growth and survival of infected cells (Young and Rickinson, 2004).

1.5.4 Genetic alterations

Accumulations of genetic alterations in the cells of the upper aerodigestive tract, including those in tumour suppressor genes and/or proto-oncogenes, disrupt vital homeostatic cell signalling pathways, thereby promoting the development of HNSCC (Section 1.3). Three genetic events known to be mutated in HNSCC will be discussed briefly below, but it should be noted that there are a vast array of candidate genes which

have also been associated with tumourigenesis of the head and neck region, a discussion of these is beyond the scope of this thesis.

1.5.4.1 TP53

The *TP53* gene encodes the p53 tumour suppressor protein which regulates the cell cycle and, if required, induces apoptosis in response to DNA damage. The percentage of HNSCC cases harbouring a *TP53* mutation varies in the literature between 50 and 80% (Balz *et al.*, 2003; Poeta *et al.*, 2007; van Houten *et al.*, 2002). Consequently, inactivation of *TP53* within a cell results in the accumulation of genetic mutations, continuous replicative potential and the ability to evade induced cell death (Fan, 2001).

A significant increase in the frequency of *TP53* mutations has been demonstrated amongst HNSCC patients who smoke and/or consume alcohol (Brennan *et al.*, 1995). Furthermore, mutation of the tumour suppressor gene has been linked with decreased survival in HNSCC, with a median survival of 3.2 years for patients with a *TP53* mutation and 5.4 years for patients with wild-type tumours (Poeta *et al.*, 2007).

1.5.4.2 p16

Inactivation or absence of the *p16* gene, located on chromosomal region 9p21, and encoding the tumour suppressor protein p16, is regarded to be one of the most common genetic aberrations in HNSCC, with over 70% of cases displaying allelic loss or absence of *p16* (Reed *et al.*, 1996; van der Riet *et al.*, 1994). Furthermore, loss of 9p was reported at the same frequency in pre-invasive and invasive lesions suggesting that loss of *p16* occurs early in the progression of HNSCC (van der Riet *et al.*, 1994). In the cell cycle the p16 protein acts to inhibit cell cycle progression by preventing cyclin-dependent kinases 4 and 6 phosphorylating the pRb, subsequently the cell cycle is arrested (Kim and Califano, 2004). As with *TP53*, the absence or inactivation of *p16* results in the loss of cell cycle control and has been associated with poor prognosis in HNSCC (Namazie *et al.*, 2002).

1.5.4.3 Epidermal growth factor receptor

Upon ligand binding the epidermal growth factor receptor (EGFR) is involved in the initiation of a signalling cascade which influences many downstream pathways including cell proliferation, migration, apoptosis and angiogenesis (Argiris *et al.,* 2008). Over expression of this membrane bound tyrosine kinase receptor along with elevated levels of mRNA and amplification of the chromosomal region that encodes the *EGFR* gene has been reported in head and neck tumours (Grandis and Tweardy, 1993; Hitt *et al.,* 2005; Laimer *et al.,* 2007; Roman *et al.,* 2008; Thomas *et al.,* 2012). Due to methodological differences the percentage of HNSCC cases positive for EGFR expression, or showing elevated levels of expression, can vary between studies, however, the majority of publications report that over 90% of cases are positive for EGFR expression (Ang *et al.,* 2002; Grandis and Tweardy, 1993; Hitt *et al.,* 2005; Thomas *et al.,* 2012). High expression of the EGFR has been associated with poor prognosis (Ang *et al.,* 2002; Chung *et al.,* 2006; Hitt *et al.,* 2005; Laimer *et al.,* 2007; Rubin Grandis *et al.,* 1998) and the receptor can be targeted by the monoclonal antibody cetuximab during the treatment of advanced staged tumours (Section 1.7).

1.6 Staging of HNSCC

Staging of HNSCC is used by clinicians to express the severity or extent of the disease and assists with predicting patient prognosis whilst also providing a useful guide for the planning of treatment strategies. In addition, staging can be used to share information between NHS Trusts and research centres enabling incidence, prognosis, treatment plans and survival rates to be assessed and monitored in the UK (Scott-Brown *et al.,* 2008).

For head and neck cancer, staging is determined by the TNM system which is composed of three categories; the extent of the primary tumour (T; referred to also as T stage), the absence or presence and extent of regional lymph node metastases (N; termed nodal status) and the absence or presence of distant metastases (M). Both the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC), who publish systems of cancer classification, reported the same TNM classification system in their 2002 publications.

The anatomical location of HNSCC is important during the staging of head and neck cancer, the AJCC divides the region by subsite to include: lip and oral cavity, pharynx, larynx, nasal cavity-paranasal sinuses, salivary glands and thyroid gland (Choong and Vokes, 2008; Scott-Brown *et al.*, 2008), with each location having a separate staging system for the primary tumour (T). For example, the T stage of the larynx is dependent on the region of the larynx involved (supraglottis, glottis or subglottis), how far the cancer has spread within and outside the larynx and the movement of the vocal cords (American Cancer Society, 2011a). The pharynx is also divided by location: oropharynx, nasopharynx or hypopharynx (American Cancer Society, 2011b). However, the classification of T stage for all HNSCC subsites can be generalised (Table 1.3).

One of the clinicopathological parameters HNSCC patients were grouped by in this thesis was T stage; patients with T1 or T2 staged tumours were classified as having early stage disease whilst patients with T3 or T4 staged tumours were denoted as having advanced stage disease.

Table 1.3 Staging of the primary tumour in HNSCC

T	Definition
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1, T2, T3, T4	Increasing size and/or local extent of the primary tumour

Reproduced from Scott-Brown *et al.*, 2008

With the exception of nodal classification for nasopharyngeal carcinoma, the definitions for regional lymph node status and the presence of distant metastases are the same, irrespective of HNSCC subsite (Table 1.4 and Table 1.5; Choong and Vokes, 2008). In the studies conducted for this thesis HNSCC patients were grouped into patients with no evidence of nodal involvement (N0) and those with involvement of the regional lymph nodes (N^+).

Table 1.4 Classification of regional lymph node metastases in HNSCC

N	Definition
Nx	Regional lymph nodes cannot be assessed
N0	No evidence of regional lymph node metastases
N1, N2, N3	Increasing involvement of regional lymph nodes

Reproduced from Scott-Brown *et al.*, 2008

Table 1.5 Classification of distant metastases in HNSCC

M	Definition
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Reproduced from Scott-Brown *et al.*, 2008

Classifying HNSCC patients by the three components of the TNM system additionally enables the patients to be subsequently placed within a TNM stage, which is designed to group patients with regard to survival rates (Table 1.6; Scott-Brown *et al.*, 2008).

Table 1.6 TNM group staging for HNSCC

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T1, T2	N1	M0
	T3	N0, N1	M0
IVa	T1, T2, T3	N2	M0
	T4a	N0, N1, N2	M0
IVb	Any T	N3	M0
	T4b	Any N	M0
IVc	Any T	Any N	M1

Reproduced from Scott-Brown *et al.*, 2008

1.7 Management of HNSCC

Following diagnosis and staging of a patient's HNSCC their treatment strategies, which are influenced by both the site and stage of the cancer (Figure 1.5), are considered by a multidisciplinary team, including a head and neck surgeon, a radiation oncologist and a medical oncologist. A major challenge in treating HNSCC is the complex anatomical regions where the cancer arises, as the subsites can not only be affected by the presence of disease but also by the therapies used to increase the probability cure. For HNSCC functional outcomes such as speech and swallowing are carefully considered alongside curative intent, and therefore each patient has an individualised treatment plan.

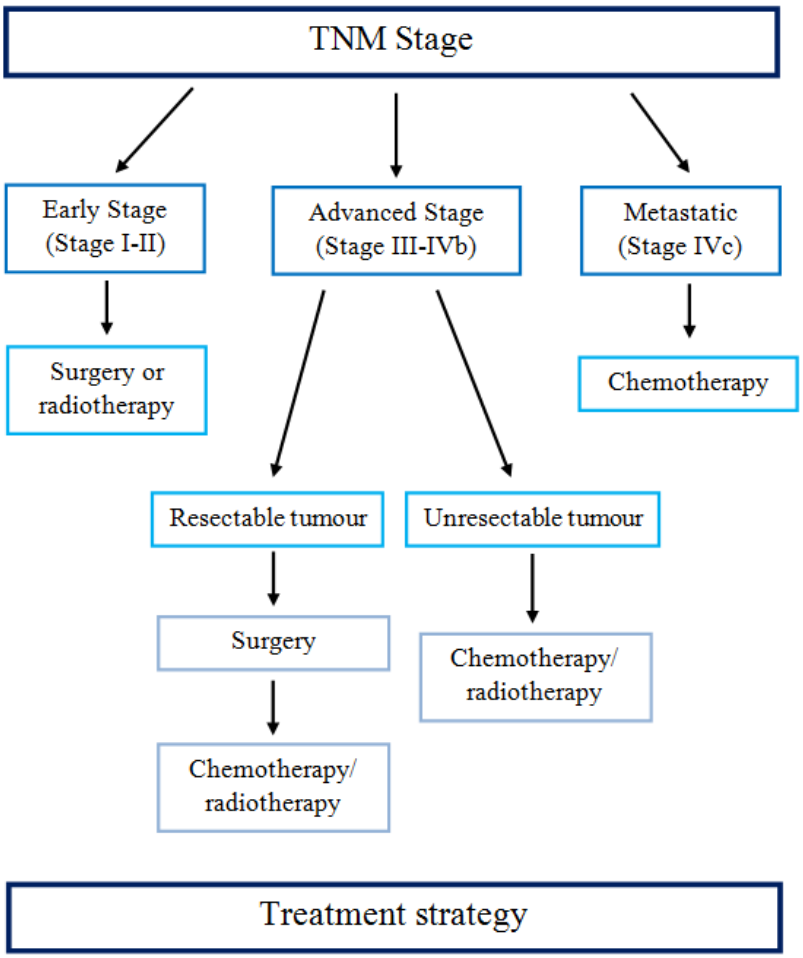


Figure 1.5 General treatment strategies dependent on staging of HNSCC

Adapted from Choong and Vokes, 2008

Approximately one third of patients with HNSCC present with early stage disease and their choice of treatment includes surgery or radiotherapy with curative intent (Figure 1.5; Argiris et *al.*, 2008). During surgery a limited amount of 'healthy' tissue is exposed to treatment and in comparison with radiotherapy the length of time of the treatment is short and avoids the toxicities associated with radiotherapy (Choong and Vokes, 2008). However, surgery is limited by the anatomical locations of the tumour and the desire to preserve organ function. Nevertheless, advances in microsurgical techniques have enabled the excision of accessible small tumours of the oral cavity and larynx which allows organ preservation whilst also providing good oncological outcomes (Argiris *et al.*, 2008). For the Ear, Nose and Throat (ENT) department in Hull, endoscopic laser surgery is a preferred treatment strategy, particularly in the management of early stage laryngeal cancer where there is a risk that conventional surgery may affect the function of the larynx. Radiotherapy however, avoids the possibilities of complications associated with surgery and potentially maintains the function of treated organs without the negative aesthetic impact associated with surgery. Additionally, radiotherapy may be tolerated better compared with surgery for patients who have poor health (Choong and Vokes, 2008). For early stage glottic and base of tongue cancer, radiotherapy results in high tumour control and cure rates (Argiris *et al.*, 2008), consequently this strategy is one of the main treatment options employed by the ENT department in Hull for patients presenting with primary tumours at an early stage. However, tumour radioresistance, which contributes to recurrence and a poor prognosis, is a significant problem in up to 25% of early stage tumours (Nix *et al.*, 2005; Yang *et al.*, 2011), therefore additional treatment strategies are continually being sought.

The majority of HNSCC patients present with locally advanced disease and usually require combined therapy (Figure 1.5). Initially it is determined whether the tumour is resectable by surgery, which includes considering the potential functional compromise along with survival benefit. In particular, the main aim of treatment for laryngeal cancer is to provide voice preservation with a high cure rate; surgery tends to be reserved for patients with persistent or recurrent disease (Marur and Forastiere, 2008). Patients will be treated with chemoradiotherapy if the tumour is unresectable or organ preservation is desired, and postoperatively for resectable tumours (Choong and Vokes, 2008); this is the standard care for patients with advanced stage laryngeal and oropharyngeal cancers (Marur and Forastiere, 2008). The cytotoxic drugs, cisplatin, docetaxel and 5-fluorouracil are standard

chemotherapeutic agents in HNSCC treatment, as well as novel targeted therapies including cetuximab; a monoclonal antibody against the EGFR which has a role in the growth and division of cells (Argiris *et al.,* 2008; Harari *et al.,* 2009). The concurrent administration of chemo- and radiotherapy has been a major advancement in the treatment of advanced stage HNSCC (Argiris *et al.,* 2008), with the improvement of chemotherapy regimens reducing the excision of primary tumours year on year in Western Europe and North America.

Depending on the stage, subsite and nodal status of the patient's head and neck tumour, removal of the regional lymph nodes are a further treatment consideration. In approximately one third of cases, although nodal involvement may not have been clinically confirmed, lymph nodes can contain micrometastases and therefore a selective neck dissection is performed to remove the likely chance of cancer developing in the lymph nodes (Argiris *et al.,* 2008). For patients with extensive lymph node involvement a radical neck dissection is performed which can include the removal of some nerves and muscles associated with neck and shoulder movement; this is to ensure that all tissue likely to harbour cancer cells is removed (American Cancer Society, 2011a).

For HNSCC patients with recurrent or metastatic disease there is the option of surgery if the tumour is resectable. However, for many the cure rate is low and the standard choice of treatment is chemotherapy with the aim of providing symptom palliation and extending survival (Argiris *et al.,* 2008; Choong and Vokes, 2008).

Despite the current treatment strategies the five year survival rate for HNSCC remains poor. To develop treatment regimes, establish new therapeutic targets and improve prognostic information it is important that research continues to broaden our understanding of the progression of head and neck cancer; including the immunosuppressive mechanisms that target components of the immune system and the host's anti-tumour immune responses that create a favourable environment for continued tumour growth and development.

1.8 Cancer and the immune system

The immune system is a complex network of components that functions to simultaneously discriminate between self and non-self, remove damaged cells, maintain tissue homeostasis and provide protection from infectious or invading pathogens (de Visser

et al., 2006). Cancers are a damaging mass of malignant cells which form due to uncontrolled growth following mutations in key control genes and in order for the immune system to inhibit and eradicate this growth it must generate an attack against these cells.

The immune system can recognise the presence of cancer cells via the molecules they express, for instance molecules that are expressed solely on cancer cells and are absent on normal cells are termed tumour specific antigens. In contrast there are molecules which are expressed by normal cells and tumours, but in a different manner, these molecules are known as tumour associated antigens. Recognition of these antigens allows an anti-tumour T cell attack to be mounted by the immune system (Fairey *et al.*, 2002; Finn, 2008).

The host's anti-tumour immune response is driven predominately by interferon-γ (IFN-γ) producing $CD4^+$ helper cells and cytotoxic $CD8^+$ T cells which act to impede tumour progression by inhibiting and killing cancerous cells (Zamarron and Chen, 2011). Natural killer (NK) cells also aid the anti-tumour attack by targeting tumour cells that are attempting to escape immune recognition through the down regulation of MHC class I molecules (Simonson and Allison, 2011). However, the presence of anti-tumour immunity is not sufficient to eradicate the cancerous mass as many tumours continue to grow with often fatal consequences for the patient. The reason for this is because the tumour employs various immunosuppressive strategies, a number of which are discussed below, which influence the function of immune components, both within the tumour microenvironment and systemically around the body and have the ability to thwart an effective immune attack and hinder the implementation of successful tumour immunotherapies (Rabinovich *et al.*, 2007).

Further to the strategies covered below, the tumour can also create a favourable environment for growth by stimulating myeloid-derived suppressor cells (MDSC), that assist with suppressing T cell function and proliferation, as well as influencing the differentiation of $CD4^+$ T cells and macrophages to subtypes that promote tumour cell progression (Section 5.1; Duray *et al.*, 2010; Tong *et al.*, 2012). The roles of various immune components along with the influence of cytokine secretions, involved in both tumour progression and inhibition, will be discussed further during the relevant result chapters (Section 5.1 and Section 6.1).

In addition cytotoxic $CD8^+$ T cells can lose effector function due to chronic stimulation by tumour antigens. These T cells are termed 'exhausted' and are characterised

by decreased cytokine expression, poor effector function and the expression of inhibitory receptors; consequently hindering the clearance of tumour cells (Wherry, 2011). T cell exhaustion was originally observed in mice chronically infected with the lymphocytic choriomeningitis virus, and although the majority of studies focus on virus-specific $CD8^+$ T cells, exhausted $CD4^+$ T cells due to chronic viral infection have also been reported (Baitsch *et al.*, 2012; Wherry, 2011). In melanoma patients, tumour-specific $CD8^+$ T cells isolated from tumour infiltrated lymph nodes were shown to express a large variety of genes associated with T cell exhaustion, including the expression of inhibitory receptors, as well as a reduction in the secretion of IFN-γ compared with circulating tumour specific T cells (Baitsch *et al.*, 2011).

1.8.1 Immune tolerance

One of the main obstacles for the immune system to overcome and generate an attack against the tumour is immune tolerance; the absence of an immune response against specific antigens. For transplant patients this is the ultimate aim however, the opposite is true for cancer patients where an inadequate immune response allows continued tumour growth. A major element of immune tolerance is the fact that the cancer has arisen from the host's own tissue and is consequently expressing a large number of self antigens to which the immune system's T lymphocytes have been tolerised, either by central or peripheral tolerance (Mapara and Sykes, 2004). Therefore one of the main regulatory mechanisms employed by the immune system to prevent responses against self constituents, and therefore autoimmune diseases, is also one of the major strategies that contributes to cancer cells escaping a T-cell mediated attack (Rabinovich *et al.*, 2007).

1.8.2 Dendritic cells

Dendritic cells are antigen presenting cells which take up, process and present antigens to T lymphocytes to enable a tumour specific response to be generated (Rabinovich *et al.*, 2007). It has been reported in head and neck cancer that the number and function of dendritic cells in the peripheral circulation and tumour draining lymph nodes of

patients was significantly reduced compared with healthy controls (Almand *et al.*, 2000). Additionally, the number and activity of dendritic cells in the periphery of patients with advanced stage HNSCC was significantly lower than that in patients with early stage disease (Almand *et al.*, 2000). A further study has shown that patients with cancer of the breast, prostate and malignant glioma have a significantly larger proportion of immature dendritic cells in their circulation which had a reduced ability to capture and present antigens (Pinzon-Charry *et al.*, 2005). Decreased numbers or function of dendritic cells in cancer patients will lead to reduced antigen presentation and immune stimulation, enabling the tumour to escape a T cell mediated response.

1.8.3 Transforming growth factor-β

The production of the immunoregulatory cytokine transforming growth factor-β (TGF-β) by malignant cells of the tumour is another potential way that cancer is able to suppress the actions of the immune system. TGF-β is a multifunctional immunosuppressive cytokine which aids immune homeostasis by maintaining tolerance through the regulation of lymphocyte proliferation, differentiation and survival (Li *et al.*, 2006). In HNSCC elevated levels of TGF-β have been reported within the carcinoma, adjacent tissues (Lu *et al.*, 2004) and secreted by carcinoma associated fibroblasts (Rosenthal *et al.*, 2004). Although Logullo and colleagues (2003) reported no clinicopathological association with TGF-β in HNSCC tissue samples, it has been shown in other malignancies, including non small cell lung, gastric and bladder carcinoma, that raised TGF-β levels in tumour specimens and plasma samples correlate with poor prognosis (Hasegawa *et al.*, 2001; Saito *et al.*, 1999; Shariat *et al.*, 2001). The role TGF-β plays in assisting the tumour to escape immune surveillance includes the reduction of MHC I and II expression on carcinoma cells, decreasing NK cell activation as well as disrupting the function and proliferation of tumour infiltrating lymphocytes (TIL; Section 6.1; Bierie and Moses, 2006). Thomas and Massague (2005) has demonstrated this to include TGF-β having the ability to specifically repress the gene expression of cytotoxic mediators (perforin, granzyme A, granzyme B, interferon γ and FasL) in cytotoxic T lymphocytes (CTL).

1.8.4 Indoleamine 2,3-dioxygenase

The enzyme indoleamine 2,3-dioxygenase (IDO) catalyses the initial and rate limiting step in the oxidative breakdown of the essential amino acid tryptophan (Rabinovich *et al.*, 2007; Uyttenhove *et al.*, 2003). It has been shown that local shortage of tryptophan and the presence of its catabolites can impair T lymphocyte activation and arrest their proliferation (Frumento *et al.*, 2002; Munn *et al.*, 1999), enhancing the ability of the tumour to evade the host immune response. IDO has been shown to be expressed in numerous tumour types, including HNSCC, with high/diffuse IDO expression also being correlated with poor prognosis in colorectal, invasive cervical and ovarian cancer (Brandacher *et al.*, 2006; Inaba *et al.*, 2010; Okamoto *et al.*, 2005; Uyttenhove *et al.*, 2003).

1.8.5 Regulatory T cells

Over the past few years a subset of suppressive T lymphocytes (regulatory T cells; Tregs) that have an essential role in the maintenance of peripheral tolerance (Section 1.9), have received significant interest for their role in the suppression of anti-tumour immunity. The level of $CD4^+$ Tregs has been reported to be elevated in a large number of malignancies, including head and neck cancer (Section 1.13). The role, presence and function of Tregs in HNSCC is investigated in this thesis in an attempt to gain a greater understanding of the regulatory population in the hope of contributing towards new immunotherapeutic strategies and improved prognostic information.

1.9 Tregs and peripheral tolerance

The immune system is finely balanced through its ability to discriminate between self and non-self, to prevent autoimmunity and preserve immune homeostasis. This equilibrium is maintained by both central and peripheral tolerance (Dieckmann *et al.*, 2001).

Central tolerance is the primary mechanism employed to delete self reactive T lymphocytes and occurs during T lymphocyte development in the thymus. For those

autoreactive T lymphocytes that escape thymic deletion or are reactive to self antigens expressed only extrathymically, peripheral tolerance is used to render these harmful populations unresponsive. Tregs are a population of T lymphocytes that play a major role in the maintenance of peripheral tolerance by suppressing the activation and effector function of autoreactive T cells, preventing the development of autoimmune diseases (Dieckmann *et al.,* 2001; Thornton and Shevach, 1998). Numerous studies in both mice and humans have shown that deletion or inactivation of this T cell subset results in the development of severe autoimmune diseases (Bennett *et al.,* 2001; Gavin *et al.,* 2006; Itoh *et al.,* 1999; Sakaguchi *et al.,* 1995; Wildin *et al.,* 2002).

1.10 History of Tregs

It has been known for many years that the immune system is regulated in some way to prevent excessive immune activity and in 1971, a short time after the two major lymphocyte groups (B and T) of the adaptive immune system had been identified, Gershon and Kondo (1971) suggested that T lymphocytes had the ability to 'shut off' the activity of other cells through the production of an immunosuppressive substance. In conjunction with publications presenting evidence for the existence of different functional subsets of T cells, as identified by Ly and Ia antigens (Cantor and Boyse, 1975; Vadas *et al.,* 1976), as well as the logical idea that the immune system needed to be tightly controlled to prevent over activity, the notion and presence of a suppressive T cell population quickly became accepted (Germain, 2008).

Immunologists of this era however did not possess the necessary tools and techniques required to conduct precise experiments, and perhaps due to this, their results have not stood the test of time. Consequently, this led to a lack of transferable skills and variations in results between different laboratories and research groups (Waldmann, 2008). As research continued in this rapidly growing field, concepts surrounding suppressor T cells began to become complicated and the accumulation of discrepancies and negative findings led a once rapidly moving field to one that was tainted with doubt. As a result, the volume of work decreased and publications on suppressor T cells became scarce (Germain, 2008).

After several years of scepticism surrounding suppressor T cells, interest began to increase during the 1990s as experimental evidence accumulated on a specific regulatory T cell population. In both syngenic nude mice and rats it was demonstrated that a lethal wasting disease with multiple organ inflammation developed via inoculation with $CD4^+$ T cells expressing high levels of CD45RB (naïve T cells) whereas injection with $CD4^+$ T cells expressing low levels of CD45RB (memory T cells) did not (Powrie *et al.*, 1993; Powrie and Mason, 1990). In addition it was shown that when both subsets were administered no wasting or colitis developed (Powrie *et al.*, 1993; Powrie and Mason, 1990). These studies indicated that there was a $CD4^+$ T cell population present within normal animals which was capable of causing harm to self. However, a T cell subset existed which regulated the pathological T cells thereby preventing the generation of disease.

The same trend was reported in mice by Sakaguchi and colleagues (1995) who demonstrated that $CD4^+$ cells that were positive for the expression of the IL-2 receptor α chain (CD25) prevented the development of autoimmune diseases, which had been shown to occur in mice inoculated with $CD4^+CD25^-$ T cells. The co-transfer of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells effectively showed that the presence of $CD25^+$ cells prevented the development of an autoimmune disease through interaction with the $CD25^-$ cells. The publication of the Sakaguchi study is regarded by many as the beginning of a plethora of investigations that reignited interest in the field of Tregs (Bettini and Vignali, 2010; Fontenot and Rudensky, 2004; Shevach, 2002; Wilke *et al.*, 2010).

1.11 Treg subtypes

$CD4^+$ Tregs have been categorised into two broad groups according to their different pathways of development and the methods employed to induce suppression; naturally occurring Tregs (nTregs) and induced or adaptive Tregs (iTregs; Figure 1.6). nTregs develop in the thymus and enter the periphery with a functionally suppressive phenotype expressing the forkheadbox transcription factor p3 (Foxp3; Wing *et al.*, 2006), and for healthy individuals this subset of Tregs is present from birth (Roncarolo and Battaglia, 2007). In contrast, iTregs arise in the periphery where regulatory capacity is generated in naïve T cells in response to environmental signals (Shevach, 2006; Wing *et*

al., 2006). iTregs can be divided further into T regulatory type 1 (Tr1) cells, T helper-3 (Th3) cells or induced $CD4^+CD25^+Foxp3^+$ Tregs depending on their method of development and secretion of cyokines (Section 1.11.2; Figure 1.6).

Tregs with a $CD8^+$ phenotype have also been identified and may be able to be divided further dependent on phenotype or function (Shevach, 2006). However, due to the ability of $CD4^+$ Tregs to suppress the activation and function of $CD8^+$ T cells (Piccirillo and Shevach, 2001) and difficulties in the characterisation of this regulatory population (Wang and Alexander, 2009) there has been limited research into CD8-specific Tregs (Shevach, 2006).

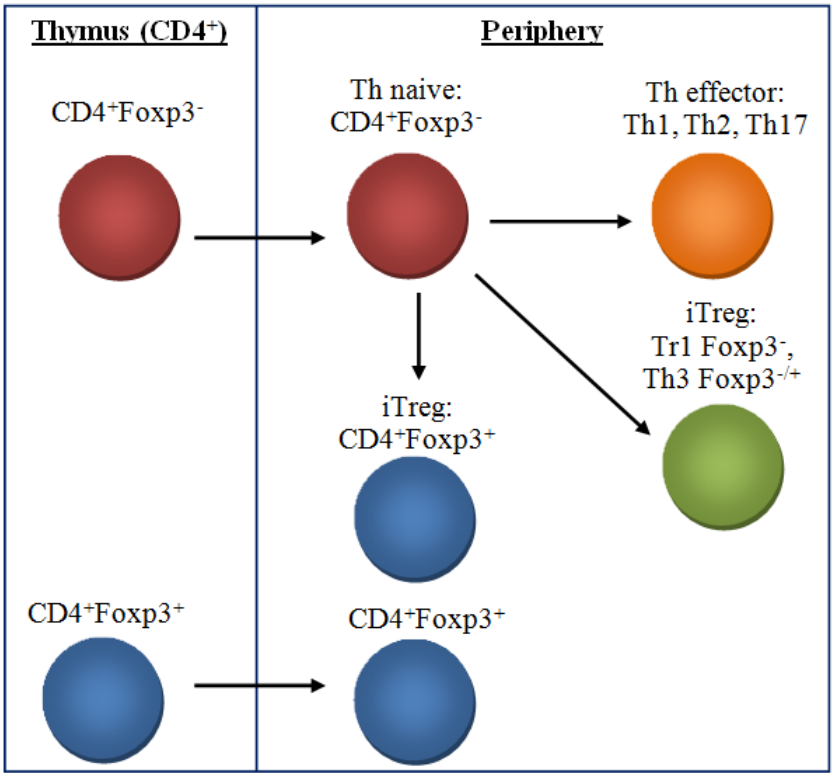


Figure 1.6 Generation of different $CD4^+$ Treg subtypes

Adapted from Curotto de Lafaille and Lafaille, 2009

1.11.1 nTregs

When the literature discusses Tregs it is commonly referring to nTregs, although this is not always clearly explained. nTregs are a $CD4^+$ T cell population that develop and

obtain their suppressive phenotype ($CD4^+CD25^+Foxp3^+$) in the thymus and migrate to the periphery (Fontenot and Rudensky, 2004). This naturally occurring regulatory population plays a major role in the maintenance of peripheral tolerance and deficiency in nTregs leads to the development of severe autoimmune diseases (Section 1.9). It has been known for many years that a suppressive T cell population develops in the thymus following a number of studies reporting the induction of multi organ autoimmune diseases after neonatal thymectomy in a murine model (Kim and Rudensky, 2006; Taguchi *et al.,* 1985; Tung *et al.,* 1987). These investigations demonstrated that central tolerance does not completely delete all autoreactive T cells and in addition, highlighted that the thymus produces a population of suppressor T cells which are able to regulate the action of these harmful T cells.

Tregs are anergic *in vitro*, i.e. they do no proliferative or produce IL-2 upon T cell receptor (TCR) stimulation, however, this anergy can be broken by the addition of high doses of IL-2 or anti-CD28 (Fehervari and Sakaguchi, 2004a; Thornton *et al.,* 2004). In contrast, Treg anergy is not observed *in vivo*, with studies demonstrating proliferation and expansion of the regulatory population (Klein *et al.,* 2003; Yamazaki *et al.,* 2003). For Tregs to exert their suppressive function they must first be activated through their TCR with a specific antigen, although once activated their suppressive activity is not antigen specific (Dieckmann *et al.,* 2001; Itoh *et al.,* 1999; Takahashi *et al.,* 1998; Thornton and Shevach, 1998). It is known that Tregs specific for self-antigens are primed in the thymus, it is therefore perhaps not surprising that Tregs have been shown to be specific for tumour associated antigens due to the cancer arising from the host's self tissue (Mougiakakos *et al.,* 2010; Piersma *et al.,* 2008). Furthermore, in a murine study it was demonstrated that the concentration of antigen required to activate and exert Treg function was approximately 10-100 fold lower than the concentration required to induce the proliferation of $CD4^+CD25^-$ T cells (Takahashi *et al.,* 1998).

Although this T cell population is vital in preserving immune homeostasis through the maintenance of peripheral tolerance, Tregs have been shown to be elevated in a number of different cancers types (Section 1.13) and are regarded as an important mechanism by which the tumour impairs the host's anti-tumour response and successfully evades the immune system, thus making them a potential therapeutic target.

1.11.2 iTregs

The induction of Tregs allows the immune system to adapt and respond to immunogenic conditions (Feuerer *et al.,* 2009). As previously mentioned the iTreg population can be divided into several different subsets, including Tr1 cells, Th3 cells or induced $CD4^+CD25^+Foxp3^+$ Tregs. Many iTregs are found at mucosal sites where tolerance to commensal flora and foreign antigens, such as food and inhaled antigens, needs to be maintained in order to prevent pathological responses being generated in the gut, lungs or airways (Yuan and Malek, 2012).

Tr1 cells are characterised by the lack of Foxp3 expression and the production of high levels of IL-10 (Vieira *et al.,* 2004). Secreting high levels of IL-10, low levels of IL-2 and no IL-4, their cytokine profile is distinct from that of Th1 and Th2 effector T cells (Groux *et al.,* 1997). IL-10 has also been shown to be responsible for the generation of this population; Groux and colleagues (1997) demonstrated that naïve antigen specific $CD4^+$ T cells continuously stimulated by the TCR in the presence of IL-10 produced a T cell population that had low proliferative capacity and were functionally suppressive. Additionally, in a murine model of inflammatory bowel disease, the transfer of Tr1 cells prevented the development of the disease (Groux *et al.,* 1997). Although IL-10 is regarded as the 'driving force' for the differentiation of Tr1 cells, in the *in vitro* induction of human Tr1 cells from naïve $CD4^+$ cells, several experimental studies have shown that IL-10 is required but probably not sufficient for Tr1 cell generation (Battaglia *et al.,* 2006; Roncarolo and Battaglia, 2007). It has also been reported that Tr1 cells exert their suppressive capacity through the production of IL-10 and TGF-β in an antigen non-specific manner (Battaglia *et al.,* 2006; Groux et al., 1997).

In contrast, suppressive Th3 cells are characterised by the primary production of TGF-β with varying amounts of IL-4 and IL-10 (Wing *et al.,* 2006) and were first identified during investigations into oral tolerance (Weiner, 2001). Although experimental evidence is less available for Th3 cells compared with Tr1 cells, it has been demonstrated that TCR and TGF-β co-stimulation converted naïve murine $CD4^+CD25^-Foxp3^-$ T cells into a $CD25^+Foxp3^+$ regulatory population that was characteristically anergic, expressed TGF-β and had suppressive activity towards T cell proliferation (Chen *et al.,* 2003). Due to the gut microenvironment having high levels of TGF-β the differentiation of naïve $CD4^+$ T cells to Th3 cells is promoted, associating the regulatory population with tolerance against food and

commensal bacterial antigens (Chen and Konkel, 2010; Weiner, 2001). However, with the positive expression of Foxp3, this TGF-β generated murine regulatory population may also be classified as induced $CD4^+CD25^+Foxp3^+$ Tregs.

The stimulation of naïve $CD4^+$ T cells to express Foxp3 in the presence of TGF-β has also been demonstrated in humans (Fantini *et al.*, 2004; Tran *et al.*, 2007). Although the distinction between Th3 cells and induced $CD4^+CD25^+Foxp3^+$ Tregs is unclear and probably overlaps, it is now generally accepted, in both mice and humans, that TGF-β signalling coupled with TCR stimulation is essential for the induction of Foxp3 expression in naïve $CD4^+$ cells and conversion of them into iTregs (Chen and Konkel, 2010). However, unlike in the murine model where Foxp3 expression generates an immunosuppressive T cell population, Foxp3 induction in human T cells does not necessarily confer a population with suppressive activity (Section 1.12).

At the beginning of the work conducted by this thesis, and to date, the iTreg populations, in particular Tr1 and Th3, remain poorly phenotypically defined. The studies conducted for this thesis have therefore focussed on the more characterised nTreg population (Section 1.12), enabling identification, isolation and functional characterisation of the regulatory population in HNSCC patients. However, it is noted that identifying nTregs by their positive expression of Foxp3 may include a population of $Foxp3^+$ iTregs.

1.12 Identification of Tregs

In attempts to understand the role of Tregs in various human diseases and the maintenance of peripheral tolerance, identification of this suppressive T cell population is important for isolation, characterisation, functional analysis, *ex vivo* expansion and detecting their existence *in vivo*. However, despite the numerous studies performed on Tregs and efforts to identify a unique marker, a definitive label has yet to be discovered. Consequently, a number of cell surface and intracellular markers are used in combination for their identification.

The most widely recognised surface marker used to identify Tregs is CD25 (IL-2 receptor α chain), with functional Tregs easily isolated from mice using the phenotype $CD4^+CD25^+$ (Sakaguchi *et al.*, 1995). Based on the murine Treg findings, several groups

employed the $CD4^+CD25^+$ phenotype to demonstrate the presence of Tregs in humans (Dieckmann *et al.,* 2001; Jonuleit *et al.,* 2001; Levings *et al.,* 2001). Approximately 10-15% of the $CD4^+$ T cell population expresses CD25 (Baecher-Allan *et al.,* 2001; Baecher-Allan *et al.*, 2005; Levings *et al.,* 2001), however, in humans this phenotype is also adopted by activated effector T cells, necessitating further characterisation of Tregs. It has subsequently been found that $CD4^+$ cells with high CD25 expression levels ($CD25^{high}$) have the ability to reduce the proliferation of $CD4^+CD25^-$ cells by 69-98%. In contrast $CD4^+CD25^{low}$ cells did not possess this suppressive activity (Baecher-Allan *et al.,* 2001; Cesana *et al.,* 2006). $CD4^+CD25^{high}$ cells, now a commonly used phenotype to identify Tregs, represent approximately 1-2% of the $CD4^+$ population; however, the exact definition of $CD25^{high}$ does vary between studies. For example, Whiteside and colleagues define the $CD4^+CD25^{high}$ population by $CD4^+$ cells expressing CD25 with a mean fluorescence of $\geq$120 (Figure 1.7a); this boundary was selected following suppression studies which indicated that it was only this population that had suppressive activity (Strauss *et al.,* 2007a; Strauss *et al.,* 2007b). Other groups distinguish $CD25^{high}$ and $CD25^{low/intermediate}$ populations based on the expression of CD25 by $CD4^-$ T cells (Figure 1.7b); $CD4^+$ T cells exceeding the level of CD25 expression by $CD4^-$ T cells are defined as the $CD4^+CD25^{high}$ population (Cesana *et al.,* 2006; Chi *et al.,* 2010; Hoffmann *et al.,* 2004; Yokokawa *et al.,* 2008). It was this method of CD25 separation that was used by the studies in this thesis to identify Treg populations with varying levels of CD25 expression.

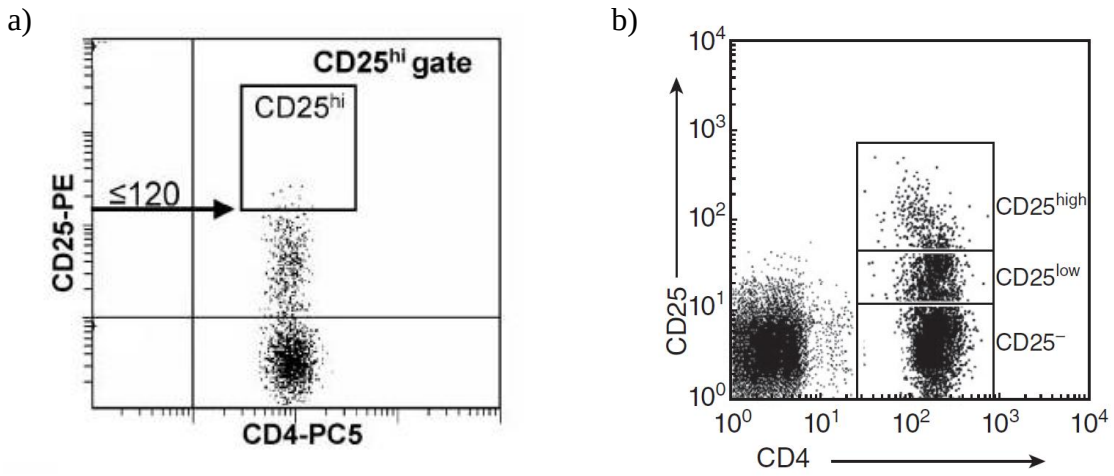


Figure 1.7 Different gating strategies to identify $CD4^+CD25^{high}$ Tregs
(a) PBMC were acquired and the lymphocyte population gated by forward and side scatter properties. From the lymphocyte population the $CD3^+CD4^+$ cells were identified and used to determine the $CD4^+CD25^{high}$ Tregs, based on the expression of CD25 having a mean fluorescence intensity ≥120, Strauss *et al.,* (2007a) and (b) $CD4^+$ cells were divided into $CD25^{high}$, $CD25^{low}$ and $CD25^-$ populations. The $CD4^+CD25^{high}$ gate was determined on the $CD4^+$ cells whose expression of CD25 exceeded that of $CD4^-$ cells, Chi *et al.,* (2010).

The variable levels of CD25 expression and the fact that the receptor is generally expressed on activated T cells fuelled the search for markers unique to Tregs. It was the similarities observed between the symptoms suffered by the Scurfy mutant mouse strain, individuals suffering from immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and the depletion of Tregs in animal models, which led several groups to investigate the causative gene of these two syndromes; Foxp3 (Fehervari and Sakaguchi, 2004b). Subsequent research demonstrated that Foxp3 plays an important role in both the development and function of Tregs (Fontenot *et al.,* 2003; Gavin *et al.,* 2006; Hori *et al.,* 2003). Murine studies reported that the mRNA level of *Foxp3* was 100 times greater in $CD4^+CD25^+$ cells than $CD4^+CD25^-$ cells, with *in vitro* activated $CD4^+CD25^-$ T cells failing to express the transcription factor (Fontenot *et al.,* 2003; Hori *et al.,* 2003). It was also demonstrated that mice lacking expression of Foxp3 could be rescued from autoimmune diseases by inoculation of naturally occurring $CD4^+CD25^+$ cells or $CD4^+CD25^-$ T cells transduced to express Foxp3 (Fontenot *et al.,* 2003; Hori *et al.,* 2003). A subsequent study observed that $CD4^+CD25^{high}Foxp3^+$ and $CD4^+CD25^{low}Foxp3^+$ cells were both equally suppressive towards $CD4^+CD25^-Foxp3^-$ T cell proliferation, indicating

that Foxp3, regardless of CD25 expression, is a useful marker for the identification of suppressive murine Tregs (Fontenot *et al.*, 2005).

Investigations into Foxp3 expression in human Tregs are a little more complex. Wang *et al.* (2007) reported that 93.3% of $CD4^+CD25^{high}$ cells expressed Foxp3 whereas only 14.2% of $CD4^+CD25^{inter}$ and 1.8% of $CD4^+CD25^-$ cells expressed the transcription factor; the same trend has been observed by a number of studies comparing $CD4^+CD25^+$ and $CD4^+CD25^-$ populations (Gavin *et al.*, 2006; Morgan *et al.*, 2005; Walker *et al.*, 2003). In contrast to murine investigations, several groups have demonstrated that human activated $CD4^+CD25^-$ effector T cells gain Foxp3 expression, along with CD25 (Allan *et al.*, 2007; Gavin *et al.*, 2006; Morgan *et al.*, 2005; Walker *et al.*, 2003; Wang *et al.*, 2007). However, it was shown by flow cytometry that activated Tregs expressed a higher level of Foxp3 than activated effector T cells (Allan *et al.*, 2007; Wang *et al.*, 2007). Additionally, the majority of activated $CD4^+CD25^-$ T cells did not have the capacity to suppress $CD4^+$ T cell proliferation or IFN-γ secretion and expressed Foxp3 only transiently (Gavin *et al.*, 2006; Tran *et al.*, 2007; Wang *et al.*, 2007). In direct contrast, Walker and colleagues (2003) reported that activated $CD4^+CD25^-$, up regulating CD25 and Foxp3, acquired suppressive activity demonstrating the ability to suppress the proliferation of $CD4^+CD25^-$ cells. It has therefore been concluded that, as with CD25, Foxp3 can be used as a marker of T cell activation but is not solely sufficient to induce suppressive activity in human T cells. Consequently, Foxp3 is not a unique marker for the identification of human Tregs but remains one of the most specific markers found to date and may be used in combination with other markers to identify this T cell subset. However, the intracellular location of Foxp3 does mean that this transcription factor cannot be used to isolate Tregs for functional studies or *in vitro* culturing, therefore limiting its use in both research and clinical applications.

In an attempt to find a marker that would identify Tregs in humans and allow for subsequent functional studies, Liu *et al.* (2006) observed that the IL-7 receptor α chain (CD127) was down regulated on $Foxp3^+$ T cells. In fact $CD4^+CD25^{high}$ cells, the commonly accepted phenotype for human Tregs, were shown to have a three fold lower level of mRNA expression for CD127 in comparison to $CD4^+CD25^-$ T cells. Liu and colleagues also demonstrated, through multiparameter flow cytometry, that the expression of CD127 was inversely correlated to Foxp3 expression. In fact for one individual, 94% of

the $CD4^+CD25^+CD127^{low/-}$ T cells expressed Foxp3 and these cells were shown to be as suppressive, if not more, than the classic $CD4^+CD25^{high}$ Treg population. Additionally, the mean percentage of $Foxp3^+$ expression in the $CD4^+CD25^{inter}CD127^{low/-}$ population was 25.5% and further analysis showed these cells to possess suppressive activity (Liu *et al.*, 2006). The inverse correlation between Foxp3 and CD127 expression and the suppressive activity of $CD127^{low/-}$ T cells has also been supported by Hartigan-O'Connor *et al.*, (2007), Seddiki *et al.*, (2006) and Shen *et al.*, (2009). Highlighting further the relationship between CD127 and Foxp3, it has been demonstrated by Jeffrey Bluestone's laboratory that Foxp3 binds to the IL-7 receptor promoter region, suggesting repression of the CD127 gene by Foxp3 (Liu *et al.*, 2006). Using the Treg markers CD25 and CD127 approximately 6-8% of $CD4^+$ T cells express the phenotype $CD4^+CD25^+CD127^{low/-}$, at least threefold more than the $CD4^+CD25^{high}$ Treg phenotype, thereby providing a greater number of Tregs which could be expanded to a sufficient number of cells for use in immunotherapy (Hartigan-O'Connor *et al.*, 2007; Seddiki *et al.*, 2006).

Klein *et al.* (2010) have reported that a proportion of $CD4^+CD25^+CD127^+$ cells are also positive for Foxp3, however, due to the intracellular location of the transcription factor, it is not possible to isolate and assess the suppressive activity of this sub-population. It has also been observed that $CD4^+CD25^-$ T cells immediately down regulate CD127 upon activation before expression of the IL-7 receptor reappears in both Foxp3 positive and negative cohorts. This illustrates that activation induced expression of Foxp3 is not sufficient to maintain the low expression of CD127, whereas Foxp3 expression in nTregs is associated with a $CD127^{low/-}$ phenotype (Allan *et al.*, 2007).

In addition to the markers discussed, there are an array of molecules that have been shown to be important to Treg function or are able to be employed to identify a subset of Tregs in combination with other markers (Table 1.7). However, the expression of these markers are not specific to the suppressive T cell population and therefore the search for a marker that will definitively and reproducibly enable the identification and isolation of Tregs continues.

Table 1.7 Markers important to Treg function or used to identify Treg subsets

Marker	Role/Marker of	Reference
Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4; CD152)	Inhibits T cell function	Birebent *et al.*, 2004
CD39	Hydrolyses ATP and ADP	Borsellino *et al.*, 2007
CD45RA	Naïve T cells	Miyara *et al.*, 2009
CD45RO	Memory T cells	Baecher-Allan *et al.*, 2001
Glucocorticoid-Induced TNF Receptor (GITR)	Suppressive function	McHugh *et al.*, 2002
Inducible Co-Stimulatory Receptor (ICOS)	Associated with IL-10 and TGF-β induced suppression	Ito *et al.*, 2008
Helios	nTregs	Thornton *et al.*, 2010

To enable the frequency and suppressive activity of Tregs from HNSCC patients to be investigated, the studies conducted in this thesis will utilise the inverse correlation between Foxp3 and CD127 expression; using the $CD127^{low/-}$ phenotype to identify, isolate and functionally characterise the regulatory populations which have both intermediate and high levels of CD25 expression.

1.13 Tregs and cancer

The notion that a suppressive T cell population is involved in tumour immune evasion is not a new concept. In the 1970s and 1980s it was suggested that one of the methods employed by the tumour, to enable its continued progression, was to favour the generation of a suppressor T cell population (Greene *et al.*, 1979; Treves *et al.*, 1974). In 1980, Berendt and North demonstrated that a thymus dependent mechanism prevented the regression of tumours and this was accredited to a subset of T cells that prevented the host from mounting a successful anti-tumour response. More than twenty years later, and following the identification of Tregs in murine models, it was reported that elimination or inhibition of Tregs with an anti-CD25 monoclonal antibody (PC61) resulted in the regression of several different murine tumours (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999), highlighting their role in sustaining tumour growth.

The presence of Tregs in cancer patients was demonstrated by Woo *et al.* (2001) who showed that there was a significant increase of Tregs ($CD4^+CD25^+$) in the tumour microenvironment of both early stage non small cell lung cancer (NSCLC) and late stage ovarian cancer (OVC) compared with the circulation of healthy controls. The elevation of Tregs in the peripheral blood of OVC patients compared with healthy controls was also demonstrated but this significant trend was not shared by the peripheral blood of NSCLC patients. Subsequently, similar results have been observed in a number of malignancies, including HNSCC, with numerous studies reporting an elevation of Tregs in the peripheral circulation of cancer patients compared with healthy controls (Table 1.8).

The elevation of Tregs in cancer patients, impairing the host's anti-tumour response, has caused numerous groups to investigate the prevalence of Tregs in the tumour microenvironment as a prognostic indicator. In one of the first studies to associate Tregs with cancer patient prognosis, Curiel and colleagues (2004) demonstrated a significant correlation between the number of Tregs present in the tumour tissue and patient survival; patients with a high tumour Treg count had a 4.2 fold reduction in survival when compared to those patients with a low Treg count. However, investigations into other cancer types have not always agreed with these initial reports, with publications reporting conflicting results over whether the presence of tumour infiltrating Tregs are associated with positive or negative prognosis, or of no relevance at all (Table 1.9). In particular, for HNSCC and ovarian carcinoma, there are studies which have presented all three outcomes (Table 1.9). With each cancer type presenting varying results regarding the prognostic association of Tregs, it is important that each type of malignancy is assessed individually, including the different subsites in the head and neck region.

Table 1.8 Representative studies demonstrating increased prevalence of Treg phenotypes in the peripheral circulation of cancer patients compared with healthy controls

Treg phenotype	Cancer type	Mean percentage of Tregs in cancer patients	Mean percentage of Tregs in healthy controls	Reference
$CD4^+CD25^+$	Pancreas/ breast	13.2/16.6	8.6	Liyanage *et al.* (2002)
	Gastric/ oesophageal	18.8/26.5	8.9	Ichihara *et al.* (2003)
	HNSCC	10.1[a]	5.4[a]	Schaefer *et al.* (2005)
$CD4^+CD25^{high}$	Hepatocellular	3.9	1.2	Ormandy *et al.* (2005)
	Gastric/ oesophageal	4.9/5.2	1.9	Kono *et al.* (2006)
	HNSCC	1.2	0.5	Mandapathil *et al.* (2009)
	Bladder	8.7	2.4	Chi *et al.* (2010)
$CD4^+CD25^+FOXP3^+$	Colorectal	5.2	3.0	Chaput *et al.* (2009)
	HNSCC*	86.0[b]	65.0[b]	Strauss *et al.* (2009)
	Oesophageal	7.0	2.5	Maruyama *et al.* (2010)
	Hepatocellular*	2.0	1.2	Feng *et al.* (2011)
$CD4^+CD25^+CD127^{low/-}$	Gastric	7.8[c]	5.5[c]	Shen *et al.* (2009)
	HNSCC*	3.4	1.8	Schott *et al.* (2010)
	Acute myeloid leukemia	9.20	5.44	Shenghui *et al.* (2011)

* Identified by stated phenotype but with $CD25^{high}$ expression

All values expressed as a percentage of $CD4^+$ cells, apart from [a] lymphocytes [b] $CD4^+CD25^{high}$ cells and [c] total peripheral T cells

Table 1.9 Representative studies demonstrating association of tumour infiltrating Tregs with prognosis in cancer

Cancer Type	Prognostic association	Reference
HNSCC	Positive	Badoual *et al.* (2006)
Ovarian	Positive	Leffers *et al.* (2009)
Colorectal	Positive	Salama *et al.* (2009)
Bladder	Positive	Winerdal *et al.* (2011)
Ovarian	Negative	Curiel *et al.* (2004)
Pancreatic	Negative	Hiraoka *et al.* (2006)
Breast	Negative	Bates *et al.* (2006)
Hepatocellular	Negative	Kobayashi *et al.* (2007)
NSCLC	Negative	Shimizu *et al.* (2010)
Renal	Negative	Liotta *et al.* (2010)
HNSCC	Negative	Sun *et al.* (2012)
Ovarian	None	Sato *et al.* (2005)
Anal	None	Grabenbauer *et al.* (2006)
Renal	None	Siddiqui *et al.* (2007)
HNSCC	None	Distel *et al.* (2009)
Colon	None	Sinicrope *et al.* (2009)
Oesophageal	None	Zingg *et al.* (2010)
NSCLC	None	Liu *et al.* (2012)

1.14 Aims of study

The presence of Tregs is thought to be an important mechanism by which HNSCC successfully evades the immune system and the host's anti-tumour attack. However, the exact role of Tregs in HNSCC has yet to be defined.

Head and neck tumours arising from different subsites are frequently grouped together in research studies; however, although HNSCC are united by general location and histology, the different primary sites vary in presentation, pathogenesis and survival, and therefore should be investigated separately in a research setting. Tregs in the peripheral circulation of HNSCC patients have been investigated previously, however the patient populations often include patients who have had prior treatment and have been grouped as a single entity (Schaefer *et al.*, 2005; Schott *et al.*, 2010; Strauss *et al.*, 2007a). In addition, numerous cancer studies examine the frequency of Tregs in the peripheral circulation of patients, however, when assessing the Treg population it is important not only to examine

their prevalence, but also to investigate their suppressive capacity; as it is the functional activity of Tregs which will determine how effective a host's anti-tumour response will be in combating the growth and progression of a tumour. The current study will recruit newly-presenting patients that have received no previous diagnosis or treatment for cancer, to enable the direct influence of the head and neck tumour on the Treg population to be assessed. By using the $CD127^{low/-}$ phenotype, both the frequency and function of Tregs in the circulation and tumour microenvironment of HNSCC patients will be assessed in relation to tumour stage, subsite and nodal status.

To investigate the role Tregs have in HNSCC the suppressive T cell population firstly needs to be isolated; several cancer research groups have used magnetic activated cell sorting (Gasparoto *et al.,* 2010; Shen *et al.,* 2010; Yokokawa *et al.,* 2008) or fluorescence activated cell sorting (Chi *et al.,* 2010; Shen *et al.,* 2009; Strauss *et al.,* 2007a). This study will compare the use of both methods to determine which of the two procedures is optimal for isolating Tregs from peripheral blood and tumour samples, taking into account the length of the experiment and the purity and yield of the Treg population. Once a method has been established a carboxyfluorescein diacetate succinimidyl ester (CFSE) assay to follow the proliferation of effector T cells will be optimised, to enable the suppressive activity of Tregs in both the periphery and the tumour microenvironment of HNSCC patients to be assessed. Additionally, the immunosuppressive environment established by HNSCC will be examined further by analysing the secretion of cytokines and investigating the effect of tumour secretions on the functional activity of Tregs.

Increasing our understanding of the regulatory population will help to establish whether Tregs could be used as a prognostic determinant in head and neck cancer or be targeted by future immunotherapeutic strategies to reduce the Tregs responsible for the HNSCC-specific immune suppression; with the hope of improving the quality of life and survival rate for HNSCC patients.

Chapter 2.

Materials and methods

2.1 Culture of head and neck cancer cell lines

Head and neck cancer cell lines were cultured in 75cm^2 culture flasks (Sarstedt, Leicester, UK) containing approximately 12ml of complete medium: Dulbecco's Modified Eagle's Medium (DMEM; PAA, Yeovil, UK), supplemented with 10% foetal bovine serum (FBS; Biosera, Ringmer, UK), 0.4mM L-glutamine and penicillin/streptomycin (final concentration: 0.1U/ml and 0.1mg/ml respectively; PAA). Cells were maintained in a humidified incubator at 37°C with an atmosphere of 5% CO_2 (Galaxy 170 S, New Brunswick Scientific, Stevenage, UK).

Upon reaching 80-100% confluence the cell monolayer was passaged in a class II biological safety cabinet (Airstream, Esco, Downton, UK). The medium was removed and the cell monolayer washed with sterile phosphate buffered saline (PBS; Appendix; Oxoid Limited, Thermo Fisher Scientific, Basingstoke, UK). Trypsin/ethylenediaminetetra acetic acid (EDTA; 0.5mg/0.22mg/ml respectively; PAA) was added to the flask (2ml) for approximately 5 minutes at 37°C and agitated to ensure thorough removal of the adherent cells. Complete medium (10ml) was added to the flask to inhibit the trypsin and the cell suspension was recovered and centrifuged at 300 x *g* for 3 minutes to pellet the cells. Following removal of the supernatant the pellet was re-suspended in complete medium and the appropriate volume of cells, dependent on the cell line and the time of passage, was added to a sterile, labelled flask containing 12ml of medium.

2.2 Cryogenic storage of head and neck cancer cell lines

To maintain a store of the head and neck cancer cell lines, to be used when required in future experiments and minimise antigenic drift, the cell lines were cryogenically stored following culture. The HNSCC cell lines were cultured for no longer than 2 months before returning to a cryogenic aliquot. Briefly, the confluent culture of cells in a 75cm^2 culture flask (Sarstedt) was removed by trypsin/EDTA (PAA) as outlined in Section 2.1. Following centrifugation, the supernatant was discarded and the resulting cell pellet re-suspended in 3ml of freeze medium (culture medium containing 10% v/v dimethylsulphoxide; DMSO; Sigma, Gillingham, UK). The cell suspension was aliquoted

into 1ml labelled cryovials and frozen at a rate of 1^{o}C per minute in a cryo freezing container (Thermo Scientific Nalgene, Loughborough, UK) at -80^{o}C before transfer in to liquid nitrogen storage.

2.3 Mycoplasma testing and treatment

Head and neck cancer cell lines were routinely tested for mycoplasma infection as outlined in Section 2.3.1 and if found to be positive were treated to eradicate the infection as described in Section 2.3.2

2.3.1 Mycoplasma testing

The presence of mycoplasma was determined using a MycoProbe^{TM} Mycoplasma Dectection Kit (R&D Systems, Abingdon, UK) following the manufacturer's instructions. Briefly, cell culture supernatants (35µl) were diluted 10 fold in cell lysis diluent (provided in the kit) and vortexed for 15–20 seconds. Following washing of the hybridisation plate twice with the 1x wash buffer provided, and the addition of 50µl of diluted probes to each designated well, 150µl of positive control (synthetic DNA oligonucleotide), negative control (sample diluent) and diluted cell culture samples were added to appropriate wells in duplicate. The diluted probes included biotin-labelled capture oligonucleotide probes and digoxigenin-labelled detection probes that target the 16S ribosomal RNA of the eight most common mycoplasma contaminants that infect cell cultures. The plate was incubated for 60 minutes in a 65^{o}C oven whilst covered with a plate sealer. Throughout the assay procedure each wash step involved aspiration and washing of each well with 400µl of 1x wash buffer, using an automated plate washer (Wellwash 4 Mk 2 Microplate Strip Washer, Thermo Scientific), before a final aspiration.

Following incubation, 150µl from each well of the hybridisation plate was transferred to a streptavidin-coated plate that had been washed twice with 1 x wash buffer, to capture the ribosomal RNA/probe hybrid. After a further 60 minutes incubation on a horizontal orbital shaker (500 ± 50rpm) at room temperature the plate was washed four times and 200µl of anti-digoxigenin alkaline phosphatase conjugate was added to each

well. The plate was incubated under the same conditions for a further 60 minutes, washed six times and 50µl of substrate solution (NADPH) added to each well. Following 60 minutes of incubation, 50µl of amplifier solution (INT-violet) was added to each well and incubated for 30 minutes before the reaction was stopped with the addition of 50µl of stop solution (2N H_2SO_4). The optical density for each well was determined at 490nm with wave length correction at 620nm.

The average of the duplicate optical density readings was determined for each control and sample tested before the average negative control optical density was subtracted. Average optical density values below 0.05 were considered negative for mycoplasma whilst values over 0.1 were positive indicating that the cell culture was infected with mycoplasma. It was recommended that if values between 0.05 and 0.1 were obtained the procedure be repeated as the results were inconclusive.

2.3.2 Mycoplasma treatment

Head and neck cancer cell lines found to be infected with mycoplasma were cultured in complete medium (Section 2.1) supplemented with MycoKill AB (10µg/ml; PAA) for two weeks. The culture was then assayed for the presence of mycoplasma (Section 2.3.1) to ensure the infection had been cleared. If the infection persisted the manufacturer recommended repeating the treatment with increased Mycokill AB concentrations; however for the current study mycoplasma was removed from the cell cultures after a single treatment.

2.4 Cell counting and viability determination

The concentration of cells in suspension was calculated using an improved neubauer haemocytometer (Hawksley, Lancing, UK). Cell suspension (10µl) was added to an equal volume of 0.4% (w/v) Trypan blue (Sigma), which enters non-viable cells through their compromised membrane resulting in blue cytoplasm. The cell/dye suspension was added to the haemocytometer chamber and the number of viable cells in the 25 squares

(volume = $1x10^{-4}cm^3$) were counted under a light microscope. An average of two counts was taken and the concentration of cells was calculated using the following equation:-

Cell concentration = Average cell count x 2 (dilution factor) x 10^4 (cells/ml)

2.5 Collection of conditioned medium from head and neck cancer cell lines

Adherent cells were recovered from the culture flask using trypsin/EDTA (PAA) as outlined in Section 2.1. Cell concentration and viability were determined using trypan blue exclusion (Section 2.4) and 1 x 10^6 cells were added to each well of a 6 well plate (Sarstedt) in 1ml of complete growth medium (Section 2.1). For each cell line a minimum of four wells was cultured to enable a 96 hour time course to be followed. Conditioned medium samples were collected for each head and neck cancer cell line so that the release of cytokines could be examined by enzyme-linked immunosorbent assays (ELISA; Section 2.6) as well as assessing the effect of cancer cell line conditioned medium on the suppressive activity of Tregs (Section 2.17).

At appropriate time points (24hrs, 48hrs, 72hrs and 96hrs) growth medium was collected from cultured cells and centrifuged at 300 x *g* for 3 minutes to remove any cell debris. Aliquots (250µl) were prepared from the supernatant and stored at -80°C in appropriately labelled 0.5ml micro tubes (Sarstedt) for use in subsequent experiments (Section 2.6 and Section 2.17).

2.6 Determination of cytokine production by ELISA

The concentrations of cytokines (TGF-β and IL-10) present in the conditioned medium of head and neck cancer cell lines (Section 2.5) or overnight cultured tumour specimens (Section 2.14.1) were determined using DuoSET ELISA Development kits (Figure 2.1; R&D Systems) following the manufacturer's instructions.

Briefly, the 96 well maxisorp plate (Nunc, Thermo Scientific, Loughborough, UK) was coated with the capture antibody overnight at room temperature (2µg/ml anti-IL-10, anti-TGF-β) before being washed with 0.05% Tween 20 (Sigma) in PBS and each well incubated for 1 hour with 300µl of reagent diluent (IL-10) or block buffer (5% Tween 20 in PBS with 0.05% sodium azide; TGF-β). Throughout the ELISA each wash step was performed using an automated plate washer (Wellwash 4 Mk 2 Microplate Strip Washer, Thermo Scientific) and involved aspiration and washing of each well with 400µl of 0.05% Tween 20 in PBS three times before final aspiration.

Measurement of TGF-β in the conditioned medium samples involved pre-treatment to convert latent TGF-β to the immunoreactive form. To do this 1N HCl was added to each sample (20µl/100µl sample), mixed, and incubated for 10 minutes at room temperature. The acid was neutralised by the addition of 1.2N NaOH/0.5M 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, Sigma; 20µl/100µl sample), the sample was then mixed and assayed immediately.

Following washing, 100µl of conditioned medium from cell lines (Section 2.5) or overnight cultured tumour specimens (Section 2.14.1) and standards (seven point standard curve using two-fold serial dilutions; high standard 2000pg/ml IL-10, TGF-β) were added to appropriate wells in duplicate and incubated for 2 hours at room temperature. The wells were washed and incubated with 100µl of biotinylated detection antibody (150ng/ml IL-10; 300ng/ml TGF-β) for 2 hours at room temperature and following further washes each well was incubated for 20 minutes with 100µl of streptavidin-HRP (1:200) avoiding direct light. Detection was achieved, after another wash step, by incubation for 20 minutes with 100µl of tetramethylbenzidine (TMB) substrate solution (Vector Laboratories, Peterborough, UK). The reaction was stopped by the addition of 50µl of 2N H_2SO_4 to each well, the plate was mixed and the absorbance determined at 450nm with wavelength correction at 570nm. A standard curve was generated using a four parameter logistic curve-fit and used to determine the level of the cytokine present in the conditioned medium from cell lines or in the overnight cultured tumour specimens. For TGF-β the read off concentrations were multiplied by a dilution factor of 1.4 due to the pre-treatment step described above.

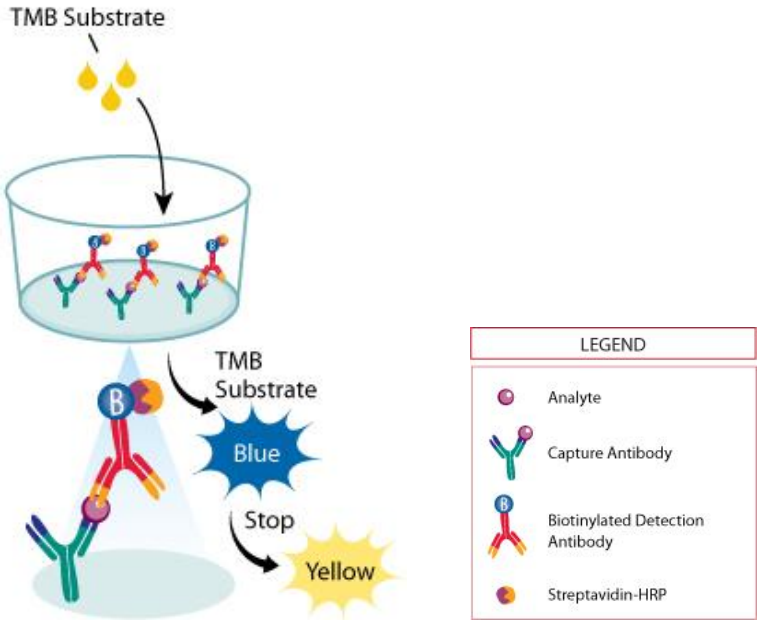


Figure 2.1 The different stages of the DuoSET sandwich ELISA method
The capture antibody is immobilised on to the 96 well plate surface and binds the analyte of interest, in this case the cytokine present in the conditioned medium of cell lines, overnight cultured tumour specimens or standards, which subsequently binds to a biotinylated detection antibody. Streptavidin-HRP binds to the biotin and the addition of the TMB substrate solution produces a blue colour which turns yellow following addition of 2N H_2SO_4. This enables the concentration of the cytokine present in the sample to be determined from the standard curve using a four parameter logistic curve-fit following absorbance measurements at 450nm with wavelength correction at 570nm. Image taken from R and D Systems, DuoSet ELISA Development Systems Assay Principle

2.7 Fluorescence Activated Cell Sorter (FACS) Aria™ II operation

The BD FACSAria™ II (BD Biosciences, Oxford, UK) was set up following the manufacturer's instructions. Briefly, the FACSFlow™ (BD Biosciences) and ethanol (VWR International, Lutterworth, UK) levels were checked and an empty waste container attached before the FACSAria™ II and computer were switched on. The FACSDiva software (BD Biosciences) was opened and the fluidics start up procedure followed. Once the stream had been turned on and stabilised the cytometer setup and tracking beads were

run (BD Biosciences), which allows the cytometer to automatically characterise, track and report measurements enabling the cytometer to be used reproducibly.

2.8 Setting compensation on the FACSAria™

When samples were labelled with more than one antibody conjugated to different fluorochromes it was necessary to 'compensate' for the spill over of one fluorochrome into the detection channel of another fluorochrome.

2.8.1 Preparing the compensation samples

PBS/bovine serum albumin (BSA; Fisher Scientific, Thermo Scientific)/sodium azide (100µl; appendix) was added to appropriately labelled 5ml polystyrene tubes (BD Biosciences). Following thorough vortexing, one full drop of the BD CompBeads negative control and 1 full drop of the BD CompBeads anti-mouse Ig κ was added to each tube. For each individual fluorescently conjugated monoclonal antibody to be used in the experiment the volume required to stain $1x10^6$ cells was added to the appropriate tube and mixed. A negative control tube was set up containing 100µl of PBS/BSA/sodium azide and 1 full drop of the BD CompBeads negative control. All samples were incubated for 20 minutes protected from direct light. Following incubation, 2ml of PBS/BSA/sodium azide was added to each tube to wash the beads and the sample was centrifuged at 200 x *g* for 10 minutes before the supernatant was discarded. The bead pellet was re-suspended in 500µl filtered PBS/BSA/sodium azide and compensation determined by acquisition on the FACSAria™ II (Section 2.8.2).

2.8.2 Calculating compensation values

Following the set up of the FACSAria™ II (Section 2.7) an experiment book in the FACSDiva software was opened, a specimen added and the fluorochromes not used during the multi colour experiment were deleted. A compensation specimen was selected from the

software's experiment programmes and the labelled cell sample to be analysed during the flow cytometry or cell sorting experiment was loaded and acquired. The voltage settings for forward and side scatter were altered to visualise the cell population of interest on the dot plot, which was subsequently encompassed by the gate P1 (Figure 2.2a). The voltage settings for each fluorochrome were altered to ensure that the peak representing cells that were negative for the fluorochrome were within the first segment of the histogram grid, whilst the cells positive for the fluorochrome remained in the 2nd to 4th segments of the histogram (Figure 2.2b). Next the negative control tube containing the unstained beads (Section 2.8.1), was loaded, encompassed by the P1 gate and 5000 total events recorded. This was repeated for all the compensation tubes, as directed by the software, and the compensation was automatically calculated using the FACSDiva compensation programme; these values were used during the experiment.

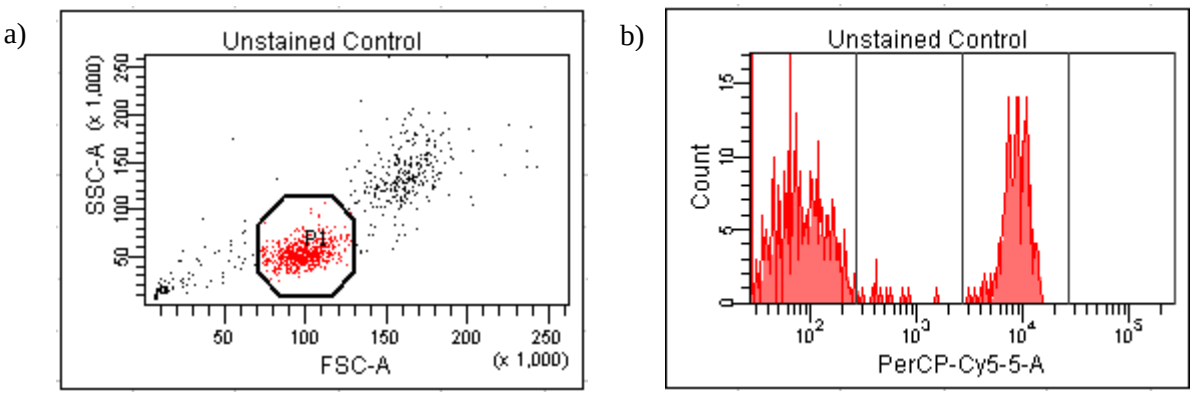


Figure 2.2 Calculation of compensation values for flow cytometry and cell sorting experiments
(a) The labelled cell sample to be used during the experiment was acquired and the forward and side scatter voltages were altered to display the cell population of interest which was encompassed by the gate P1. (b) The fluorochrome voltages were changed to ensure the peak representing the cells (P1) that were negative for the fluorochrome were within the first segment of the histogram grid and the cells positive for the fluorochrome were within the 2nd to 4th sections of the histogram.

2.9 Titration of fluorescently conjugated antibodies

Equivalent concentrations of fluorescently conjugated antibodies and corresponding isotype controls were used throughout the phenotypic analysis of cells (Section 2.10), to minimise the possibility of non-specific binding. All fluorescently conjugated antibodies

were titrated to determine the lowest concentration at which a positive population of cells could clearly be identified.

The concentration and viability of cells isolated by centrifugation was determined by trypan blue exclusion (Section 2.4) before being re-suspended at 1 x 10^6 cells/100µl in PBS/BSA/sodium azide. Following filtering through a 35µm nylon mesh (BD Biosciences) the cell suspension was aliquoted into appropriately labelled 5ml polystyrene tubes (100µl/tube).

For the titration of antibodies specific for intracellular markers the cells were fixed and permeabilised using a buffer set (BD Biosciences). Briefly, cells (1 x 10^6) were fixed by incubating with 1X Buffer A (2ml), containing diethylene glycol and formaldehyde, for 10 minutes at room temperature protected from light, before being washed with 2ml of PBS/BSA/sodium azide and centrifuged at 400 x *g* for 3 minutes. Following removal of the supernatant, the cells were permeabilised in the residual volume with 0.5ml 1X Buffer C [dilution 1:50, Buffer B (50X):Buffer A (1X)] for 30 minutes protected from light. The cells were washed twice with 1 ml of PBS/BSA/sodium azide and centrifuged at 400 x *g* for 3 minutes before the supernatant was discarded and the cell pellet was re-suspended in 100µl of PBS/BSA/sodium azide in preparation for the cells to be labelled.

In separate tubes, cells were incubated with the appropriate volume of fluorescently-conjugated antibody, as recommended by the manufacturer, and the corresponding concentration of isotype control. In addition, two-fold serial dilutions of the fluorescently-conjugated antibody were also included in separate tubes. The cells were then incubated for 30 minutes protected from direct light. Following a wash with 1 ml of PBS/BSA/sodium azide and centrifugation at 400 x *g* for 3 minutes the supernatant was discarded and the cell pellet re-suspended in 500µl of filtered PBS/BSA/sodium azide. The samples were then immediately acquired and analysed on the FACSAria™ II (Section 2.7) using the FACSDiva software. During acquisition of the isotype control, the forward scatter, side scatter and fluorochrome voltages were altered to visualise and gate the cell population of interest on a dot plot (Figure 2.3a) and to ensure the histogram peak was within the first segment of the grid (Figure 2.3b). The data for 10 000 cells within the gate was recorded. A marker was placed on the isotype control histogram which encompassed ≤1% of the cells (Figure 2.3b); this marker was used to identify the positive populations in the other tubes. Following titration the minimal concentration of antibody that gave

maximal binding was used in all subsequent experiments for the phenotypic analysis of cells (Section 2.10), along with the matched corresponding concentration of isotype control.

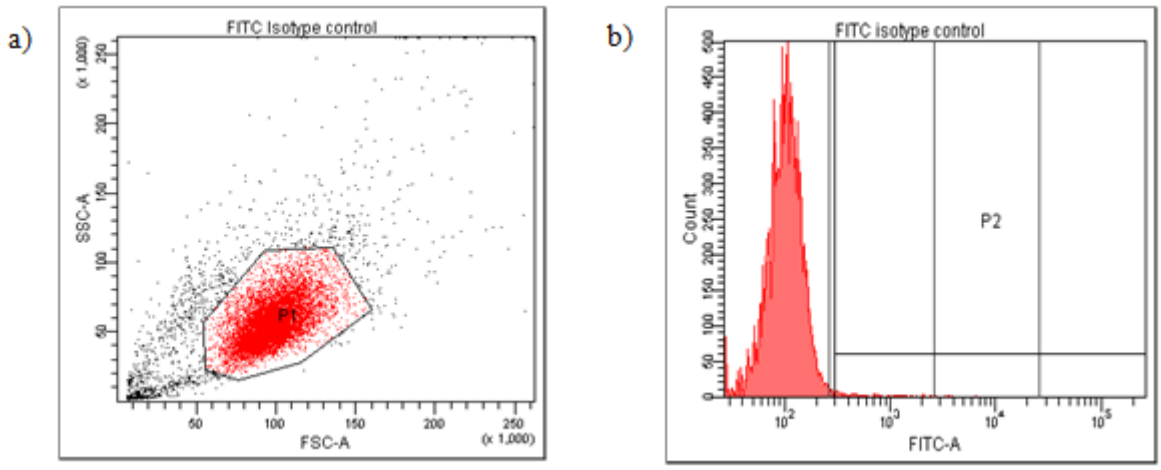


Figure 2.3 Dot plot and histogram of an isotype control sample acquired by flow cytometry
(a) Whilst acquiring the isotype control sample voltage settings for the forward and side scatter were altered to clearly show the cell population of interest. The cell population was gated (P1) and used to determine the phenotype of the cells. (b) The histogram plot of the isotype control was plotted using the P1 population. The voltage for the isotype control was altered so that the histogram was within the first segment of the grid. A marker (P2) was inserted ensuring that the percentage of cells encompassed by the marker was ≤ 1% for use in subsequent tubes to identify positive cells.

2.10 Phenotypic analysis of cells using flow cytometry

Cells isolated by centrifugation were re-suspended at 1 x 10^6 cells/100µl before being filtered and aliquoted into appropriately labelled 5ml polystyrene tubes (100µl/tube), as outlined in Section 2.9. Cells were incubated separately, with the appropriate volume of fluorescently conjugated antibody, as determined by titration (Section 2.9), or isotype control for 30 minutes protected from direct light. Cells being phenotyped additionally for an intracellular marker were subsequently fixed and permeabilised before the addition of the fluorescently conjugated antibody (Section 2.9). Following a wash with 1 ml of PBS/BSA/sodium azide and centrifugation at 400 x *g* for 3 minutes the supernatant was discarded and the cell pellet was re-suspended in 500µl of filtered PBS/BSA/sodium azide before being immediately acquired and analysed on the FACSAria™ II (Section 2.7) using

the FACSDiva software. For cell samples incubated with more than one fluorescently conjugated antibody compensation values were calculated as outlined in Section 2.8. The samples were acquired as outlined in Section 2.9, ensuring the marker placed on the isotype control histogram encompassed ≤1% of the cells (Figure 2.3b) thereby enabling the percentage of cells positive for the extracellular or intracellular molecule to be determined.

2.11 Immunohistochemistry

Formalin fixed and paraffin embedded HNSCC specimens and normal tonsil, for use as a positive control, were cut into 5µm sections and mounted onto glass slides by Hull Royal Infirmary Pathology Department. The sections were dewaxed through three changes of Histoclear II (National Diagnostics, Hull, UK) before being rehydrated through graded alcohols (100%, 90% and 70%). The sections were subsequently rinsed in running tap water before being incubated for 15 minutes in a 3% v/v hydrogen peroxide solution in methanol to block endogenous peroxidases. Following a further wash in running tap water the sections were boiled in an antigen unmasking solution (Vector Laboratories) using a pressure cooker for 3 minutes. The sections were placed in 1 x Tris-buffered saline (TBS; pH7.6; appendix) before being assembled in to a Sequenza™ slide rack and washed three times with TBS. To prevent non-specific binding of the secondary antibody and the avidin biotin detection system respectively, the sections were incubated for 20 minutes with a 100 fold dilution of normal horse serum (Vector Laboratories) before avidin and biotin binding sites were blocked as per the manufacturer's instructions (avidin/biotin blocking kit, Vector Laboratories). Diluted primary antibody (100µl; Abcam, Cambridge, UK) was added to all sections except one which had an equal concentration of irrelevant IgG1 antibody (1:20 dilution; AbD Serotec, Kidlington, UK) added and another to which an equal volume of TBS was added to act as negative controls. Following incubation for 1 hour at room temperature the sections were washed three times in TBS and incubated for a further 30 minutes with a 50 fold dilution of biotinylated secondary antibody (raised in horse; Vectastain Elite universal kit; Vector Laboratories). After rinsing in TBS the sections were incubated for 30 minutes in a streptavidin/biotin detection system linked to horseradish peroxidase (Vectastain Elite universal kit; Vector Laboratories) before undergoing a further

wash step. Positive staining was visualised by applying 3,3'-diaminobenzidine tetrahydrochloride (Sigma) to the section for approximately 5 minutes before the sections were rinsed in tap water and counterstained with Harris haematoxylin (Sigma) for 20 seconds. Subsequently the sections were rinsed in running tap water before being dehydrated through graded alcohols (70%, 90% and 100%) and three changes of Histoclear II. The sections were then mounted with a coverslip using Histomount (National Diagnostics) to prepare the stained sections for analysis.

2.11.1 Quantification of immunostaining

Positive immunostaining was identified in both the tumour-associated stroma and the tumour nests of HNSCC specimens using a light microscope under 400x magnification. Photomicrographs of five representative fields from each tissue compartment were taken and the absolute number of positive cells per field were counted using Image J software by two independent assessors (Dr. V. Green and Dr. A. Michno). The overall mean score per field for the positive staining of each compartment was calculated from the scores determined by the two assessors.

2.12 HNSCC patients and healthy controls

Newly presenting HNSCC patients and healthy controls were enrolled on to the study following ethical and NHS Trust R and D approval and obtaining informed signed consent. The HNSCC patients were recruited through the Ear, Nose and Throat (ENT) Department of the Hull and East Yorkshire NHS Trust. None of the newly presenting patients had received diagnosis or treatment for any other form of cancer, had active autoimmune or co-existing infectious disease and had received no previous radio- or chemotherapy before sample collection. Healthy controls were either healthy volunteers or were patients undergoing non-cancer related surgery, including removal of their uvula or tonsils, through the ENT department.

2.13 Isolation of peripheral blood mononuclear cells from whole blood

Blood samples (50ml) were taken by venepuncture into heparin coated syringes (2ml; 5000IU/ml) from healthy controls or HNSCC patients undergoing surgical resection of their tumour once informed signed consent was obtained. Peripheral blood mononuclear cells (PBMC) were isolated from 50ml of whole blood under sterile conditions in a class II biological safety cabinet (Airstream, Esco) using density gradient centrifugation. Blood was diluted (1:1 v/v) with PBS (Oxoid Limited) and 20ml layered on top of an equal volume of lymphocyte separation medium (LSM 1077; PAA) before centrifugation at 400 x *g* for 30 minutes without brake to generate layers as illustrated in Figure 2.4.

The clear middle layer and the cloudy layer above this, the "buffy coat" which contains the PBMC, was removed by pipette and transferred to an equal volume of PBS to wash the PBMC before centrifugation at 400 x *g* for 10 minutes, with the brake on, to pellet the cells. The supernatant was discarded and the resulting pellets combined before a further wash and re-suspension in freeze medium (FBS; Biosera, containing 10% v/v DMSO; Sigma). The PBMC suspension was aliquoted into 1ml labelled cryovials and placed in cryogenic storage as outlined in Section 2.2.

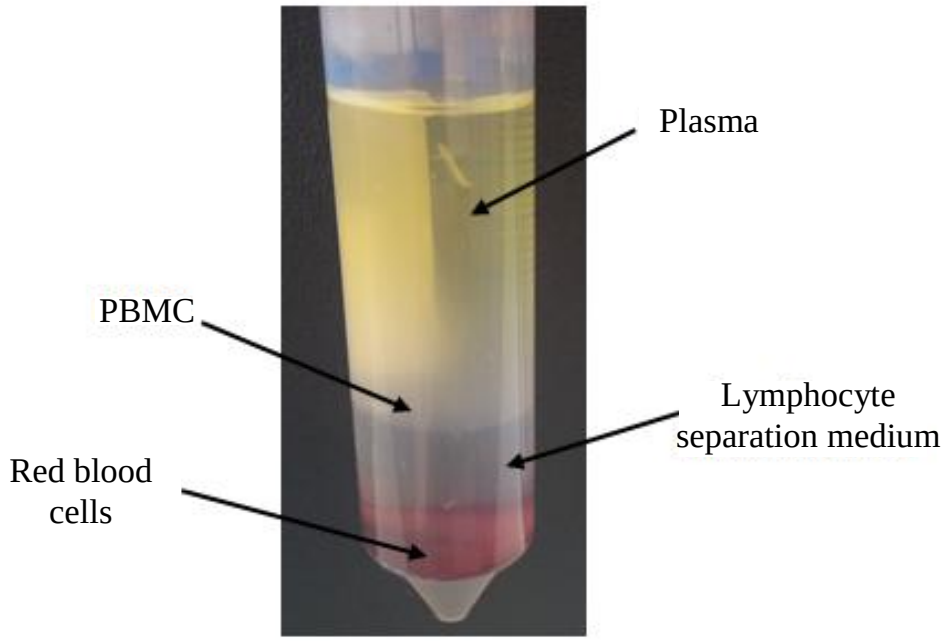


Figure 2.4 Layers formed after differential centrifugation of whole blood on lymphocyte separation medium to isolate PBMC
Blood samples were diluted (1:1 v/v) with PBS and layered on top of an equal volume of lymphocyte separation medium before centrifugation at 400 x *g* for 30 minutes without brake to generate the different layers, the PBMC are within the cloudy "buffy coat" layer.

2.14 Isolation of tumour infiltrating lymphocytes

Freshly isolated tumour specimens from HNSCC patients were collected during resection surgery at Castle Hill Hospital and transported in dissociation medium [DMEM (PAA) supplemented with 10% FBS (Biosera), 0.4mM L-glutamine, penicillin/streptomycin (PAA; final concentration: 0.1U/ml and 0.1mg/ml respectively) and fungizone (final concentration 2.5µg/ml; Invitrogen, Paisley, UK)] to the Daisy laboratories on the hospital site. Under sterile conditions in a class II biological safety cabinet (Airstream, Esco) fat, blood or necrotic areas were removed and the remaining tumour was weighed and cut in to ~1mm^3 pieces in a Petri dish (Sarstedt) using scalpels (Swann Morton, Sheffield, UK) whilst covered in dissociation medium. The tumour fragments were transferred to a 50ml polypropylene tube (Sarstedt) and following an antibiotic wash (15ml; PBS plus 2.5µg/ml fungizone and penicillin streptomycin, 0.1U/ml and 0.1mg/ml respectively) were recovered by centrifugation at 400 x *g* for 4 minutes. The specimen was

dissociated for 2–4 hours at 37^{o}C in dissociation medium containing 0.02% DNase (type 1; w/v; Roche Diagnostics, Burgess Hill, UK) and 0.02% collagenase (type IV; w/v; Sigma) whilst under constant rotation (MACSmix™ Tube Rotator; Miltenyi Biotec, Bisley, UK). Following incubation and further centrifugation at 400 x *g* for 4 minutes the supernatant was discarded and the dissociated tumour fragments were re-suspended in 10ml of dissociation medium, this was repeated a further three times. After the final wash step the dissociated tumour was plated overnight in a 25cm^2 culture flask (Sarstedt), containing approximately 5ml of dissociation medium to enable the majority of the fibroblasts, epithelial cells and monocytes to adhere to the flask whilst leaving the tumour infiltrating lymphocytes (TIL) in suspension.

2.14.1 Collection of TIL and tumour specimen conditioned medium

Following overnight incubation of the dissociated HNSCC tumour specimen, the medium was collected and centrifuged at 400 x *g* for 4 minutes. Aliquots (500µl) were prepared from the supernatant and stored at -80°C in appropriately labelled micro tubes for subsequent use in ELISA (Section 2.6) as well as analysing the effect of tumour secretions on the suppressive activity of Tregs (Section 2.17). The remaining cell pellet, containing freshly isolated TIL, was fluorescently sorted to isolate effector and Treg populations (Section 2.16). TIL not to be used immediately by FACS were counted and their viability determined by trypan blue exclusion (Section 2.4) before centrifugation at 400 x *g* for 4 minutes. The supernatant was discarded and the TIL were re-suspended (approximately 2 x 10^{7}cells/ml) in freeze medium (dissociation medium containing 10% v/v DMSO; Sigma), aliquoted into 1ml labelled cryovials and placed in cryogenic storage as outlined in Section 2.2.

2.15 Isolation of Tregs using magnetic activated cell sorting (MACS)

Tregs were isolated from PBMC using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II (Miltenyi Biotec) following the manufacturer's instructions, after optimisation as described in Chapter 3, for comparison to the fluorescently activated cell sorting method.

Briefly, three cryovials of frozen PBMC were thawed and washed with sterile filtered buffer (PBS, 0.5% BSA, 2mM EDTA) and centrifuged at 400 x *g* for 3 minutes to pellet the cells. The supernatant was discarded and the PBMC pellet was re-suspended in 5ml of buffer before cell concentration and viability were determined using trypan blue exclusion (Section 2.4).

Three separate aliquots of 1 x 10^7 cells were centrifuged at 300 x *g* for 10 minutes (throughout the isolation each centrifugation took place under these conditions unless otherwise stated). The supernatant was discarded and each cell pellet was incubated in the residual volume with 10µl of $CD4^+CD25^+CD127^{dim/-}$ T Cell Biotin-Antibody Cocktail II (biotin-conjugated monoclonal anti-human antibodies against CD8, CD19, CD123 and CD127) for 10 minutes at 4°C. Following incubation, Anti-Biotin Microbeads (20µl) were added and incubated with the cell suspension for a further 15 minutes at 4°C. Cells were washed by adding 1ml of buffer, centrifuged and the resulting cell pellets re-suspended in 500µl of buffer.

Each 1 x 10^7 cell suspension was added to an LD column which had been attached to a MidiMACS separator magnet and had been washed with 2ml of buffer (Figure 2.5). The LD column retains the unwanted magnetically labelled cells and separates them from the unlabelled cells (containing the Tregs) which pass through the column (Figure 2.6). Following the addition of cells each LD column was washed three times with 1ml of buffer and the effluents containing unlabelled target cells were collected, combined and centrifuged at 300 x *g* for 10 minutes. The supernatant was discarded and incubation of unlabelled cells with $CD4^+CD25^+CD127^{dim/-}$ T Cell Biotin-Antibody Cocktail II and Anti-Biotin Microbeads was repeated before applying to a fresh LD column to minimise the number of $CD127^+$ cells which had come through during the first incubation and LD column run through. The resulting fraction of unlabelled cells were centrifuged and the cell

pellet was re-suspended in the residual volume of buffer, and incubated with 15µl of CD25 labelled Microbeads II for 15 minutes at 4°C.

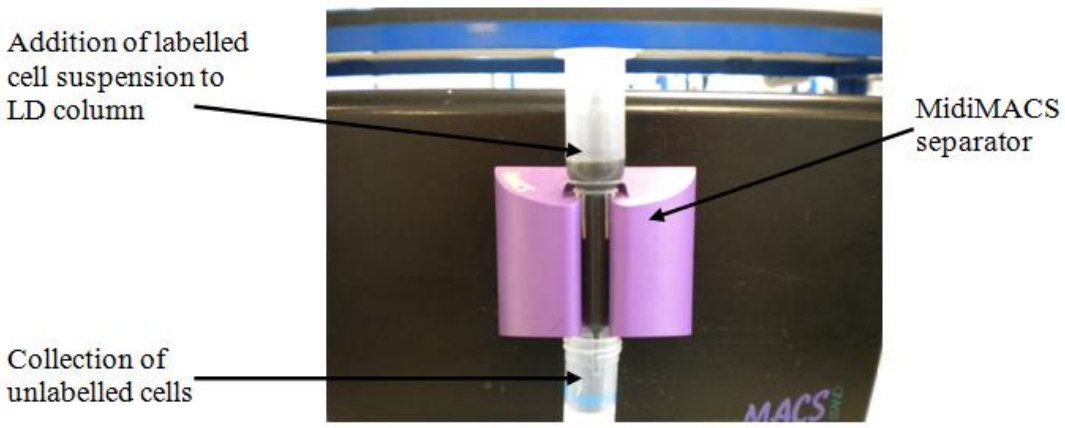


Figure 2.5 LD column and magnetic MidiMACS separator magnet
Cells labelled with the T Cell Biotin-Antibody Cocktail II and Anti-Biotin Microbeads were retained in the LD column and removed from the cell suspension, enabling the unlabelled cells, containing Tregs, to be collected in the effluent.

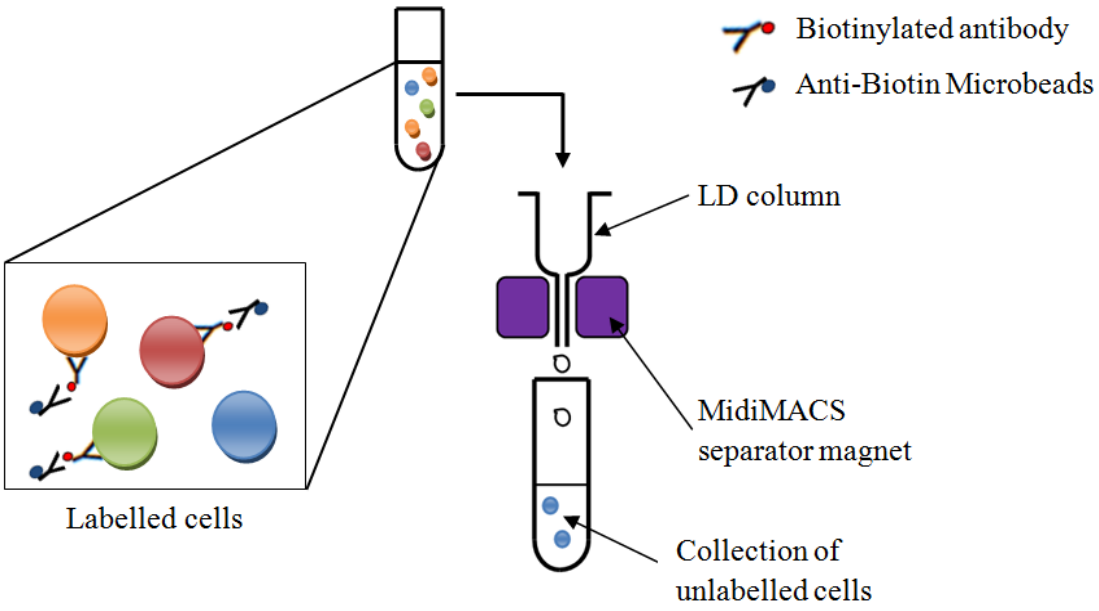


Figure 2.6 Negative depletion of non-target cells from cell suspension by LD column
The cell suspension was labelled with the $CD4^{+}CD25^{+}CD127^{dim/-}$ T Cell Biotin-Antibody Cocktail II (biotin-conjugated monoclonal anti-human antibodies against CD8, CD19, CD123 and CD127) and Anti-Biotin Microbeads which when passed through an LD column, attached to a MidiMACS separator magnet, depleted the sample of non-target cells. This allowed the unlabelled cell fraction containing Tregs to be collected and labelled further on in the protocol.

The cells were washed with 1ml of buffer and following centrifugation the resulting pellet was re-suspended in 500µl of buffer and applied to an MS column which had been attached to a magnetic MiniMACS separator and rinsed with 500µl of buffer (Figure 2.7). The MS column positively selects the target population by retaining the magnetically labelled $CD25^+$ cells (Tregs) and allowing the unlabelled non-target cells to pass through (Figure 2.8). Following the addition of the cells the MS column was washed three times with 500µl of buffer. The column was removed from the MiniMACS separator and placed onto a collection tube where 1ml of buffer was used to flush out the magnetically labelled cells by pushing the plunger into the column. The magnetically labelled fraction was added to a second newly prepared MS column, washed, flushed and collected as described above to increase the purity of the $CD4^+CD25^+CD127^{dim/-}$ Tregs.

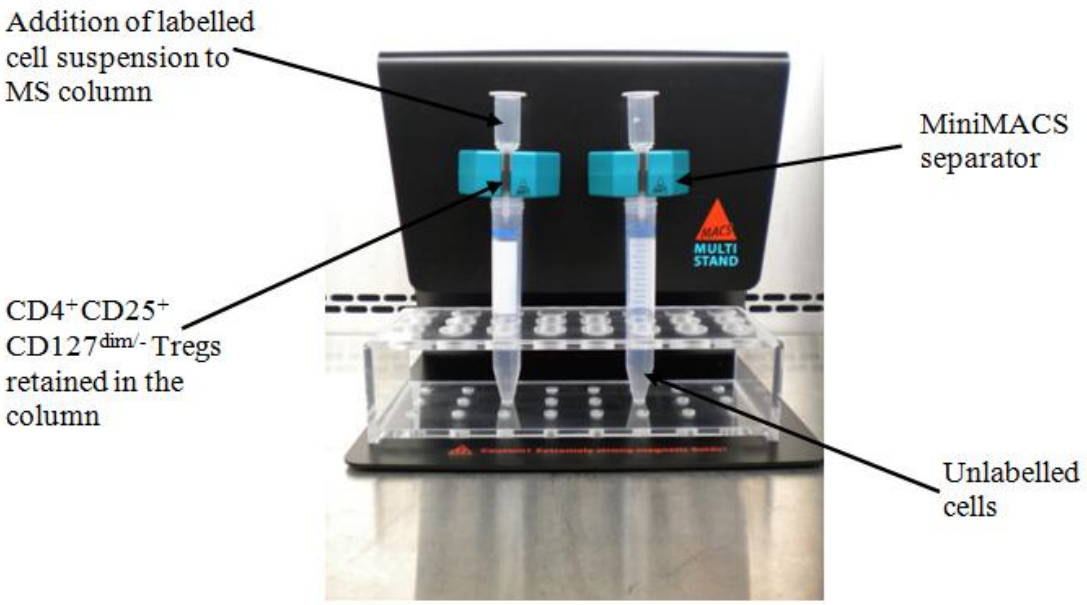


Figure 2.7 Two MS columns attached to MiniMACS separators
Cells labelled with CD25 Microbeads II were retained in the MS column which was attached to a MiniMACS separator. $CD4^+CD25^+CD127^{dim/-}$ Tregs were obtained by removing the column from the separator and flushing the Tregs out of the MS column.

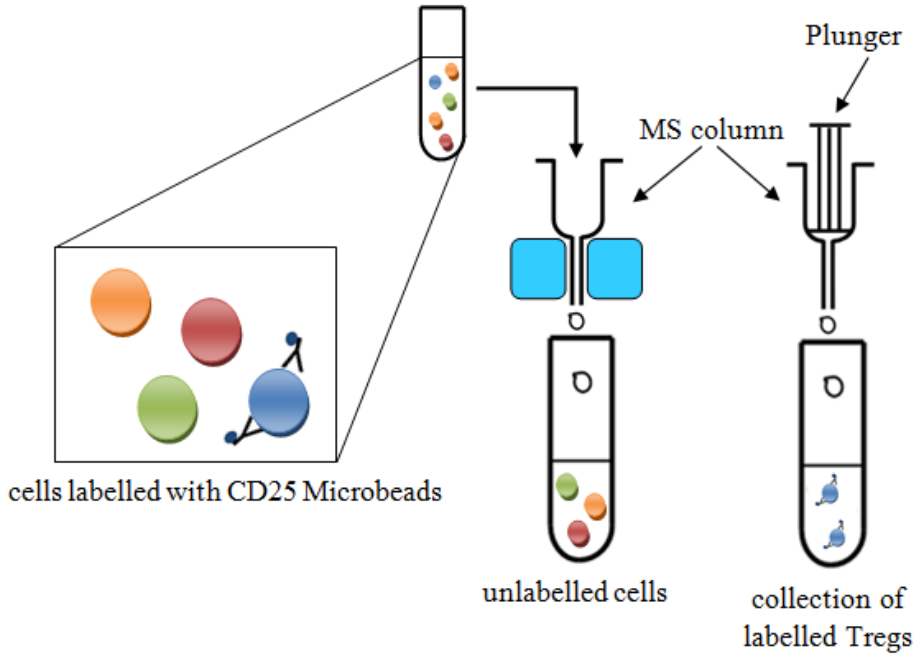


Figure 2.8 Positive selection of target cells from cell suspension by MS column
The negatively depleted cell suspension was incubated with CD25 labelled microbeads which were positively retained by the MS column. The target cell population, $CD4^+$ $CD25^+$ $CD127^{dim/-}$, was recovered by removing the column from the MiniMACS separator magnet and eluting the enriched positively selected fraction by pushing the plunger into the column with buffer.

2.16 Isolation of Tregs using fluorescence activated cell sorting (FACS)

Tregs to be isolated from PBMC or TIL using FACS were prepared using the Human Regulatory T Cell Sorting Kit (BD Biosciences) following the manufacturer's instructions.

Briefly, following washing in wash buffer (1 x PBS, 1% Human AB serum; Invitrogen) the concentration and viability of thawed PBMC (Section 2.13) or thawed/freshly isolated TIL (Section 2.14) was determined by trypan blue exclusion (Section 2.4). The sample was centrifuged in a 50ml polypropylene tube (Sarstedt) at 400 x *g* for 3 minutes to pellet the cells. For a concentration of less than 1 x 10^8 cells the resulting cell pellet was re-suspended in wash buffer to give a final staining concentration of 2 x 10^7 cells/ml, for greater than 1 x 10^8 cells the staining concentration was 1 x 10^8 cells/ml. The appropriate volume of Human Regulatory T Cell Sorting Cocktail [200µl/1 x

10^8 cells; mouse anti-human CD4-PerCP-Cy5.5 (clone L200), CD25-PE (clone 2A3), CD127-Alexa Fluor 647 (clone 4013)] was added to the cell suspension and incubated for 30 minutes protected from direct light to label the Tregs. A volume of wash buffer required to fill the 50ml polypropylene tube was used to wash the cells before centrifugation at 250 x *g* for 15 minutes. Following removal of the supernatant the cell pellet was re-suspended in wash buffer at a concentration of 7.5 x 10^6 cells/ml and filtered through a 35µm nylon mesh into a 5ml sterile polystyrene tube (BD Biosciences), to provide a single cell suspension.

2.16.1 FACSAria™ II set up for isolation of Tregs using FACS

The FACSAria™ II was set up as outlined in Section 2.7 before undergoing a programmed aseptic clean as described in the manual and was wiped down thoroughly with ethanol wipes to maintain the sterility of the sort. The sorting collection device, containing four temporary 5ml collection tubes, was installed and the amplitude of the stream was altered so that the drop 1 value was between 200 and 300 pixels; the amplitude determines the break off point of the first drop in the stream, whilst the drop 1 value is the number of pixels from the top of the video image of the stream to the centre of the first broken-off drop and is required to determine the drop delay (see below; Figure 2.9). The voltage was turned on and the test sort function activated to ensure that four collection streams and one waste stream were visible (Figure 2.10). By opening the waste draw it was checked that four streams were correctly entering the tubes in the collection device and the middle stream was passing into the waste, which during sorting would include non-target cells.

After the amplitude range, which defines the optimum values for the four streams entering the collection tubes, had been determined, an accudrop sample (BD Biosciences), containing a single population of particles that are conjugated to a fluorochrome emitting at 570nm, was run to calculate the drop delay. This value is very important to a sort as it calculates the amount of time it takes for a particle to travel from interception by the laser to the break off point of the stream, the drop containing a target cell can therefore be charged and sorted according to this value. Next the compensation values for the fluorochrome-conjugated antibodies present in the Treg sorting cocktail were calculated, as outlined in Section 2.8.

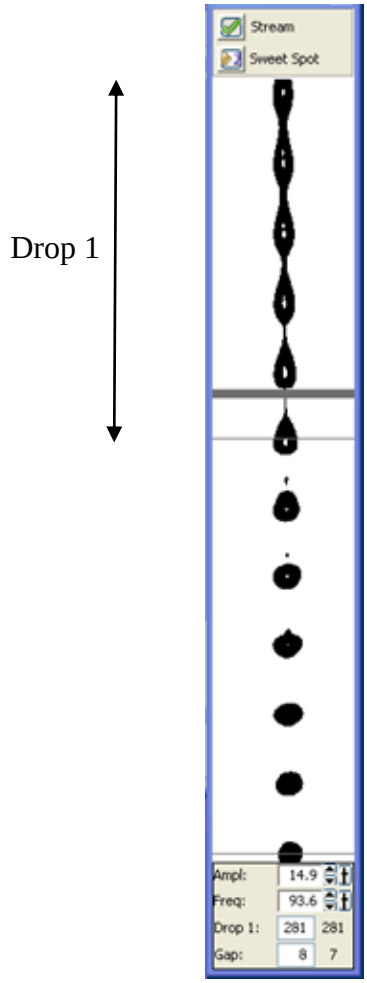
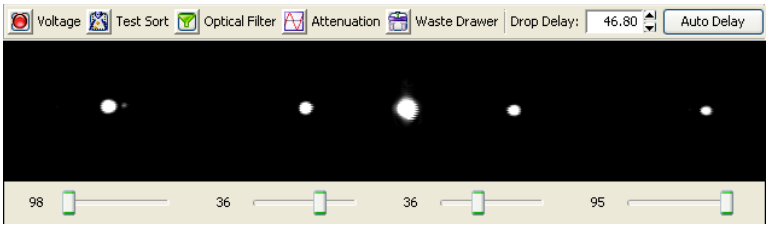


Figure 2.9 Video image of the stream
During the set up of the FACSAria for cell sorting the amplitude of the stream is altered so that the drop 1 value, measuring the number of pixels from the top of the video image of the stream to the centre of the first broken off drop, is between 200-300 pixels.



Number of stream 1 2 3 4 5

Figure 2.10 Visualisation of the five streams for FACS
Turning on the plate voltage and activating the test sort function visualises 5 streams. The central stream (3) enters into the waste and during sorting will include non-target cells. By opening the waste drawer it is ensured that streams 1, 2, 4 and 5 are entering the collection tubes.

The prepared stained PBMC or TIL to be sorted (Section 2.16) were briefly run through the flow sorter and the lymphocyte population was gated and acquired (10,000 events) as determined by forward and side scatter properties (Figure 2.11a). Doublets were subsequently excluded using FSC-W (Figure 2.11b) and SSC-W (Figure 2.11c) parameters. The resulting single cell population (Figure 2.11c; P3) was gated to encompass cells positive for the expression of CD4 (Figure 2.11d). Due to the study isolating two Treg populations differentiated by the level of CD25 expression, a dot plot was drawn to show the expression of CD4 and CD25 by the single cell population (Figure 2.11e). A quadrant was applied to the dot plot which encompassed cells negative for the expression of CD4 and CD25 in the lower left quadrant. High levels of CD25 expression ($CD25^{high}$) by the $CD4^+$ population was determined by placing a gate over the cells expressing CD25 at levels exceeding that expressed by $CD4^-$ cells and the cells expressing intermediate levels of CD25 were above the quadrant marker but below the $CD25^{high}$ gate. Subsequently, a dot plot was created to show the level of CD127 and CD25 expression by $CD4^+$ cells (Figure 2.11f). To identify the Tregs expressing low/negative levels of CD127 a gate was applied below the larger population of cells positive for the expression of the IL-7 receptor. The Tregs were then divided into two populations separated by CD25 expression ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) using the boundaries determined in the previous plot (Figure 2.11e and Figure 2.11f). Placing a gate above the Treg populations identified an effector T cell population positive for the expression of CD127 and CD25 ($CD4^+CD25^+CD127^+$). Gating $CD4^+$ cells negative for the expression of CD25 identified the second effector T cell population ($CD4^+CD25^-CD127^{-/+}$).

Following coating with culture medium (Section 2.17) sterile polypropylene 5ml tubes (Sarstedt) containing 0.2ml of the same medium were installed into the sorting collection device. Once it had been selected which tube would collect which gated population the yield and purity masks were chosen; the masks determine whether the interrogated drop is sorted based upon the location of the target cell within the drop or the presence of contaminating cells. The sweet spot was then activated which automatically adjusts the amplitude to ensure that the drop 1 and gap values are kept approximately the same so as not to alter the drop delay. Finally the prepared PBMC or TIL were loaded and sorted to isolate the identified effector T cell and Treg populations, which could take up to 6 hours depending on the cell concentration.

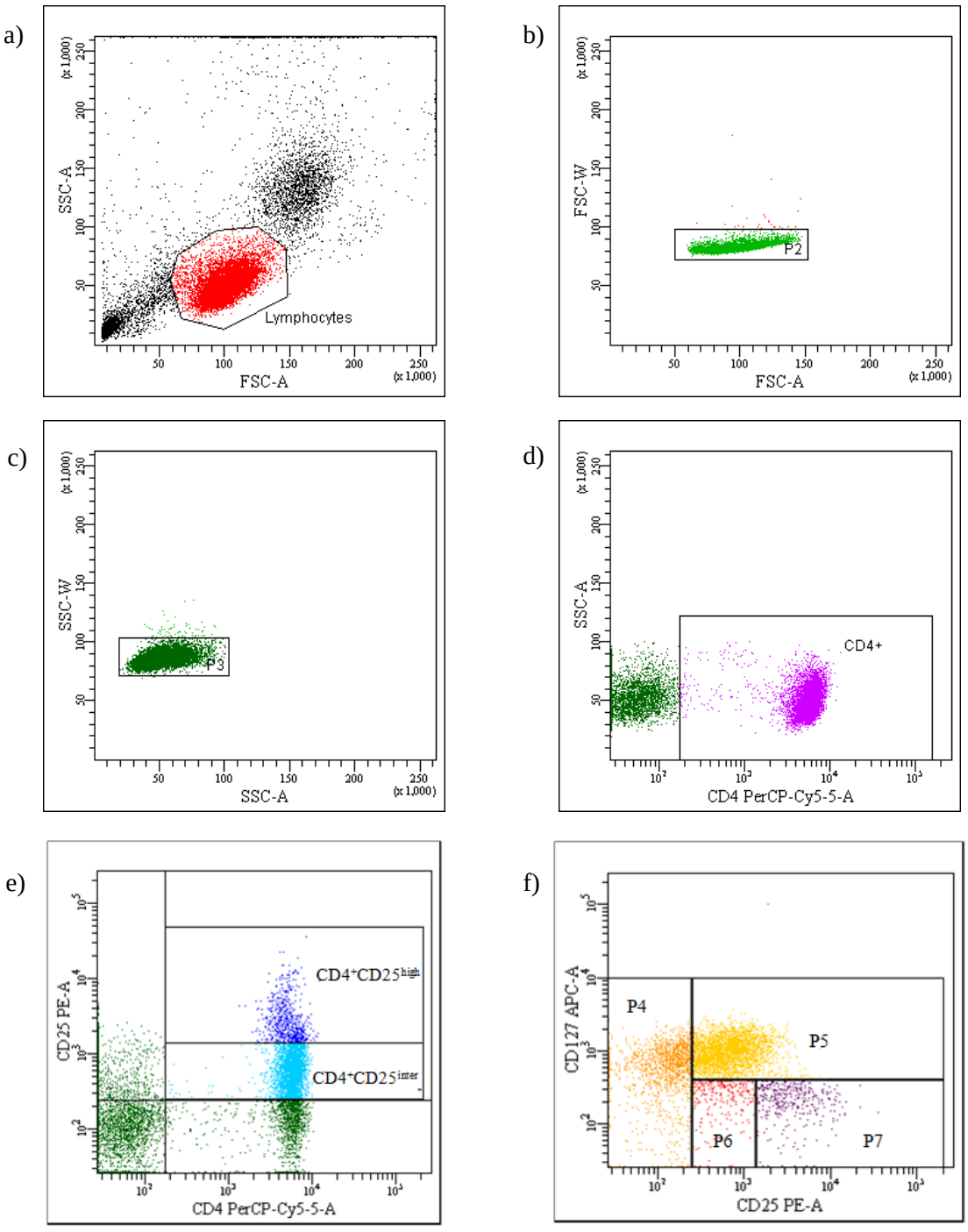


Figure 2.11 Gating strategy used to identify and isolate effector T cell and Treg populations

A PBMC or TIL sample was labelled with a Treg sorting cocktail and the lymphocyte population was gated using FSC-A vs. SSC-A parameters (a). Following doublet discrimination using FSC-W (b) and SSC-W (c) parameters, the resulting single cell population (P3) was gated to encompass cells positive for the expression of CD4 (d). The boundary for $CD25^{inter}$ and $CD25^{high}$ levels of expression was determined (e) and these boundaries were used to create the gates required to sort Tregs, $CD4^{+}CD25^{inter}CD127^{low/-}$ (P6) and $CD4^{+}CD25^{high}CD127^{low/-}$ (P7), and effector T cell populations, $CD4^{+}CD25^{-}CD127^{-/+}$ (P4) and $CD4^{+}CD25^{+}CD127^{+}$ (P5; f).

2.17 Culture of peripheral Tregs and effector T cells with conditioned medium

Immediately following isolation by flow sorting (Section 2.16), the concentration and viability of the peripheral effector T cell ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) and Treg ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) populations was determined by trypan blue exclusion (Section 2.4).

Approximately 5 x 10^5 effector T cells were cultured in a single well of a flat bottom 24 well tissue culture plate (Sarstedt) in 750µl of culture medium: X-VIVO 20 (Lonza, Slough, UK), supplemented with 5% heat inactivated AB serum (Invitrogen) and penicillin/streptomycin (final concentration, penicillin 0.1U/ml, streptomycin 0.1mg/ml; PAA) along with 100U/ml recombinant human IL-2 (AbD Serotec). Approximately 2 x 10^5 Tregs were cultured in 134µl of the same culture medium and 100U/ml recombinant human IL-2, based on the final culture volume (200µl), in a single well of a round bottom 96 well tissue culture plate (Sarstedt). Tregs and effector T cells were maintained in a humidified incubator at 37°C with an atmosphere of 5% CO_2 (LEEC, Nottingham, UK). After 24 hours half of the wells containing Tregs had 66µl (1:3 dilution) of conditioned medium, collected following either 48 hours of incubation with HNSCC cell lines (Section 2.5) or following overnight incubation with a dissociated tumour specimen (Section 2.14.1), added to the appropriate wells. The remaining half of the Treg cultures had the same volume of culture medium added to provide a control.

Following 48 hours incubation with conditioned or culture medium a CFSE assay was set up (Section 2.18) to analyse whether incubation with cell line or tumour conditioned medium influences the suppressive activity of Tregs.

2.18 Determination of cellular proliferation using a carboxyfluorescein succinimidyl ester (CFSE) assay

Immediately following isolation by flow sorting (Section 2.16) or 48 hour incubation with or without HNSCC conditioned medium (Section 2.17), the concentration and viability of the T cell populations were determined using trypan blue exclusion

(Section 2.4). Effector cells ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) were centrifuged at 400 x *g* for 3 minutes, supernatant discarded and the cell pellet re-suspended in 1ml of pre-warmed (37°C) sterile PBS/0.1% BSA; throughout the protocol each centrifugation took place under these conditions. An aliquot of effector cells (5×10^4) not to be stained with CFSE was transferred to a clean tube and along with the remaining effector cells to be labelled with the dye, centrifuged and re-suspended again in 1ml of pre-warmed PBS/0.1% BSA. Following a further centrifugation the effector cells to be stained with CFSE were re-suspended in pre-warmed PBS/0.1% BSA at a concentration of 1×10^6 cells/ml. CFSE (Sigma) was added to the effector T cells to be stained at a final concentration of 5µM before all cell suspensions, labelled and unlabelled, were incubated for 10 minutes at 37°C. The staining was quenched by the addition of 2.5ml of ice cold culture medium (Section 2.17). All cell suspensions were incubated on ice for 5 minutes and following centrifugation the supernatant was discarded and the cell pellet washed three times with pre-warmed culture medium before being re-suspended at a concentration of 5×10^4 cells/100µl.

The Treg populations ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) were centrifuged and following removal of the supernatant re-suspended in pre-warmed culture medium (5×10^4 cells/100µl) and co-cultured with the CFSE labelled effector T cell populations, $CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$, at various ratios [Treg:effector, 1:1 ($5x10^4 : 5x10^4$), 1:2 ($2.5x10^4 : 5x10^4$), 1:5 ($1x10^4 : 5x10^4$) and 1:10 ($5x10^3 : 5x10^4$)] in a single well of a round bottom 96 well tissue culture plate (Sarstedt) in 200µl of culture medium. Human T-Activator CD3/CD28 dynabeads (Invitrogen) were added to the culture at a cell:beads ratio of 1:3 along with 100U/ml recombinant human IL-2 (AbD Serotec). The single well of unlabelled effector T cells was cultured under the same stimulatory conditions. To determine the maximal level of CFSE retained by cells without cellular proliferation, one well containing labelled effector T cells received no stimulation. Table 2.1 illustrates all the culture combinations required to set up a CFSE assay in order to assess the suppressive activity of Tregs on the proliferation of effector T cells.

Following four days of co-culture cells were transferred to 5ml polystyrene tubes (BD Biosciences) and recovered by centrifugation. The supernatant was collected and stored in appropriately labelled micro tubes at -80°C. The cell pellets were re-suspended in

250µl of PBS/BSA/sodium azide before being analysed for CFSE content using the FACSAria™ II with FACSDiva software (Section 2.7). The control well, containing cells without CFSE staining, was used to set up forward scatter and side scatter voltages to provide a clear dot plot of the cell population (Figure 2.3a). The population was gated and the voltage of the channel detecting the CFSE dye emitting at 517nm was altered to ensure that the histogram peak was within the first segment of the grid, as described in Section 2.9. Following set up using the control tube, 10, 000 events of the cell suspensions incubated with CFSE were acquired.

Table 2.1 Combinations of cells cultured during the CFSE assay to determine the suppressive activity of Tregs on the proliferation of effector T cells

Number	Culture combinations	
1	Non-labelled effector T cells	
2	CFSE labelled effector T cells (no stimulation)	
3	CFSE labelled effector T cells	
4	Tregs ($CD25^{inter}$) and CFSE labelled effector T cells (1:1)	Tregs ($CD25^{high}$) and CFSE labelled effector T cells (1:1)
5	Tregs ($CD25^{inter}$) and CFSE labelled effector T cells (1:2)	Tregs ($CD25^{high}$) and CFSE labelled effector T cells (1:2)
6	Tregs ($CD25^{inter}$) and CFSE labelled effector T cells (1:5)	Tregs ($CD25^{high}$) and CFSE labelled effector T cells (1:5)
7	Tregs ($CD25^{inter}$) and CFSE labelled effector T cells (1:10)	Tregs ($CD25^{high}$) and CFSE labelled effector T cells (1:10)

All wells, except number 2, were stimulated with Human T-Activator CD3/CD28 dynabeads and 100U/ml IL-2.

2.19 Analysis of CFSE proliferation to determine the suppressive activity of Tregs

All CFSE experimental data were analysed using ModFit LT™ software (Verity Software House, Topsham, USA). The percentages of suppression were calculated based

on the proliferation index (PI) for effector cells cultured alone (100% proliferation, 0% suppression) compared to the PI of effector cells co-cultured with Tregs.

Initially the lymphocyte population was gated based on characteristic forward and side scatter parameters (Figure 2.3a). The fluorescent intensity of the CFSE labelled effector T cells that had been cultured without stimulation was then determined (Figure 2.12a); this represents the parent population i.e., cells that have not undergone cellular proliferation. The CFSE assay is based on the assumption that a single CFSE labelled parent cell will divide into two daughter cells, thereby causing the level of fluorescence intensity to be reduced by half. Each division (generation) can be visualised on a histogram plot as a 'peak', with the number of peaks representing the number of rounds of division that have taken place in the culture (Figure 2.12b).

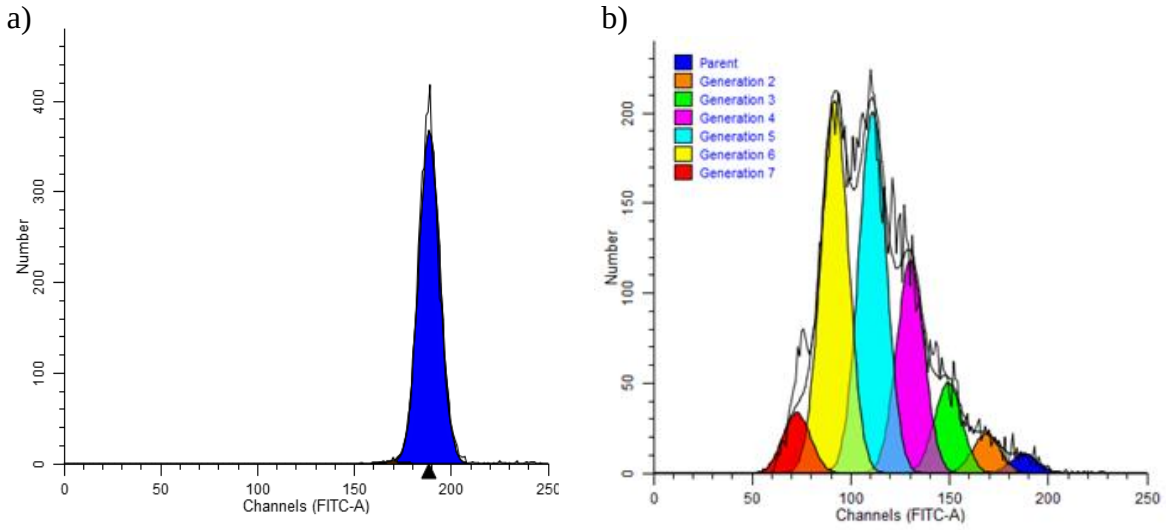


Figure 2.12 CFSE data analysed by ModFit™ software
The lymphocyte population was gated by forward and side scatter properties and the level of fluorescence intensity of CFSE labelled effector T cells cultured without stimulation was determined; this represents the parent population (a). The rounds of division undergone by stimulated CFSE labelled effector T cells are visualised as individual peaks on a histogram plot (b). In this plot seven generations are observed indicating that 6 rounds of division have taken place.

Applying this concept, the PI, a measure of the increase in cell number over the duration of culture, is determined by dividing the total number of cells in each generation by the original number of calculated parent cells present at the beginning of culture

(Section 3.2.3). The PI for effector T cells cultured alone represents 100% proliferation and 0% suppression, comparing this to the subsequent PI calculated from the co-culture of effector T cells and Tregs at varying ratios enabled the percentage of proliferation, and consequently the percentage of suppression induced by Tregs to be determined (Table 2.2).

Table 2.2 Calculating the percentage of suppression induced by Tregs using proliferation indexes

Treg : Effector T cell ratios	Proliferation index (ModFit)	Percentage of proliferation	Percentage of suppression
Effectors alone	5.64	100	0
1:1	3.47	61.3	38.7
1:2	4.27	75.8	24.2
1:5	5.07	90.1	9.9
1:10	5.29	93.5	6.5

Chapter 3.

Optimisation of methods for the functional characterisation of Tregs in HNSCC

3.1 Isolation of Tregs

The ability to isolate and study specific cell types has become an important goal in the clinical setting as cell based therapies are explored and developed. In order to investigate the possible benefits of Treg based immunotherapy in a clinical situation and obtain a greater biological understanding of the role of Tregs, a reproducible method first needs to be created to isolate an enriched population of this suppressive T cell subtype. The aim was to identify a robust method that would enable the maximal isolation and functional characterisation of Tregs from head and neck cancer patients which would improve the understanding of their role in HNSCC during subsequent chapters. The two main methods used in the literature to isolate Tregs are magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS).

MACS technology is a magnetic separation method that separates cells in suspension by the expression of cell surface antigens. (Miltenyi Biotec, 2010). Cells of interest are specifically labelled with super-paramagnetic MACS Microbeads and when the labelled cells are passed through a MACS column, which is placed in a strong magnet, the magnetically labelled cells are retained in the column and separated from the unlabelled cells which pass through. By removing the column from the magnet the retained cells can then be eluted enabling both fractions, labelled and unlabelled cells, to be recovered. This allows target cells to be recovered by two strategies: positive selection and depletion. Positive selection specifically labels the target cells, retaining them in the MACS column whilst the depletion method labels non-target cells, enabling the unlabelled target cells to pass through the column and remain "untouched" (Miltenyi Biotec, 2010). To isolate Tregs from HNSCC patient PBMC samples both strategies were used, depletion followed by positive selection.

FACS is a flow cytometric method that can also separate viable cells of interest from a heterogeneous population according to the expression of cell surface antigens. A broad cell population containing the target cells is identified ('gated') by forward and side scatter parameters and the target population within this gate is identified by the expression of specific cell surface molecules which are detected using fluorescently conjugated monoclonal antibodies. A stream of liquid , containing the labelled cell sample, is flowed through a cytometer so that particles are formed containing single cells. When a stream

particle, containing a single cell, is interrogated by the laser and determined to contain a cell of interest an electrical charge is applied to the stream just as the particle containing the target cell breaks off (BD Biosciences, 2009). The charged particle passes through two strongly charged deflection plates causing deflection of the particle into a sorting collection device. Uncharged particles, containing non-target cells, are not affected by the deflection plates and enter the waste container (BD Biosciences, 2009).

Although both MACS and FACS have successfully been used to isolate Tregs previously (Chi *et al.*, 2010; Gasparoto *et al.*, 2010; Shen *et al.*, 2010; Strauss *et al.*, 2007a), both techniques were used by the current study to isolate Tregs from PBMC samples to determine the method that would enable the maximal isolation of Tregs from HNSCC patients for their subsequent functional characterisation.

3.1.1 Isolation of Tregs by MACS

The final protocol used to isolate Tregs using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II is outlined in Section 2.15; however several experiments were conducted to optimise the isolation of Tregs using MACS technology.

Firstly, a trial kit which isolates $CD4^+CD25^+$ Tregs was purchased from Miltenyi Biotec to determine whether Tregs could be isolated from stored head and neck cancer patient PBMC samples (Section 2.13) using the MACS principle and following the two step procedure provided by the manufacturer. The trial kit included a $CD4^+$ T Cell Biotin-Antibody Cocktail (biotin-conjugated monoclonal anti-human antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a) and Anti-Biotin Microbeads which retained non-$CD4^+$ cells in the LD column, thereby removing them from the cell suspension (negative depletion; Figure 2.5). However, analysis of the cells retained in the LD column by flow cytometry following labelling with CD4-PerCP (Section 2.10; clone SK3 ;BD Biosciences) showed that a number of $CD4^+$ cells were also being retained. To try and reduce the number of $CD4^+$ cells being retained in the column, the number of washes of the LD column during a single experiment was increased from two up to eight and the number of $CD4^{+/-}$ cells passing through the column was determined by flow cytometry as mentioned previously. The majority of $CD4^+$ cells were found to have passed through the column after two washes however, even after three washes there were still a

number of $CD4^+$ cells coming through the column which decreased following subsequent washes (Table 3.1). After eight washes a few $CD4^+$ cells were still present in the column effluent however, increasing the number of washes also increased the number of $CD4^-$ cells passing through the column. Three washes of the LD column was chosen for subsequent isolations as this enabled $CD4^+CD25^+$ Treg isolation with minimal $CD4^-$ cell contamination in a reasonable time frame. Furthermore, it was also shown that LD columns are naturally 'sticky'; following the addition of 1 x 10^7 unlabelled PBMC to an LD column 2,272 lymphocytes were retained in the column even though the cells had not been incubated with the T Cell Biotin-Antibody Cocktail or Anti-Biotin Microbeads; of the lymphocytes retained 26.6% were $CD4^+$.

Table 3.1 The number of $CD4^+$ and $CD4^-$ cells passing through the LD column as the number of washes increases starting with 1 x 10^7 PBMC

Number of LD column washes	Number of $CD4^+$ cells passing through LD column	Number of $CD4^-$ cells passing through LD column
2	7477	2523
3	170	138
4	58	75
5	34	59
6	18	51
7	34	83
8	26	88

Results from one experiment

Having shown that Tregs could be isolated from stored HNSCC patient PBMC samples using the trial $CD4^+CD25^+$ Regulatory T Cell Isolation Kit a $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II was purchased (Miltenyi Biotec). This kit not only allows isolation of Tregs based on the expression of CD4 and CD25 markers but also by the more recently discovered low/negative surface expression of CD127, which is inversely correlated to Foxp3 (Section 1.12). The final optimised protocol is outlined in detail in Section 2.15; briefly, the PBMC sample was labelled with a T Cell Biotin-Antibody Cocktail II and Anti-Biotin Microbeads before being applied to a LD column which depleted the sample of non-target cells. The unlabelled cell fraction, containing the Tregs, was subsequently incubated with CD25 labelled Microbeads II and

applied to an MS column, positively selecting the $CD4^+CD25^+CD127^{dim/-}$ Treg population.

The protocol was optimised by analysing the cells either retained in the columns or coming through in the effluent at each of the stages using flow cytometry with the Treg marker antibodies CD4-FITC (clone M-T466), CD25-APC (clone 4E3) and CD127-PE (clone MB15-18C9; Section 2.10; Miltenyi Biotec) and the appropriate isotype controls (BD Biosciences). These antibodies had been titrated (Section 2.9) and due to the use of several fluorochrome-conjugated antibodies compensation values had been calculated too (Section 2.8). From the previous results the LD column was washed 3 times, however a number of $CD4^+$ cells were still being retained in the column (Figure 3.1a). It was concluded that this was because some of the cells retained in the column due to their expression of the markers included in the T Cell Biotin-Antibody Cocktail II (biotin-conjugated monoclonal anti-human antibodies against CD8, CD19, CD123 and CD127), to remove the non-target cells, may also express CD4. Figure 3.1b demonstrates this by showing that the $CD4^+$ cells which have been retained in the column have done so because the majority of them are positive for CD127.

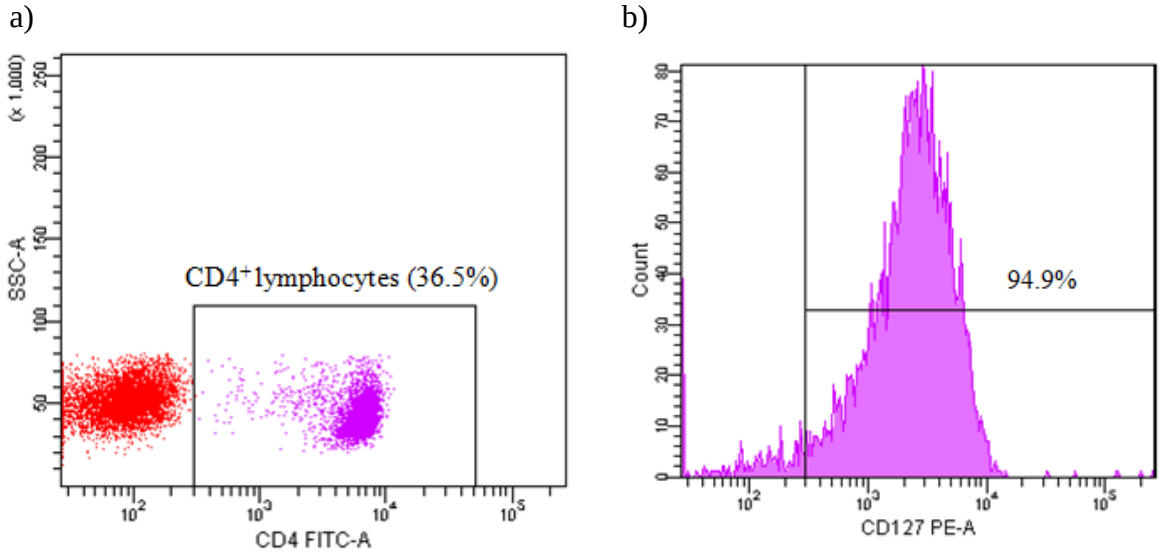


Figure 3.1 Expression of CD4 and CD127 by cells retained in the LD column
PBMC labelled with the T Cell Biotin-Antibody Cocktail II and Anti-Biotin Microbeads were passed through a LD column and washed 3 times. The cells retained in the column were removed and labelled with CD4-FITC and CD127-PE mouse anti-human antibodies. a) Following gating of the lymphocyte population by forward and side scatter properties, a dot plot was created to show that 36.5% of lymphocytes were $CD4^+$. b) Histogram plotted using the $CD4^+$ population, the majority of the $CD4^+$ cells were also positive for the expression of CD127 (94.9%).

Although an antibody against CD127 is included in the Biotin-Antibody Cocktail, it was observed that a number of $CD127^+$ cells were being eluted through the LD column instead of being retained, decreasing the purity of the $CD4^+CD25^+CD127^{dim/-}$ Treg population. To reduce this, an extra incubation with the T Cell Biotin-Antibody Cocktail II and Anti-Biotin Microbeads was included in the protocol before the sample was applied to a new LD column in attempt to remove the remaining $CD127^+$ cells. From four separate experiments using different PBMC samples the extra step was shown to retain $CD127^+$ cells in the new LD column; on average 69.7% of the retained lymphocytes were positive for the expression of CD127. This showed the importance of the extra step to remove contaminating $CD127^+$ cells that had not been retained by the first LD columns.

The protocol provided by the manufacturer started with 1×10^7 PBMC however the average number of Tregs isolated from three separate experiments was only 135 (range 49-233 Tregs isolated). The starting number of PBMC was therefore increased to 3×10^7 to try and increase the yield of Tregs isolated (Figure 3.2); along with an increase in the volume of CD25 labelled Microbeads II from 10µl to 15µl to account for the elevated number of cells needing to be stained before addition of the cell suspension to the MS column. From six experiments using different HNSCC patient PBMC samples, the average number of Tregs isolated starting with 3×10^7 PBMC was 1,660 (range 381-4,070), an increase of more than twelve fold compared with Tregs isolated from a starting number of 1×10^7 PBMC. The range of Tregs isolated from 3×10^7 PBMC also demonstrated that the number of Tregs obtained was dependent on the HNSCC patient sample.

The final optimised protocol (Section 2.15) was conducted four times; enabling the yield of Tregs isolated from PBMC which had been stored under different conditions to be compared. Table 3.2 shows the percentages of expression of CD4, CD25 and CD127 on cells isolated from fresh PBMC, overnight cryopreservation at -80ºC, 2 weeks and 1 month liquid nitrogen storage, as well as the total Treg yield. No differences were seen in the expression of the different Treg markers with the different storage methods however, the absolute yield of Tregs isolated was much higher from fresh PBMC (4, 220 Tregs isolated) compared with cryopreserved cells which decreased from 2,839 (-80ºC overnight) to 671 (1 month liquid nitrogen) Tregs isolated with increased length of storage. The isolation of Tregs from fresh PBMC was from a healthy volunteer however, the samples stored under the different conditions were all from the same HNSCC patient PBMC sample.

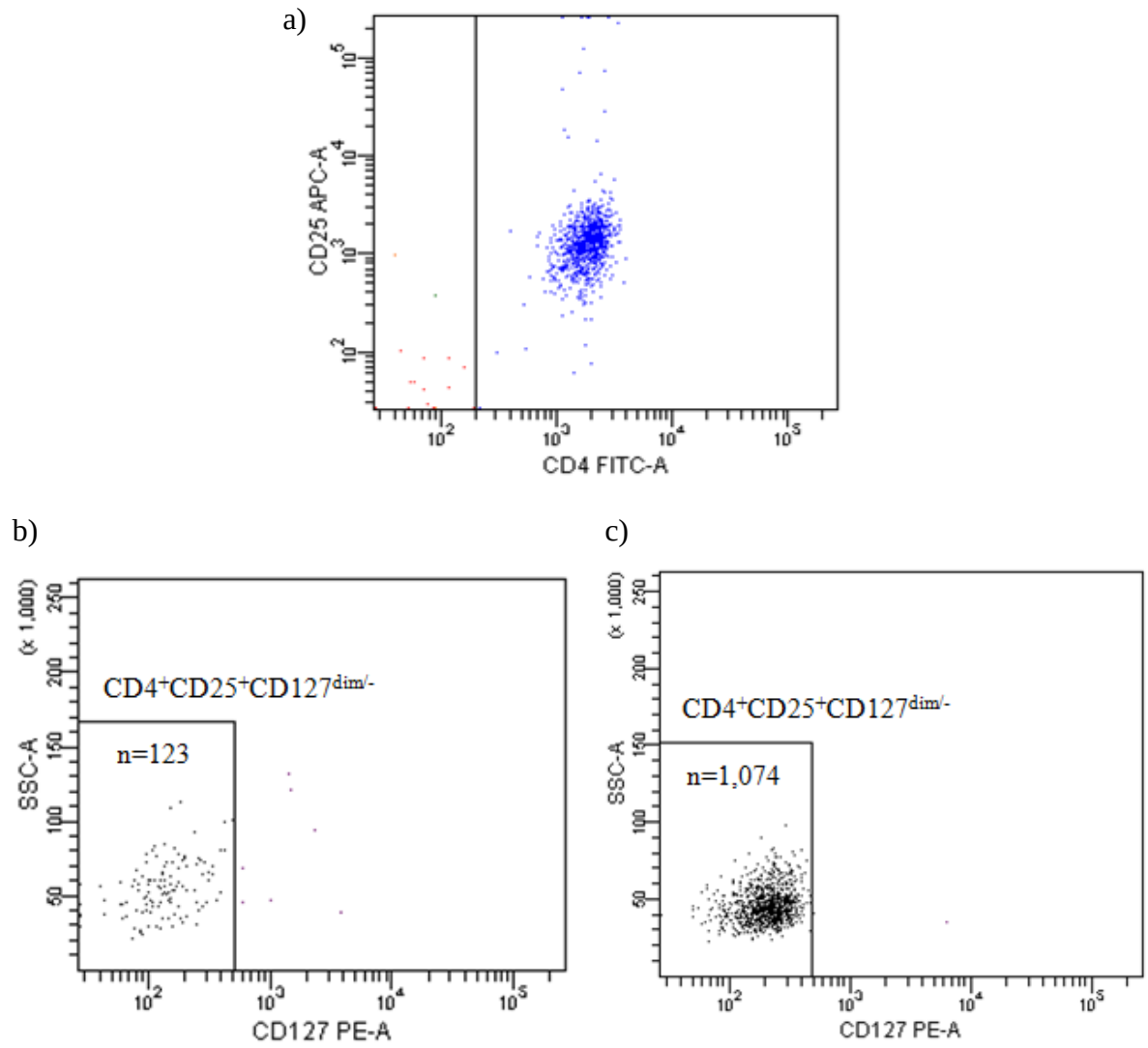


Figure 3.2 Comparison of the number of Tregs isolated by MACS with different starting numbers of PBMC

Tregs retained in the MS column were removed and labelled with the Treg marker antibodies CD4-FITC, CD25-APC and CD127-PE. The lymphocyte population was gated and the isolated $CD4^+CD25^+$ population (blue; a) was used to create a dot plot showing their dim/negative expression of CD127 and the number of Tregs ($CD4^+CD25^+CD127^{dim/-}$) isolated, starting with b) 1 x 10^7 PBMC (n=123) and c) 3 x 10^7 PBMC (n=1,074). Representative of 3 and 6 experiments, respectively.

Table 3.2 Isolation of Tregs by MACS using PBMC samples stored under different conditions from a starting number of 3 x 10^7 PBMC

Isolation	Storage of PBMC sample	Percentage of isolated lymphocytes expressing CD4	Percentage of isolated $CD4^+$ cells expressing CD25	Percentage of isolated $CD4^+$ $CD25^+$ cells expressing $CD127^{dim/-}$	Absolute number of $CD4^+CD25^+$ $CD127^{dim/-}$ Tregs isolated
1	Fresh[a]	99.5	99.6	99.9	4, 220
2	Cryo freezing container[b] (-80ºC overnight)	96.8	98.7	98.9	2,839
3	Liquid nitrogen[b] (2 weeks)	98.5	98.7	99.9	1, 074
4	Liquid nitrogen[b] (1 month)	98.3	98.1	99.3	671

[a] PBMC from one healthy control
[b] PBMC from the same HNSCC patient
Representative of one Treg isolation at each different storage condition

3.1.2 Isolation of Tregs using FACS

Tregs were isolated from cryopreserved PBMC samples using the Human Regulatory T Cell Sorting Kit (BD Biosciences) following the manufacturer's instructions as outlined in Section 2.16. To compare Treg isolation by MACS with FACS, four isolations were conducted on cryopreserved head and neck cancer patient (n=2) and healthy control (n=2) PBMC samples using the flow cytometric method. The number of $CD4^+CD25^+CD127^{low/-}$ Tregs sorted from a starting PBMC population of approximately 3 x 10^7 cells, in all cases was over 100,000 and in one experiment this increased to over 300,000 (Table 3.3). Apart from the first isolation, the $CD4^+CD25^+CD127^{low/-}$ population was divided into those having intermediate and high expression of the IL-2 receptor so that these two Treg populations could be investigated separately and compared in subsequent

chapters for their frequency and suppressive activity. From two of the isolations there were more cells expressing CD25 at a high level compared with intermediate expression.

Table 3.3 Isolation of $CD4^+CD25^+CD127^{low/-}$ Tregs by FACS

Independent isolation	Starting PBMC	Percentage of isolated lymphocytes expressing CD4	Number of $CD4^+CD25^+CD127^{low/-}$ cells isolated	Number of $CD4^+CD25^{inter}CD127^{low/-}$ cells isolated	Number of $CD4^+CD25^{high}CD127^{low/-}$ cells isolated
1[a]	2.69×10^7	99.9	199, 541	Not determined	Not determined
2[a]	2.27×10^7	99.8	119, 778	100, 642	19, 136
3[b]	3.42×10^7	98.9	336, 956	123, 261	213, 695
4[b]	3.1×10^7	99.9	252, 222	99, 420	152, 802

[a] HNSCC patients
[b] Healthy controls
Four different PBMC samples

To assess the suppressive activity of isolated Tregs on the proliferation of autologous effector T cells the effector population also had to be isolated. The original FACS gating strategy used to isolate effector T cells was set with the phenotype of $CD4^+CD25^{-/+}CD127^+$ however, this method excluded a population of cells that were negative for the expression of CD25 (Figure 3.3a). A further gate was therefore added to divide the effector T cell population based on CD25 expression; this included $CD4^+CD25^-CD127^{-/+}$ cells, frequently used by research groups to assess the suppressive activity of Tregs on effector T cells (Shen *et al.*, 2010; Strauss *et al.*, 2007a; Yokokawa *et al.*, 2008), and $CD4^+CD25^+CD127^+$ effector cells (Figure 3.3b). This enabled further investigation into the functional activity of Tregs and whether their ability to suppress the proliferation of effector T cells is effected by the expression of CD25 on the effector T cell population (Section 4.3.3.5 and Section 5.3.4.2).

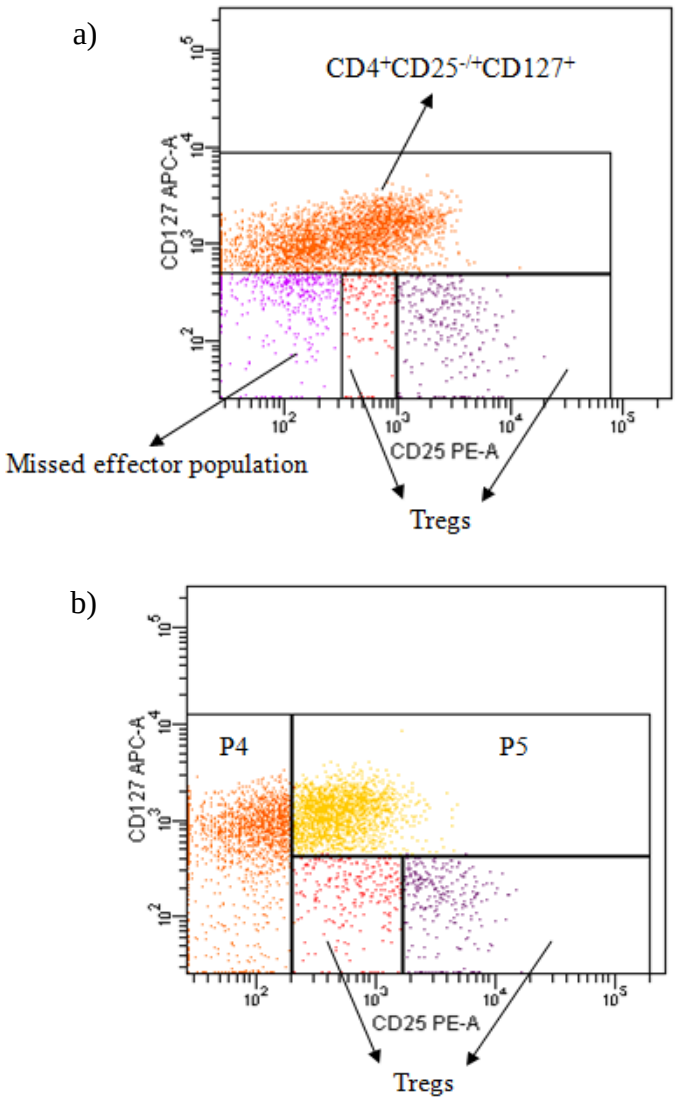


Figure 3.3 Gating strategy used to identify and isolate effector T cell populations
The gating strategy used to isolate effector T cells was altered based on the expression of CD25. A PBMC sample was labelled with a Treg sorting cocktail and following gating of the lymphocyte population and doublet discrimination $CD4^+$ cells were gated. Effector and Treg populations were identified based on the expression of CD25 and CD127 by $CD4^+$ cells. a) Effector T cells were originally isolated with the phenotype $CD4^+CD25^{-/+}CD127^+$ but a population of cells that were $CD4^+CD25^-$ were excluded. The gates shown by dot plot b) were used to separate the isolation of effector T cells based on their CD25 expression, P4 ($CD4^+CD25^-CD127^{-/+}$) and P5 ($CD4^+CD25^+CD127^+$).

Following isolation using the above strategy, the individually sorted populations were put back through the flow cytometer to check that what had been sorted was correct. Figure 3.4 shows the sorted populations, which were acquired by the FACSAria™ II with FACSDiva software (BD Biosciences). The dot plots clearly demonstrate that the sorted

populations are what were expected however, it should be noted that the sorted populations do not always exactly 'fit' in their specific gate post-sort. This could be due to contamination with other cell populations but is more than likely to be due to the loss of bound fluorochrome-conjugated antibodies during the sort process, resulting in a lower level of fluorescence, or because this is the second time the fluorochromes have been excited by the lasers of the FACSAria™ II, resulting in quenching of the fluorescence.

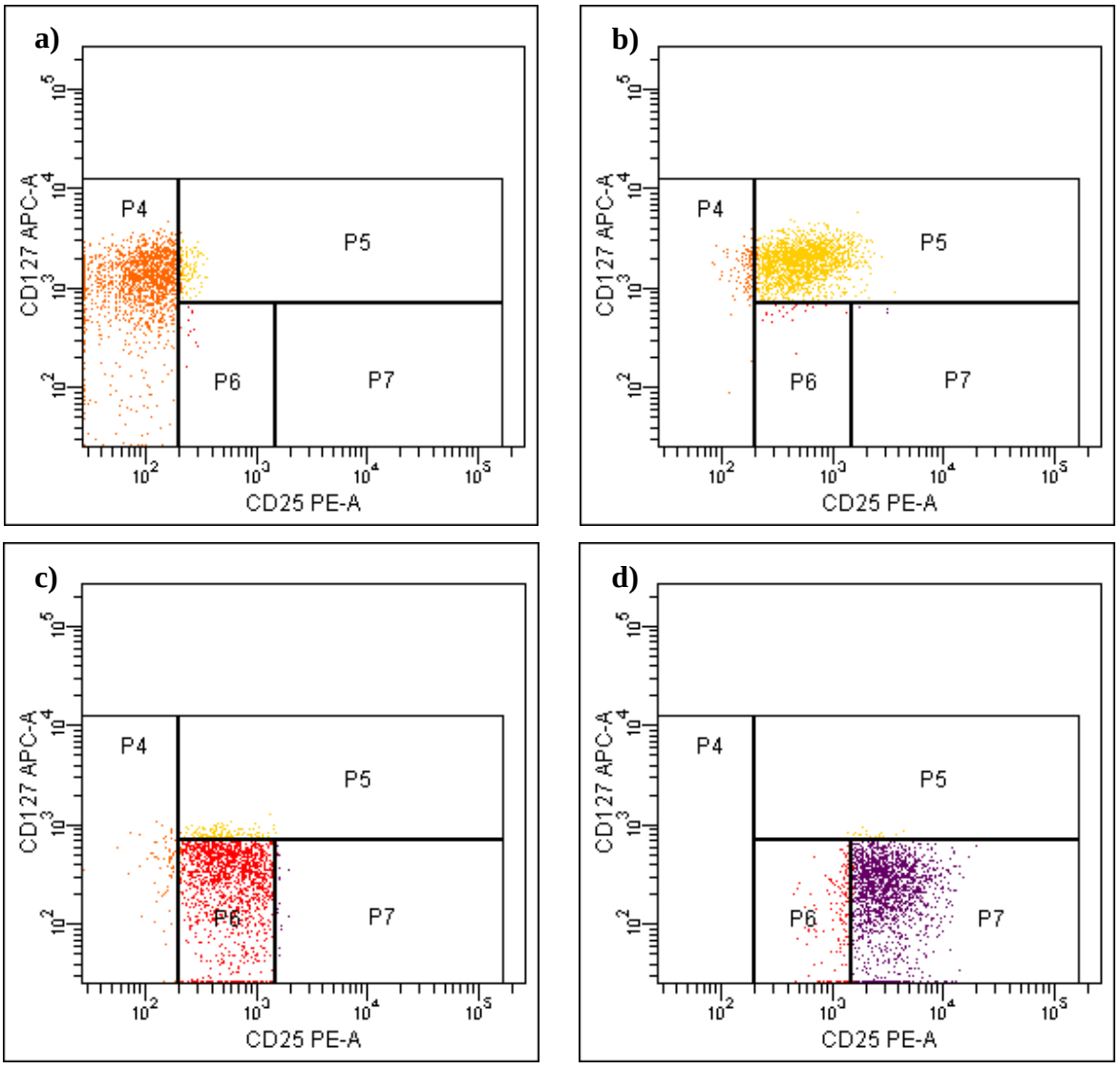


Figure 3.4 Populations following the sorting of PBMC for effector T cells and Tregs
The sort gates (P4, P5, P6 and P7) were used to isolate $CD4^{+}CD25^{-}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$ effector T cell populations and $CD4^{+}CD25^{inter}CD127^{low/-}$ and $CD4^{+}CD25^{high}CD127^{low/-}$ Treg populations, respectively. The resulting isolated populations obtained from sort were reanalysed on the flow cytometer to show the purity of the resulting populations; producing dot plots a), b), c) and d) respectively.

3.1.3 Assessing MACS and FACS for the isolation of Tregs

In the current study two methods of Treg isolation from PBMC samples, MACS and FACS, which have been used by other research groups, were compared to determine which method would optimally isolate Tregs from HNSCC patients to enable the assessment of the regulatory population's functional activity.

3.1.3.1 Assessment of MACS

Using both MACS and FACS optimised methods, $CD4^{+}CD25^{+}CD127^{low/-}$ Tregs were successfully isolated with the majority of isolated lymphocytes (98-99%) expressing CD4. Although the purity of the isolated Treg populations from both methods was similar the absolute yields differed greatly. It was found that the yield of Tregs isolated by MACS could be successfully increased over twelve fold by increasing the starting number of PBMC. However this also increased the length of the isolation considerably as well as the cost of the procedure by using more isolating columns, T Cell Biotin-Antibody Cocktail and Anti-Biotin Microbeads. In addition, although PBMC samples were readily available and therefore the starting number of cells could be increased, it had to be kept in mind that the method optimised on patient PBMC samples would be used to isolate Tregs from the tumour, where Tregs are much less abundant and the tissue less readily available. Furthermore due to the low number of Tregs isolated the suppressive activity of Tregs on the proliferation of effector T cells could not be immediately assessed following isolation.

Several research groups have successfully utilised MACS technology to analyse Tregs present in the peripheral circulation and tumour microenvironment of cancer patients. For example, Gasparoto *et al.* (2010) used the $CD4^{+}CD25^{+}$ regulatory T cell isolation kit to isolate Tregs from the PBMC and TIL of patients with oral squamous cell carcinoma and demonstrated that $CD4^{+}CD25^{+}$ T cells were able to significantly inhibit the proliferation of allogenic PBMC. However, due to the low yield of Tregs isolated via this method the suppressive T cell population was expanded for 15 days before being used in the proliferation assay. The current study attempted to expand the Treg population isolated by MACS but due to the low yield of Tregs isolated the population did not proliferate or expand. The same $CD4^{+}CD25^{+}$ Treg isolation kit and similar results to Gasparoto and

colleagues have been reported in the peripheral circulation of hepatocellular carcinoma (Shen *et al.*, 2010), gastric cancer (Ichihara *et al.*, 2003) and HNSCC (Mandapathil *et al.*, 2009) patients, additionally Yokokawa and colleagues (2008) used the same kit to isolate $CD4^{+}CD25^{high}$ Tregs in prostate cancer patients; although unlike Gasparoto *et al.* the authors from these four studies did not expand the isolated regulatory population before use in functional assays.

To the author's knowledge the $CD4^{+}CD25^{+}CD127^{dim/-}$ Regulatory T Cell Isolation Kit II, optimised by the current study, has not previously been used by cancer research groups but has been used in other research areas, including investigation into the effects of glucocorticosteroids on Langerhans cells/Tregs interactions during cutaneous inflammation (Stary *et al.*, 2011), the frequency and suppressive activity of Tregs in unexplained recurrent spontaneous miscarriage (Bao *et al.*, 2011) and the altered expression of miRNA in Tregs in multiple sclerosis (De Santis *et al.*, 2010). These three studies did not comment on the number of Tregs isolated, and whilst De Santis and colleagues did not assess the isolated $CD4^{+}CD25^{+}CD127^{dim/-}$ Tregs for suppressive activity, the remaining two studies did assess Treg function without expanding the population and demonstrated the Tregs to be suppressive on the proliferation of effector T cells.

3.1.3.2 Assessment of FACS

The number of $CD4^{+}CD25^{+}CD127^{low/-}$ Tregs isolated using FACS was over 50 times greater (per 1 x 10^{7} starting cells) than from the fresh PBMC sample using MACS. This may be attributed to the loss of Tregs during each step of the MACS process which includes the retention of $CD4^{+}$ by the LD columns as well as the loss of Tregs by the use of two MS columns which increase the purity of the isolated population. Consequently Tregs isolated by FACS could be immediately set up in a co-culture CFSE proliferation assay with isolated effector T cells to assess their suppressive activity without the need to expand the population (Section 2.18). Additionally, FACS is a more straight forward procedure taking half the time of MACS.

In cancer studies several research groups have used FACS to isolate peripheral $CD4^{+}CD25^{+/high}$ Tregs for assessment of their suppressive activity including investigations into HNSCC (Mandapathil *et al.*, 2009; Strauss *et al.*, 2007a) and bladder carcinoma (Chi

et al., 2010), additionally Shen and colleagues (2009) have examined the functional capacity of FACS isolated $CD4^+CD25^+CD127^{low/-}$ Tregs from gastric cancer patients. Outside cancer investigations, $CD4^+CD25^{+/high}CD127^{low/-}$ Tregs have been isolated by FACS and shown to be suppressive on the proliferation of effector T cells in numerous settings including healthy controls (Hartigan-O'Connor *et al.,* 2007; Seddiki *et al.,* 2006), patients with systemic lupus erythematosus (Venigalla *et al.,* 2008) and patients with type 1 diabetes (Putnam *et al.,* 2009).

3.1.3.3 MACS vs. FACS

A number of research groups have also compared the advantages and disadvantages of the MACS and FACS isolation methods. Hoffmann *et al.* (2006a) and Yan *et al.* (2009) compared the techniques for the isolation of Tregs from human leukapheresis product and murine spleen lymphocytes, respectively. Both studies reported that there was no significant difference in the ability of the isolated Tregs from either of the two different isolation methods to inhibit the proliferation of $CD4^+$ $CD25^-$ effector T cells. However, Yan and colleagues showed that the use of MACS was a far lengthier process. This is in accordance with the current study; it took 6-7 hours to isolate Tregs from 3×10^7 PMBC using the MACS technique whereas it took only 2-3 hours using FACS. In addition, Yan *et al.,* (2009) reported that the MACS isolated $CD4^+CD25^+$ Treg population was of a lower purity and with a greater percentage of cell death than that of FACS.

In contrast to the two previous investigations discussed above one study has reported that $CD4^+CD25^+$ Tregs isolated via the use of magnetic beads were unable to suppress responder $CD4^+CD25^-$ T cells after αCD3 stimulation, unlike the $CD4^+CD25^{high}$ Tregs isolated by FACS (Baecher-Allan *et al.,* 2005). However, a number of other studies have used magnetic bead isolated $CD4^+CD25^+$ Tregs to perform assays that show the suppressive activity of this T cell population (Bonelli *et al.,* 2008; Dieckmann *et al.,* 2001; Elkord *et al.,* 2006; Jonuleit *et al.,* 2001). Baecher-Allan and colleagues (2005) reported that the yield of $CD4^+CD25^+$ Tregs isolated by MACS was more than 20 fold greater than $CD4^+CD25^{high}$ cells isolated by FACS; suggesting that cells expressing all levels of CD25 are isolated by the magnetic bead sorting protocol and therefore the isolated population may contain both $CD4^+CD25^{high}$ Tregs and activated $CD4^+CD25^{intermediate}$ effector T cells.

Assessing the Treg phenotype $CD4^{+}CD25^{+}CD127^{low/-}$, the work conducted in this thesis found a greater number of Tregs to be isolated by FACS compared with MACS.

A number of studies exploring the relationship between Tregs and HNSCC have isolated the regulatory population from the peripheral circulation of patients. As already mentioned, Whiteside and colleagues used FACS to isolate $CD4^{+}CD25^{high}$ Tregs (Mandapathil *et al.,* 2009; Strauss *et al.,* 2007a; Strauss *et al.,* 2007b) whilst Gasparoto *et al.* (2010) chose to isolate $CD4^{+}CD25^{+}$ Tregs with magnetic beads using a regulatory T cell isolation kit and an autoMACS separator. Both research groups observed that the suppressive activity of Tregs, isolated by either method, from the peripheral circulation of HNSCC patients was greater compared with healthy controls thereby suggesting that both methods can work and it is up to individual laboratories to establish a method which works best in their hands.

From the optimisation of the two isolation methods and the points discussed above it was decided that for the studies conducted in this thesis the $CD127^{low/-}$ Treg population would be isolated by FACS from the peripheral circulation and tumour specimens of HNSCC patients.

3.2 Assessing cellular proliferation using a CFSE assay

During the last 30 years a range of fluorescent dyes have been employed to analyse the role of lymphocytes in adaptive immunity, this includes following their migration into tissues, their positioning within tissues as well as tracking their proliferative history (Parish, 1999). One of the most versatile dyes to be exploited for its ability to follow lymphocyte migration long term and cell division both *in vitro* and *in vivo* is carboxyfluorescein diacetate succinimidyl ester (CFDASE).

CFDASE is a non-fluorescent compound that due to the presence of two acetate side chains is highly membrane permeant (Figure 3.5). Once within the cell, intracellular esterases are able to cleave the acetate groups resulting in a fluorescent molecule (carboxyfluorescein succinimidyl ester; CFSE) with reduced lipophilicity. This prolongs the time available for CFSE to covalently couple to intracellular molecules via a stable amine bond forming conjugates that are either rapidly degraded and able to exit the cell, or

conjugates that are stable and unable to escape the cell; it is these long lived compounds that enable the stable labelling and assessment of cells with CFSE (Figure 3.5; Parish 1999). Although the compound used to label the cells is CFDASE the first publication reporting use of this dye used the acronym CFSE, to maintain consistency and avoid confusion subsequent publications have continued to use the term CFSE (Parish, 1999; Shen *et al.*, 2009; Strauss *et al.*, 2007a) and therefore this study has also adopted the same acronym.

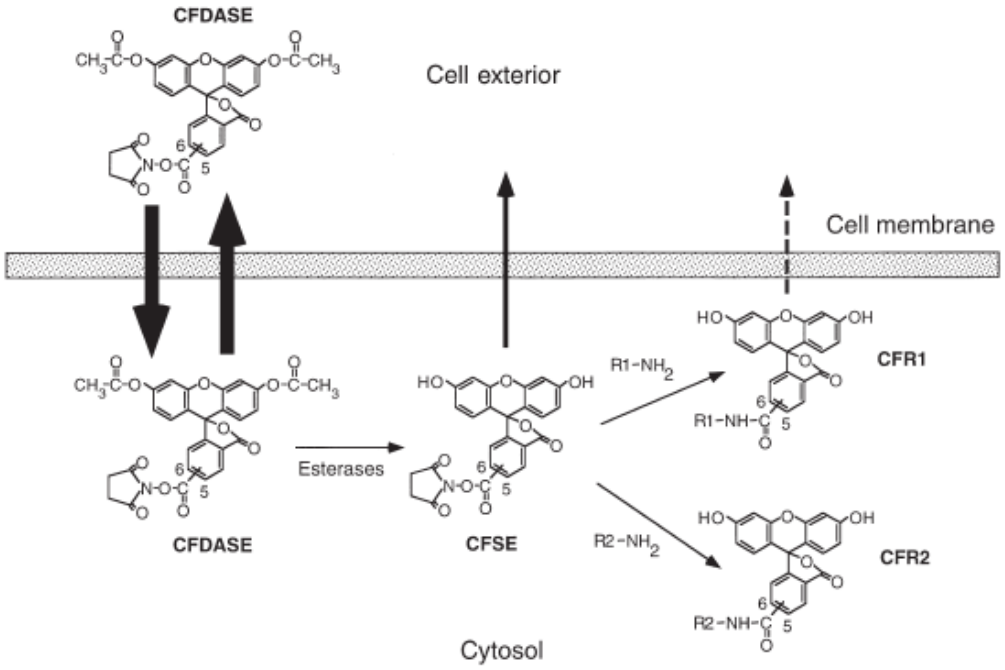


Figure 3.5 Schematic representation of the stages involved during the labelling of a cell with CFDASE
The size of the arrows represents the permeability of the molecule through the cell membrane. The acetate side chains of CFDASE make the compound highly membrane permeant, readily passing in and out of the cell. However, once in the cell, intracellular esterases cleave the two acetate groups yielding the fluorescent molecule CFSE which has reduced cell membrane permeability. The succinimidyl moiety of CFSE enables covalent binding with intracellular molecules forming conjugates that are either rapidly degraded and able to escape the cell (CFR1) or conjugates that are long living and do not exit from the cell (CFR2), thereby achieving the stable fluorescent cell labelling. Parish, 1999.

CFSE was initially developed to be used in the long term tracking of lymphocyte migration, however in 1994 Lyons and Parish presented data illustrating a further

advantageous use of the fluorescent dye for the monitoring of lymphocyte proliferation. The flow cytometric method was shown to be based on the sequential halving of fluorescence intensity with each cycle of division (Lyons and Parish, 1994); as each labelled cell divides it shares its CFSE equally between the two daughter cells resulting in each cell having half the level of CFSE labelling and consequently half the fluorescence intensity (Figure 2.12b). This is visualised as a series of peaks by the flow cytometer which detects the level fluorescence of the CFSE dye emitting at 517nm. Approximately 8–10 cycles of division can be detected before the level of fluorescence becomes equal to the autofluorescence level of unlabelled cells (Hasbold *et al.*, 1999).

The ability of CFSE to follow cycles of division accurately provided an important tool for the current study to investigate the suppressive activity of Tregs, isolated from head and neck cancer patients, on the proliferation of autologous effector T cells. In order to optimise the CFSE assay HNSCC cell lines were initially used to establish the method before the technique was transferred for use with isolated effector T cells.

3.2.1 CFSE assay with HNSCC cell lines

The head and neck cancer cell line SIHN-011A was incubated with a range of concentrations of CFSE (1.25µM, 2.5µM, 5µM and 10µM) to establish whether there was an optimal concentration for following cellular proliferation. SIHN-011A was kindly supplied by Dr. S. Eccles (The Institute of Cancer Research) and was derived from a base of tongue tumour, however, the full clinical details of the tumour specimen were not supplied in initial work (O-Charoenrat *et al.*, 2000).

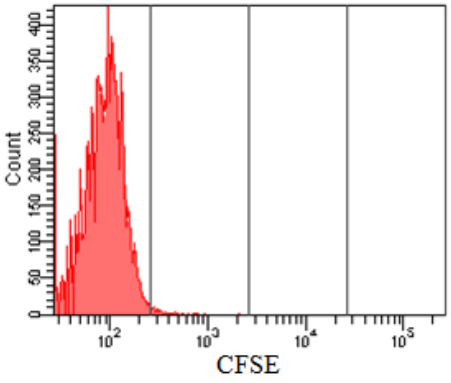
SIHN-011A (1 x 10^6 cells/ml) were labelled with an appropriate concentration of CFSE and cultured for 2 days before the level of CFSE fluorescence was measured for a total of 10 days. The histograms obtained for each day of culture showed that the level of CFSE fluorescence within the cells (mean fluorescence intensity) was higher with increasing starting concentrations of CFSE (Figure 3.6; representative plots acquired at day four of culture).

The histograms recorded over the 10 day culture period, following incubation of SIHN-011A with 10µM of CFSE, are illustrated in Figure 3.7; similar histograms were observed for all the different starting concentrations of CFSE used and showed that any of

the concentrations were able to monitor the proliferation of SIHN-011A. The mean fluorescence intensity decreased every day as the CFSE halved in each cell after division. This assay was repeated and the same trend recorded, it was also found that aliquots of the prepared CFSE stock could be stored at -20°C and used in assays months later.

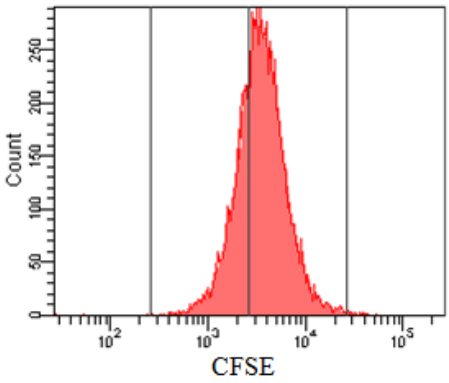
Although the mean fluorescence intensity decreased over the 10 days of culture showing that SIHN-011A was proliferating, the characteristic 'peaks' associated with the CFSE assay and cycles of division were not observed. The HNSCC cell lines represent a single cell population and therefore after continuous culture their proliferation may be synchronised; cells dividing at the same time would cause the level of CFSE to reduce at the same rate and consequently a single peak of fluorescence would be observed to 'shift', decreasing in fluorescence intensity, during the subsequent cycles of division. A similar progressive 'shift' in fluorescence during a proliferation time course (24-96 hours) was also demonstrated by Matera and colleagues (2004) using an ovarian carcinoma cell line (IGROV1).

a)



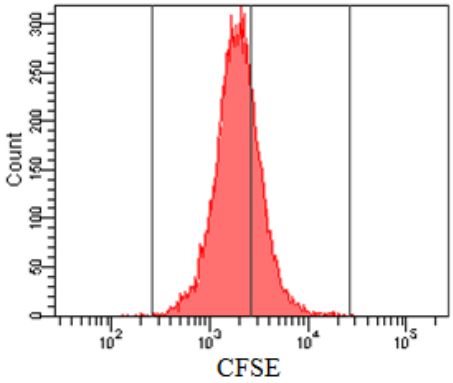
Mean fluorescence intensity: 95

b)



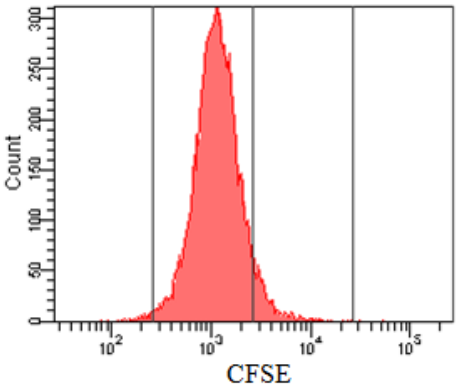
Mean fluorescence intensity: 4,149

c)



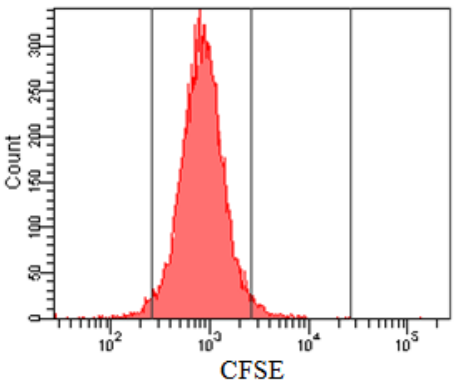
Mean fluorescence intensity: 2,253

d)



Mean fluorescence intensity: 1,353

e)



Mean fluorescence intensity: 984

Figure 3.6 SIHN-011A incubated with a range of CFSE concentrations
SIHN-011A (1 x 10^6 cells) was cultured with a range of CFSE concentrations for four days before CFSE content was measured by flow cytometry. Histogram plots from SIHN-011A labelled with a) no CFSE b) 10µM c) 5µM d) 2.5µM and e) 1.25µM. Representative of two experiments

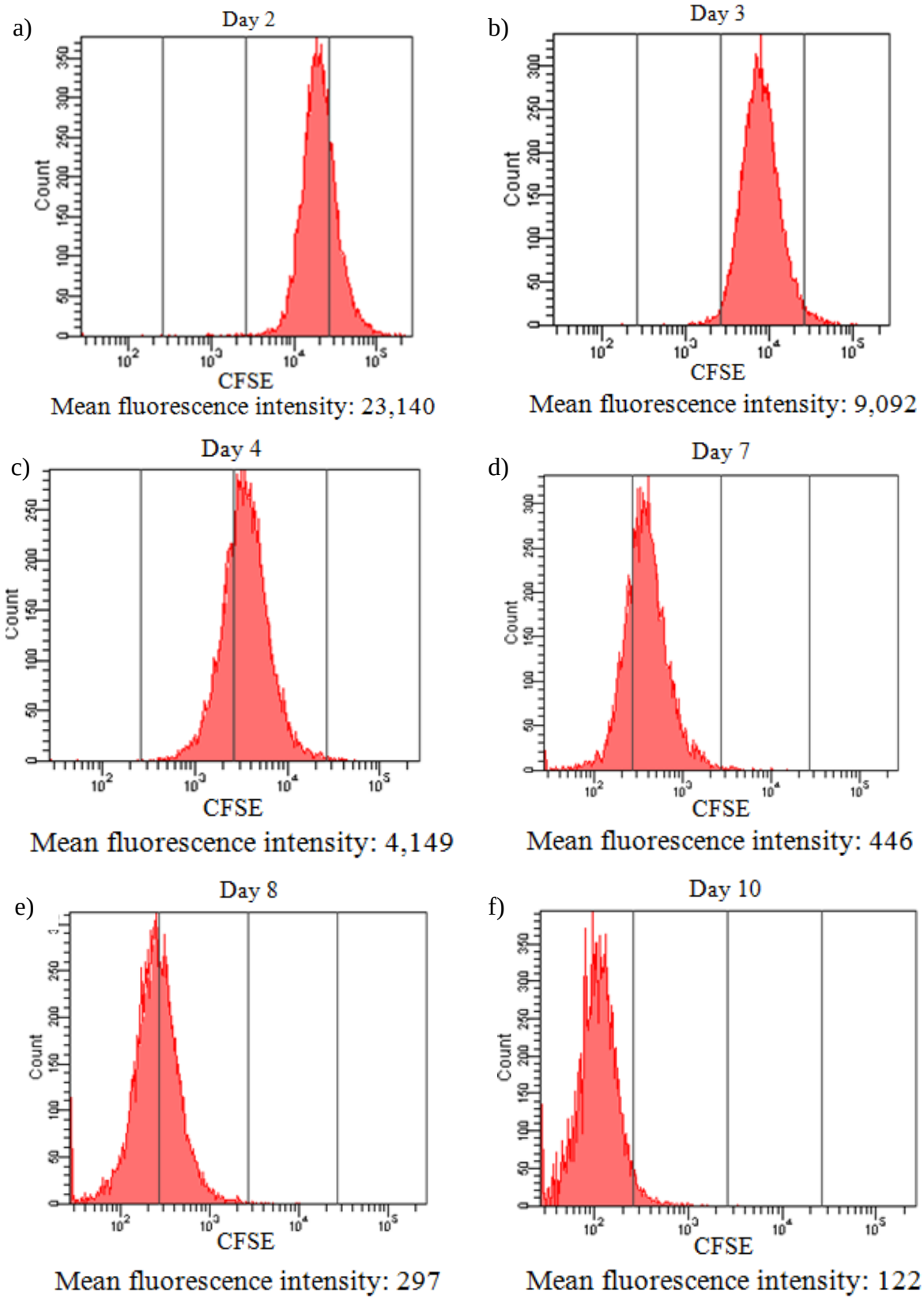


Figure 3.7 SIHN-011A CFSE proliferation assay
Histograms showing the fluorescence of 10,000 acquired cells following incubation with 10µM CFSE for between 2 and 10 days of culture. Representative of two experiments.

Two further HNSCC lines UMSCC-11b and UMSCC-12a (Table 3.4; kindly supplied by Dr. T. Carey, University of Michigan) were incubated with 10μM of CFSE to ensure that the method developed with SIHN-011A could be transferred for use with other head and neck cancer cell lines. UMSCC-11b and UMSCC-12a proliferated at similar rates with the mean fluorescence intensity of cells labelled with CFSE decreasing at similar levels over the first 4 days of culture.

Table 3.4 UMSCC cell lines 11b and 12a

HNSCC cell line name	Gender of patient	Age of patient	TNM	Anatomic site of origin	Specimen site
UMSCC-11b	Male	65	T2N2aM0	Larynx	Larynx
UMSCC-12a	Male	71	T2N1M0	Larynx	Larynx

3.2.1.1 Blocking cellular proliferation

To mimic the suppression induced by Tregs on the proliferation of effector T cells, the growth of SIHN-011A was reduced by incubating the head and neck cancer cell line with serum free (FBS) medium. SIHN-011A was labelled with CFSE and on the fourth day of culture the cell line was divided into two flasks, one flask contained complete growth medium and the other flask contained serum free medium. The removal of serum from the cells resulted in a decrease in proliferation as indicated by the higher mean fluorescence intensity of the peak compared with the cells incubated in the presence of serum; the effect was most marked three days post serum removal (Day 7; Figure 3.8). This experiment showed that alterations in the proliferation of cells could be measured using a CFSE assay.

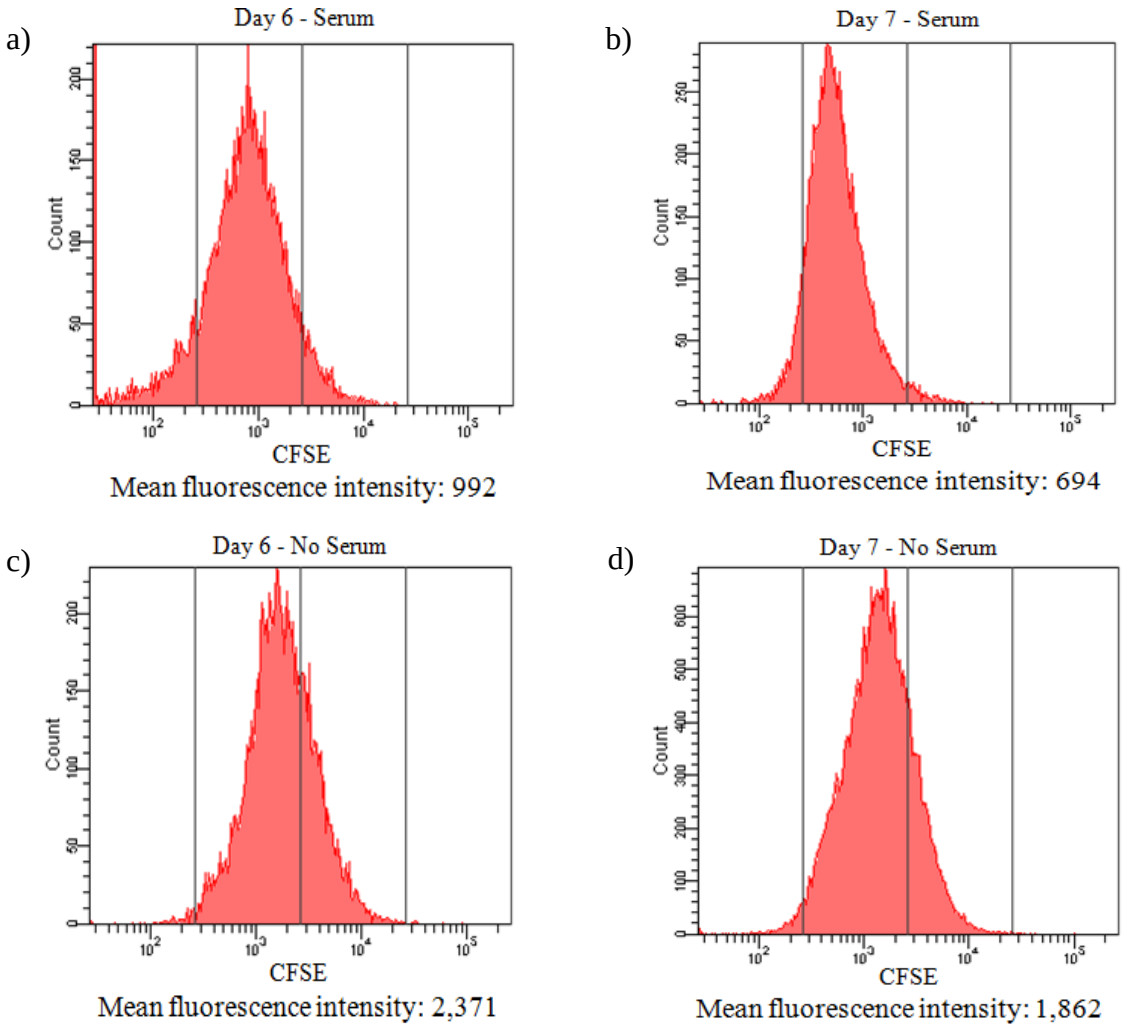


Figure 3.8 SIHN-011A proliferation with and without serum containing medium
Histograms comparing the proliferation of SIHN-011A in medium with and without serum. 1 x 10^6 SHIN-011A cells were labelled with 10µM of CFSE and cultured for 4 days before being split into two separate cultures, incubated with either complete growth medium (a, b) or growth medium without serum (c, d), the fluorescence intensity of the cells was measured on days 6 (a, c) and 7 (b, d) of culture using flow cytometry.

3.2.2 CFSE assay with effector T cells

The CFSE assay established on the head and neck cancer cell lines was transferred to isolated effector T cells. Cancer research groups using the CFSE assay to assess the suppressive activity of Tregs on the proliferation of effector T cells have labelled the effector population with a range of CFSE concentrations (1-5µM; Ju *et al.*, 2009; Lin *et al.*, 2012; Mandapathil *et al.*, 2009; Shen *et al.*, 2009; Shenghui *et al.*, 2011; Strauss *et al.*, 2007a). Due to the use of 5µM by Lyons and Parish (2004) in the initial study to determine

lymphocyte division by CFSE and the same concentration successfully following the proliferation of HNSCC cells lines, 5µM CFSE was chosen to label isolated effector T cells and monitor their proliferation.

The effector T cells (n=4) were incubated with 5µM CFSE immediately following isolation by FACS (Section 2.18) and the level of CFSE fluorescence in the cells was measured over a nine day culture period (Figure 3.9). Between day 2 and 7 several peaks with various levels of fluorescence, representing the different daughter populations, were evident and the mean fluorescence intensity of the peaks decreased consistently during the culture period. Following 7 days of culture only a single major peak with minimal fluorescence was evident, indicating that the effector T cells had lost most of their CFSE labelling presumably due to the number of cycles of division the cells had undergone (Figure 3.9). Serum starvation of effector T cells, as described for the HNSCC cell line SIHN-011A, also showed that proliferation could be reduced since three days post serum removal the mean fluorescence intensity for cells maintained in growth medium without serum was 623 whereas cells cultured in complete growth medium had a mean fluorescence intensity of 345.

The CFSE assay was subsequently adapted to include Tregs to enable their suppressive activity on the proliferation of effector T cells to be examined (Section 2.18). It was important to determine the optimum length of incubation for the co-culture of CFSE labelled effector T cells and Tregs as this has varied between research groups with some choosing five (Hartigan-O'Connor *et al.*, 2007; Strauss *et al.*, 2007a; Venken *et al.*, 2007), four (Parish *et al.*, 2009; Putnam *et al.*, 2009) or three (Ju *et al.*, 2009; Seddiki *et al.*, 2006) days. Three different co-culture CFSE assays were prepared for both of the effector T cell populations isolated, $CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$ (Section 2.18), and were incubated for either three, four or five days before the results were acquired by flow cytometry. Figure 3.10 illustrates the plots obtained for these three different days of incubation showing the $CD4^+CD25^-CD127^{-/+}$ effector T cell population cultured alone and with Tregs ($CD4^+CD25^{high}CD127^{low/-}$) at a ratio of 1:1; similar plots were recorded for the $CD4^+CD25^+CD127^+$ population. Day three and four show distinct CFSE peaks, with cells on day three having undergone four cycles of division and those on day four, six cycles of division (Figure 3.10). Following five days of culture the peaks are less defined making it difficult to determine how many cycles of division the effector T cells have gone through.

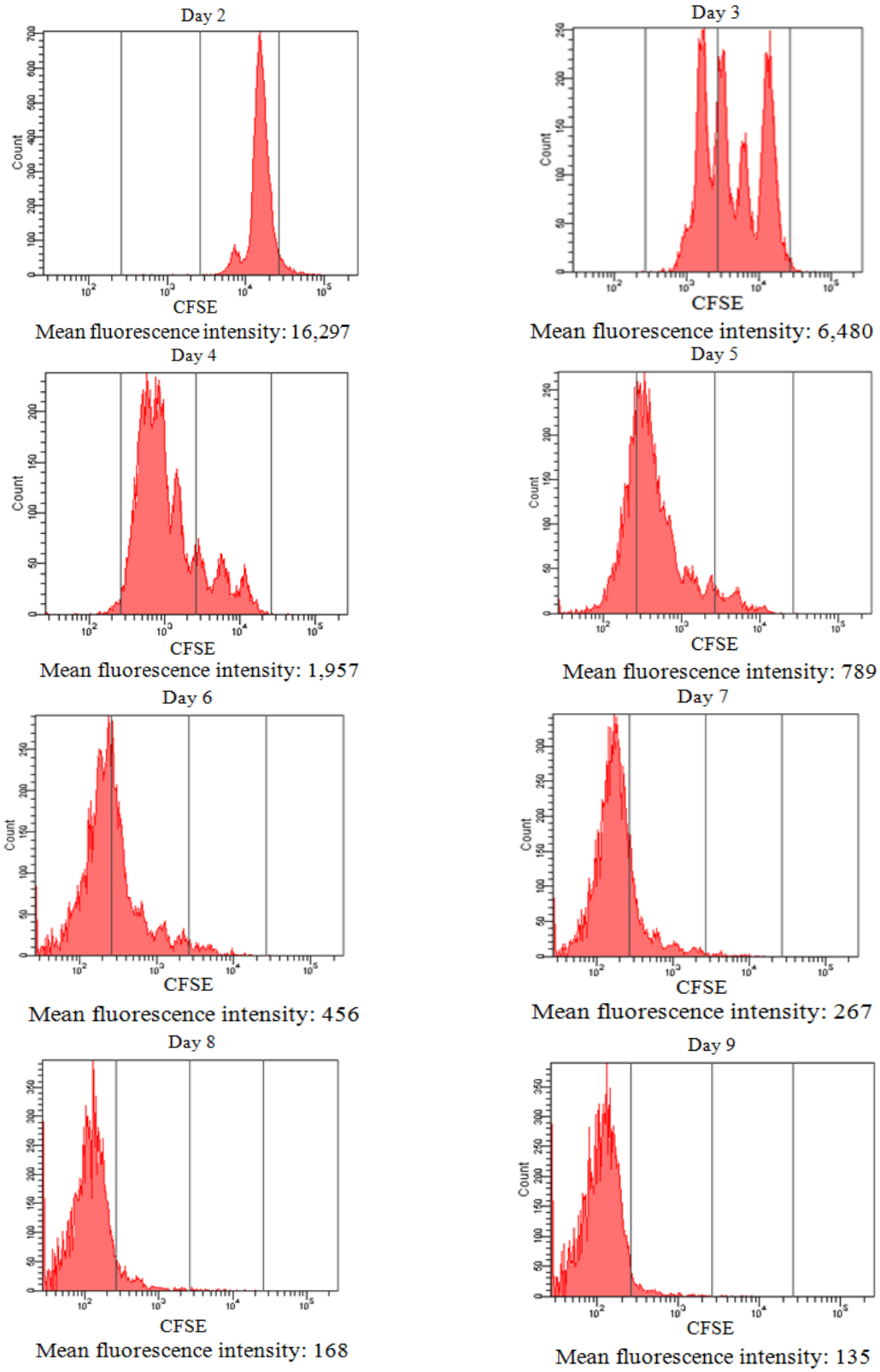


Figure 3.9 Effector T cell CFSE proliferation assay
Histograms showing the level of fluorescence generated from 1 x 10^6 CFSE labelled (5μM) effector T cells isolated by FACS from PBMC over a nine day period of culture. Representative of 4 experiments.

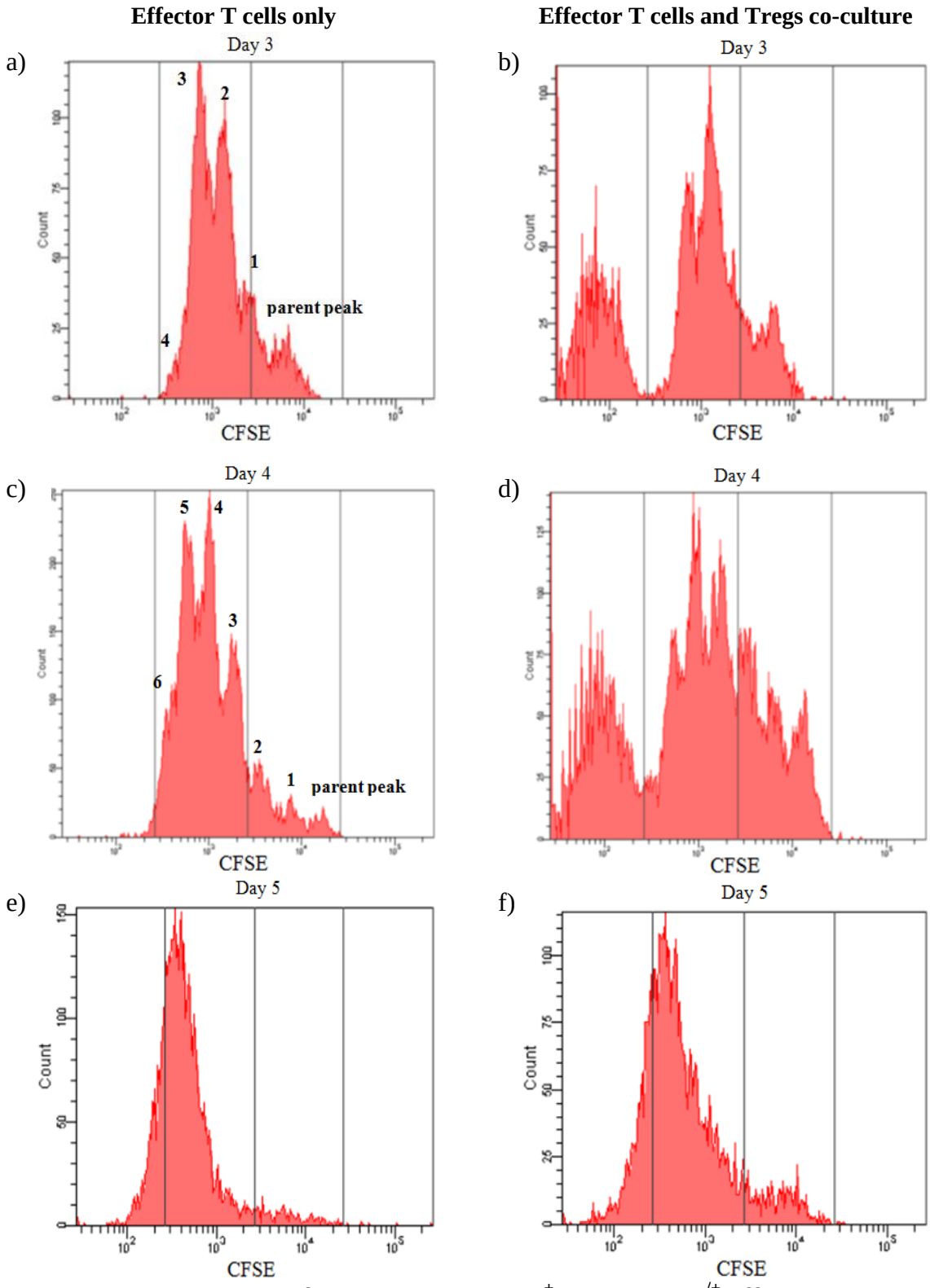


Figure 3.10 Co-culture of CFSE labelled $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells with $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs at different days of culture

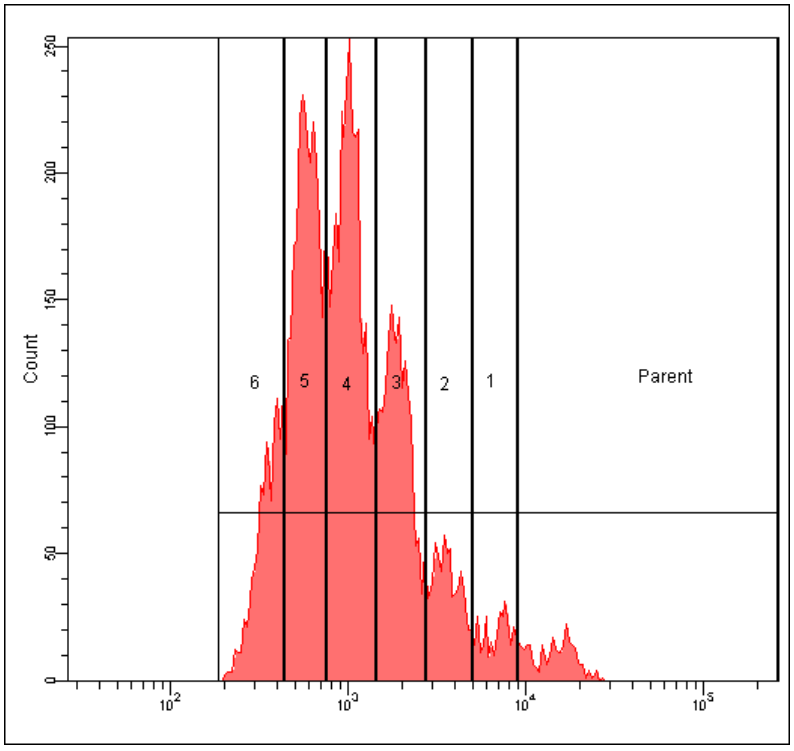
Effector T cells were immediately labelled with 5µM CFSE following isolation by FACS and either cultured alone (a, c, e) or with autologous Tregs at a 1:1 ratio (b, d, f). Following three (a, b), four (c, d) or five (e, f) days of culture the level of fluorescence generated from the proliferating effector T cells was measured. Representative of 3 experiments.

From the results illustrated by Figure 3.10 four days of co-culture of the effector T cells and Tregs appeared to be optimal for analysing the suppressive activity of Tregs, as this produced the most defined CFSE peaks showing the greatest number of cycles of division. However, upon repeating the assay with a different patient sample, the proliferation peaks were not as well defined highlighting variability between patient samples. Consequently two further CFSE assays were prepared with one patient sample and analysed following three and four days of co-culture; the results were similar to those shown by Figure 3.10 and therefore the suppressive activity of Tregs on the proliferation effector T cells was subsequently analysed on day four of co-culture.

3.2.3 Calculation of the proliferation index

To establish the proliferation of a CFSE labelled effector T cell population the proliferation index (PI) was calculated. The PI is a measure of the increase in cell number over the duration of culture and is determined by dividing the total number of cells in each generation by the original number of calculated parent cells present at the beginning of culture. The PI assumes the cell number doubles as cells proliferate through each successive daughter generation (ModFit, Proliferation Index).

Initially the PI was determined manually; as the CFSE assay is based on the assumption that a single CFSE labelled parent cell will divide into two daughter cells, each division (generation) can be visualised on a histogram plot as a ‘peak’ and by applying a gate to each 'peak' the number of cells in each generation is determined (Figure 3.11; 6 cycles of division). The peak with the greatest fluorescent intensity represents the parent peak i.e., the CFSE labelled effector T cells that have not undergone cellular proliferation (Figure 3.11).



Generation	Number of cells in each generation
Parent	291
1	298
2	652
3	1,822
4	3,056
5	2,643
6	995

Figure 3.11 Gated peaks identifying cycles of division by proliferating CFSE labelled effector T cells
Effector T cells were immediately labelled with 5µM CFSE following isolation by FACS and cultured for four days before the cells were harvested and their CFSE content measured by flow cytometry. On the histogram gates were applied to encompass each peak to visualise the cycles of division. This enables the number of cells within each generation to be determined.

To calculate the PI for a CFSE labelled effector T cell population the following equation was used: PI = sum of cells in all generations / number of original parent cells (ModFit, Proliferation Index).

The sum of cells in all generations is easily calculated by adding together the number of cells in each 'peak' (division; Table 3.5). To determine the number of original parent cells it must be calculated how many parent cells the cells in each generation originated from. For example, if after one round of division there are 200 cells the number of parent cells will have been 100 due to each parent cell dividing to give two daughter cells. If there are 200 cells after two rounds of division then they were derived from 50 original parent cells. To calculate the original number of parent cells, the number of cells the each generation (n) is divided by 2 raised to the power n, e.g. $200 / 2^2 = 50$.

Table 3.5 Calculations to determine the PI

Generation (n)	Number of cells in each generation	Divide by 2^n	Parent cell number
Parent (0)	291	1	291
1	298	2	149
2	652	4	163
3	1,822	8	228
4	3,056	16	191
5	2,643	32	83
6	995	62	16
Sum	**9,757**	-	**1,121**

From the values calculated in Table 3.5 the PI for the CFSE labelled effector T cell population analysed in Figure 3.11 can be determined:

PI = sum of cells in all generations (9,757) / number of original parent cells (1,121) = 8.7

The PI for effector T cells cultured alone represents 100% proliferation and 0% suppression, comparing this value to the PI for the co-culture of effector T cells and Tregs the percentage of proliferation and subsequently percentage of suppression can be determined as outlined in Table 2.2.

Determining the peaks of division manually is arbitrary and dependent on the researcher's interpretation of the flow cytometric data. To remove human analysis many research publications that use the CFSE dye to assess the proliferation of effector T cells use ModFit LT™ software (Verity Software House; Mandapathil *et al.*, 2009; Putnam *et al.*, 2009; Shen *et al.*, 2009; Strauss *et al.*, 2007a). The ModFit LT™ software also works on the assumption that the daughter cells will have half the fluorescence of the parent generation. By establishing the fluorescence of the parent population each generation (peak) is subsequently determined based on a 50% decrease in fluorescent intensity (Figure 2.12). The applied peaks determine the number of cells present within each generation and the software calculates the PI by the same equation used in the manual method.

The ModFit LT™ software (Verity Software House) was used throughout the studies conducted in this thesis to assess the suppressive activity of Tregs, isolated from the peripheral circulation (Chapter 4), tumour microenvironment (Chapter 5) as well as the influence of HNSCC secretions (Chapter 6), on the proliferation of autologous effector T cells.

Chapter 4.

Phenotype, frequency and suppressive activity of T cell subsets in the peripheral circulation of HNSCC patients and healthy controls

4.1 Introduction

Tregs are a suppressive population of T lymphocytes that help preserve immune homeostasis by maintaining peripheral tolerance through the suppression of auto reactive T cells (Section 1.9). Based on origin and development, the $CD4^+$ Treg population has been divided into two main groups: nTregs and iTregs (Section 1.11). nTregs (normally referred to as "Tregs") are the most well defined regulatory population and because of the relative ease of identification, through CD25 and Foxp3 positivity, their role in cancer has been widely studied. It has been shown in a number of different cancer types, including HNSCC, that Tregs are elevated in the peripheral circulation of patients compared with healthy controls (Table 1.8). The work in this thesis has identified and isolated Tregs from newly-presenting HNSCC patients and healthy controls using the inverse relationship between CD127 and that of Foxp3 (Section 1.12); however it is known that Foxp3 can be expressed by a proportion of iTregs (Section 1.11.2) and also by activated $CD4^+$ helper cells (Section 1.12).

Relationships between Tregs and clinical parameters have been investigated previously in the peripheral circulation of cancer patients, with the majority of studies focusing on the percentage of Tregs. In gastric, oesophageal and renal cancer, significant differences were observed between early and advanced stage tumours, with the latter showing increased levels of Tregs (Kono *et al.,* 2006; Liotta *et al.*, 2010; Mizukami *et al.*, 2008b; Shen *et al.*, 2009). In contrast, no significant differences in the frequency of Tregs were observed in HNSCC patients presenting with early or advanced stage tumours (Schott *et al.,* 2010; Strauss *et al.,* 2007a). When groups have studied the relationship with survival disparate results have been presented, for example Kono *et al.* (2006) and Sasada *et al.* (2003) reported that gastric cancer patients with a high prevalence of peripheral Tregs had significantly poorer survival rates than those with low Treg levels; whereas the opposite trend was found in renal cancer patients (Liotta *et al.,* 2010). To date, although several research groups have attempted to understand the prevalence of Tregs and their association with prognosis (Section 1.13), the lack of agreement between studies means the use of Tregs as a prognostic marker remains controversial.

One of the reasons for the discrepancies between studies is likely to be due to the absence of a single definitive marker to identify this suppressive T cell subset. Initially, the

murine $CD4^+CD25^+$ Treg phenotype was translated into the human setting (Section 1.12) and $CD4^+CD25^+$ was the first phenotype employed by research groups to demonstrate the increased frequency of Tregs in human cancer (Section 1.13). Although CD25 is one of the most common markers for the regulatory T cell subset, it is also expressed by activated effector T cells, resulting in a heterogeneous mix of Treg and effector T cell populations. Functional studies subsequently identified the $CD4^+CD25^{high}$ phenotype as being the major suppressive population (Section 1.12) and this remains one of the most commonly investigated phenotypes in cancer studies to date.

Further research for a specific Treg marker led to the discovery that the transcription factor, Foxp3, plays an important role in the development and function of Tregs and consequently Foxp3 remains one of the commonest Treg markers used. Unfortunately, due to the intracellular location of Foxp3, this marker cannot be used to isolate Tregs for functional studies and in the quest to find additional markers, more recent studies have demonstrated an inverse relationship between the surface expression of the interleukin 7 receptor (CD127) and Foxp3, allowing the use of this marker to assess both the level and functional capacity of Tregs.

In addition to frequency it is also important to investigate the suppressive capacity of Tregs as it is their functional activity, not just their presence, which will determine how effective a host's anti-tumour immune response will be in combating the growth and progression of a tumour. In cancer patients it has been reported that, not only are Tregs elevated in frequency, but they are also more suppressive in comparison to those from healthy controls (Table 4.1). However, the results from studies investigating the suppressive function of Tregs in cancer patients in relation to tumour stage did not show any statistically significant differences associated with progression (Shen *et al.*, 2009; Shen *et al*., 2010; Strauss *et al.*, 2007a).

Table 4.1 Treg phenotypes demonstrating increased suppressive activity of Tregs in the peripheral circulation of cancer patients in comparison with healthy controls

Treg phenotype	Cancer type	Research group
$CD4^+CD25^+$	Nasopharyngeal HNSCC Oral SCC Acute myeloid leukemia	Lau *et al.* (2007) Mandapathil *et al.* (2009) Gasparoto *et al.* (2010) Shenghui *et al.* (2011)
$CD4^+CD25^{high}$	HNSCC Prostate HNSCC	Strauss *et al.* (2007a and b) Yokokawa *et al.* (2008) Mandapathil *et al.* (2009)
$CD4^+CD25^+CD127^{low/-}$	Gastric	Shen *et al.* (2009)

In order to try and clarify the role of Tregs in HNSCC, the current study used the $CD127^{low/-}$ phenotype to identify and isolate Tregs from the peripheral circulation of HNSCC patients and healthy controls. Previous studies have used the $CD4^+CD25^+CD127^{low/-}$ phenotype to isolate Tregs, however, for this investigation two Treg populations, separated by the expression of CD25, were also identified and isolated: $CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$ Tregs. This allowed an assessment into whether the level of CD25 expression would influence the suppressive activity of $CD127^{low/-}$ Tregs which, to the author's knowledge, has not before been investigated in cancer patients. Two distinct effector T cell populations: the classic $CD4^+CD25^-$ population, frequently used by research groups to assess the suppressive activity of Tregs (Shen *et al.*, 2010; Strauss *et al.*, 2007a; Yokokawa *et al.*, 2008) and a population of activated T cells expressing the IL-7 receptor α chain, $CD4^+CD25^+CD127^+$ were also identified and isolated. To the author's knowledge this is the first study to assess the suppressive activity of $CD127^{low/-}$ Tregs in HNSCC patients. The clinical objective of the study was to assess Treg frequency and function in the peripheral circulation of HNSCC patients in relation to tumour stage [early (T1 and T2) and advanced (T3 and T4)], nodal status [no nodal involvement (N0) and nodal involvement (N^+)], head and neck tumour subsites, principally the larynx and oropharynx, and healthy controls.

4.2 Materials and methods

4.2.1 HNSCC patients and healthy controls

Following ethical (Yorkshire and the Humber research ethics committee; REC reference 10/H1304/7 and 05/Q1105/55) and NHS Trust R and D (R0988 and R0220) approval, and having obtained written informed consent, 51 newly-presenting HNSCC patients and 14 healthy controls [undergoing non cancer related surgery (n=11) and healthy subjects (n=3); Section 2.12] were recruited on to the study. Of these, 12 HNSCC samples had poor viability and/or low cell number and were therefore not used for flow cytometry or cell sorting. Due to the low number of cells sorted and limitations of the CFSE assay, it was only possible to study 28 HNSCC patients and 10 healthy controls for the suppressive activity of Tregs (Figure 4.1).

4.2.2 Assessing the phenotype and prevalence of T cell subsets in the peripheral circulation of HNSCC patients and healthy controls

Following retrieval from cryopreservation, approximately 3 x 10^7 PBMC isolated by density gradient centrifugation from HNSCC patient and healthy control venous blood samples (Section 2.13), were stained with a Treg sorting cocktail (BD Biosciences) containing antibodies against CD4, CD25 and CD127, to enable the isolation of Treg and effector T cell populations by FACS (Section 2.16). During this process the gating strategy employed during FACS (Figure 2.11) enabled the prevalence of $CD4^+$ cells (Figure 4.2a), $CD4^+CD25^+$, $CD4^+CD25^{inter}$ and $CD4^+CD25^{high}$ cells (Figure 4.2b), Tregs and effector T cells (Figure 4.2c) in the HNSCC patient cohort and healthy controls to be examined. $CD4^+$ cells expressing high and intermediate levels of the IL-2 receptor were gated based on the expression of CD25 by the $CD4^-$ population (Section 1.12) and it was the combination of these two gates that gave the percentage of $CD4^+$ cells expressing CD25 (Figure 4.2b). By the same method, combining the $CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$ effector T cell populations gave the total percentage of $CD4^+$ cells expressing $CD25^{-/+}CD127^{-/+}$ and the combination of the Treg populations

$CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$ provided the percentage of $CD4^+$ cells expressing $CD25^+CD127^{low/-}$ (Figure 4.2c).

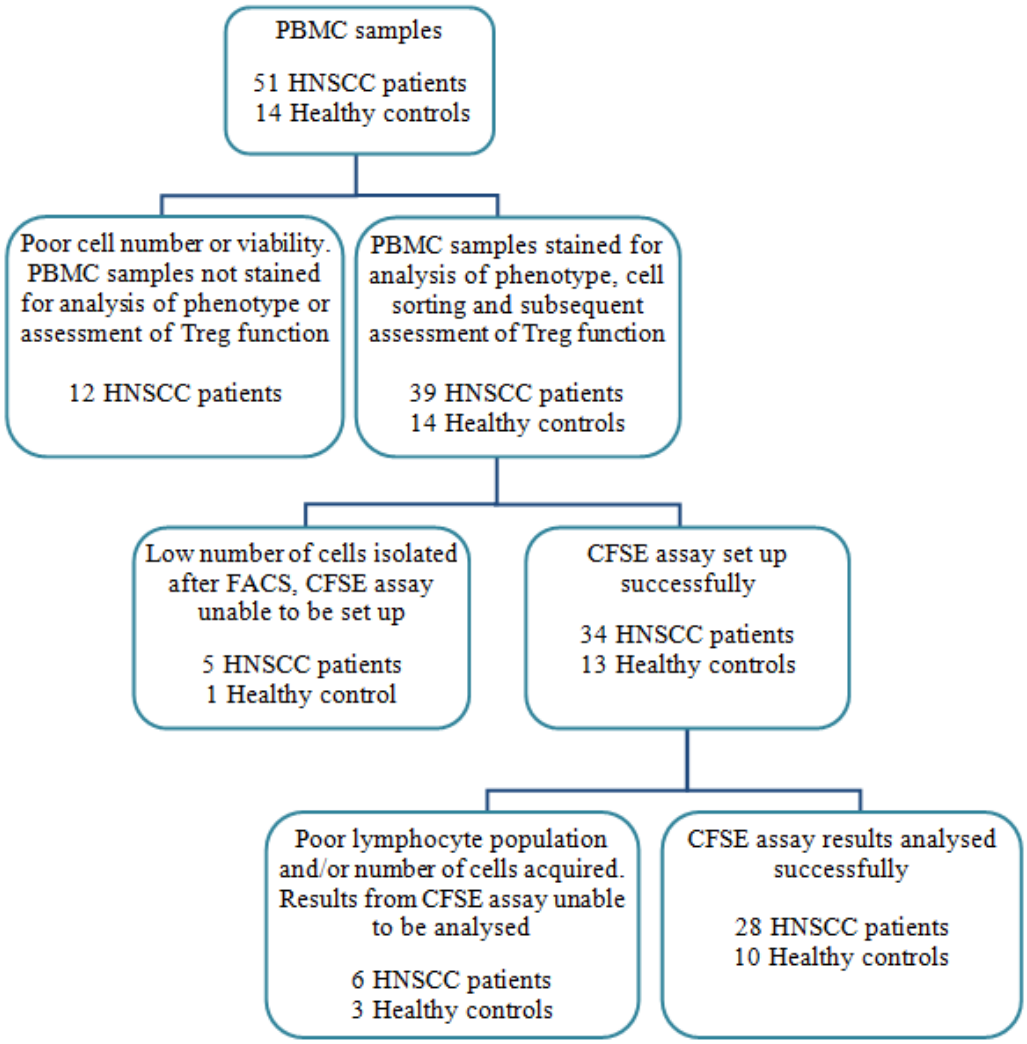


Figure 4.1 CONSORT diagram to illustrate the PBMC samples that were analysed for phenotype and assessed for Treg function

HNSCC patients (n=51) and healthy controls (n=14) were recruited for analysis of the phenotype of specific T cell subsets and assess Treg function in the peripheral circulation. However, due to viability, cell number and the limitations of the CFSE assay not all samples yielded data.

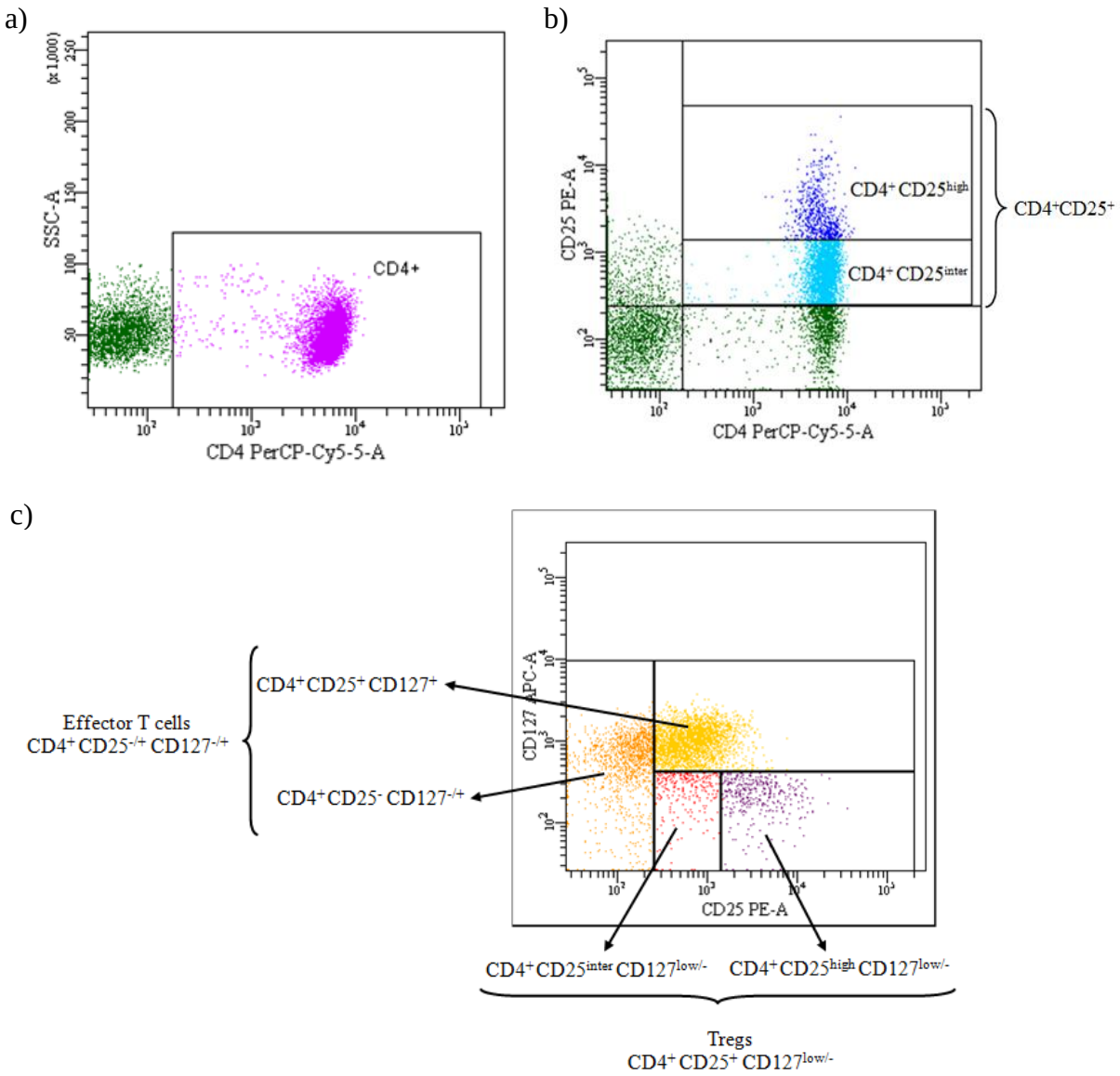


Figure 4.2 Gating strategy to detect the prevalence of $CD4^+$ cells, Tregs and effector T cells to be assessed in healthy controls and HNSCC patients

A PBMC sample was labelled with antibodies against CD4, CD25 and CD127 and, following doublet discrimination, a) the lymphocyte population positive for the expression of CD4 was gated. b) A dot plot showing the expression of CD4 and CD25 was created and a quadrant was set encompassing the $CD4^-CD25^-$ cells in the lower left quadrant. $CD4^+$ cells expressing high and intermediate levels of CD25 were gated using the expression of CD25 on the $CD4^-$ population as a guide. Combining the high and intermediate gates gave the percentage of $CD4^+$ cells expressing CD25. c) The $CD4^+$ population was used to create a dot plot of CD25 and CD127 expression and two Treg populations were identified, $CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$, which when combined gave the percentage of $CD4^+$ cells expressing $CD25^+CD127^{low/-}$. In addition, two effector T cell populations were identified, $CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$, combining to show the percentage of $CD4^+$ cells expressing $CD25^{-/+}CD127^{-/+}$.

4.2.3 Assessment of Foxp3 expression by T cell subsets in the peripheral circulation of HNSCC patients and healthy controls

T cell subsets identified within PBMC samples (Figure 4.2) from healthy controls, oropharyngeal and laryngeal cancer patients (one early stage and one advanced stage from each HNSCC subsite) were assessed for the expression of the transcription factor Foxp3. PBMC samples were stained with a Treg sorting cocktail (BD Biosciences; Section 2.16) before being fixed, permeabilised and incubated with a fluorescently conjugated monoclonal mouse anti-human antibody specific for the intracellular marker Foxp3 (AF488, clone 259D/C7; BD Biosciences) or its corresponding isotype control (Section 2.10). The same gating strategy employed to isolate Treg and effector T cells by FACS (Figure 2.11) was applied during the acquisition of the samples. From the dot plots generated, histograms were created for both positive and negative samples. Markers were set on the isotype controls to encompass ≤ 1% of cells (Figure 2.3b), and the percentage of Foxp3 expression by different T cell subsets, including the T cell populations isolated to assess the function of Tregs (Figure 4.3), was determined.

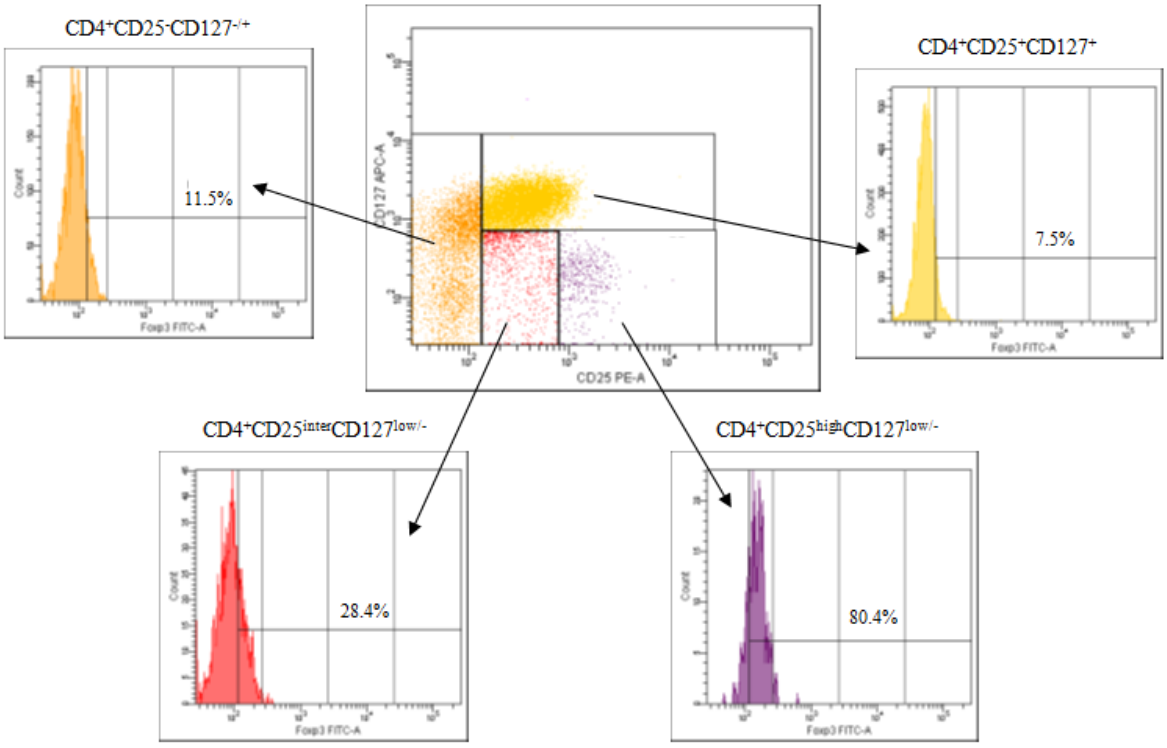


Figure 4.3 Histograms used to assess the expression of Foxp3 by different T cell subsets in the peripheral circulation
A PBMC sample was stained with antibodies against CD4, CD25 and CD127 followed by intracellular labelling with an anti-Foxp3 antibody. Following doublet discrimination, lymphocytes positive for the expression of CD4 were gated and used to identify $CD4^+$ T cell populations expressing varying levels of CD25 and CD127; $CD4^+CD25^{inter}CD127^{low/-}$, $CD4^+CD25^{high}CD127^{low/-}$, $CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$. Histograms plotted specifically with these T cell populations determined their percentage of Foxp3 expression.

4.2.4 Assessment of the suppressive activity of Tregs isolated from the peripheral circulation of HNSCC patients and healthy controls using a CFSE assay

Immediately following isolation by FACS (Section 2.16) Tregs from 34 HNSCC patients and 13 healthy controls were assessed for their suppressive activity on the proliferation of autologous effector T cells via a CFSE assay (Section 2.18).

The concentration and cell viability of each isolated population was determined using trypan blue exclusion (Section 2.4) to establish whether sufficient viable cells had

been obtained to set up a CFSE assay. In the majority of cases the CFSE assay was run with 5 x 10^4 effector T cells cultured in each well of a 96 well plate (Section 2.18), however, when insufficient cells were isolated the number of effector cells plated was successfully scaled down to 1 x 10^4. Effector T cell populations ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) were incubated with 5µM of CFSE (Sigma) before being washed and co-cultured with Tregs ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) at various ratios (Treg:effector, 1:1, 1:2, 1:5 and 1:10) depending on the number of cells available. The 1:1 ratio was always prepared and other ratios were set up where possible. Lymphocyte stimulation was provided by Human T-Activator CD3/CD28 dynabeads (Invitrogen) and 100U/ml recombinant human IL-2 (AbD Serotec). On day four of co-culture, cells were analysed for their CFSE content using the FACSAria™ II with FACSDiva software (Section 2.18) and the results analysed using ModFit LT™ software (Verity Software House). The percentages of suppression were calculated based on the proliferation index for effector cells cultured alone (100% proliferation, 0% suppression) compared with the proliferation index of effector cells co-cultured with Tregs (Section 2.19).

4.2.5 Statistical analysis

Statistical analysis was performed using SPSS version 19. The normality of data was assessed by the Shapiro-Wilk test. Differences between independent data sets, with normal distribution, were analysed using the Student's unpaired T test with the assumption of equal variance assessed by the Levene's test. The Mann-Whitney *U* test was used for independent data sets that did not follow a normal distribution. Differences between dependent data sets that were normally distributed were analysed using the Student's paired T test. The Wilcoxon Signed Rank test was used for dependent data sets that were not of normal distribution. Values were considered significant when $p<0.05$.

4.3 Results

4.3.1 Phenotype and frequency of T cell subsets in HNSCC patients and healthy controls

The PBMC from 39 HNSCC patients and 14 healthy controls (Table 4.2) were characterised phenotypically using flow cytometry following staining with a Treg sorting cocktail. The frequency of various T cell subsets amongst healthy controls and HNSCC patients was assessed and related to tumour stage, subsite and nodal status.

Table 4.2 Clinicopathological characteristics of HNSCC patients and healthy controls whose PBMC were phenotypically characterised

Characteristics	Number
HNSCC patients sex and age	
Male	35
Female	4
Total	39
Mean age (years)	62
Age range (years)	40-87
Healthy controls sex and age	
Male	12
Female	1
Total	14*
Mean age (years)	45
Age range (years)	23-63
Tumour stage	
Early (T1 and T2)	19
Advanced (T3 and T4)	20
Tumour site	
Laryngeal	23
Oropharyngeal	16
Tumour stage and site	
Early laryngeal	10
Advanced laryngeal	13
Early oropharyngeal	9
Advanced oropharyngeal	7
Nodal status	
N0	14
N^+	25

* The sex and age of one healthy control was not recorded.

4.3.1.1 Percentage of lymphocytes expressing CD4

The HNSCC patients, as a whole cohort, showed significantly elevated levels of $CD4^+$ T cells in comparison with healthy controls (Table 4.3). This significance arose principally from patients with no nodal involvement (N0) who had significantly higher levels of $CD4^+$ cells compared with those having nodal involvement (N^+) and from the laryngeal cohort who had a significantly higher proportion of $CD4^+$ cells compared with the oropharyngeal cohort (Table 4.3). These two subgroups were also observed to have significantly increased levels of $CD4^+$ T cells compared with healthy controls, p=0.003 and p=0.01, respectively.

Table 4.3 Percentage of cells expressing specific T cell markers in healthy controls and HNSCC patients in relation to tumour stage, subsite and nodal status

		Percentage of $CD4^+$ cells expressing		
Cohort	Percentage of lymphocytes expressing CD4	$CD25^+$	$CD25^{inter}$	$CD25^{high}$
Healthy controls (n=14)	38.14 ± 2.47	56.82 ± 4.35	42.55 ± 3.69	14.27 ± 1.77
HNSCC patients (n=39)	44.03 ± 1.46	60.91 ± 2.38	48.32 ± 2.15	12.59 ± 0.98
p value	**0.04**	0.39	0.18	0.43
Early stage (n=19)	44.62 ± 2.36	59.46 ± 3.58	48.96 ± 3.43	10.49 ± 1.10
Advanced stage (n=20)	43.48 ± 1.79	62.29 ± 3.23	47.70 ± 2.73	14.59 ± 1.49
p value	0.70	0.56	0.77	0.06
Laryngeal (n=23)	46.61 ± 1.91	58.90 ± 3.38	47.14 ± 2.83	11.76 ± 1.24
Oropharyngeal (n=16)	40.33 ± 1.96	63.80 ± 3.17	50.01 ± 3.39	13.79 ± 1.57
p value	**0.03**	0.32	0.52	0.31
Early Laryngeal (n=10)	49.30 ± 2.90	56.05 ± 5.43	46.23 ± 5.11	9.82 ± 1.45
Advanced Laryngeal (n=13)	44.55 ± 2.49	61.09 ± 4.38	47.84 ± 3.28	13.25 ± 1.84
p value	0.23	0.47	0.79	0.18
Early Oropharyngeal (n=9)	39.41 ± 3.10	63.24 ± 4.54	52.00 ± 4.59	11.24 ± 1.72
Advanced Oropharyngeal (n=7)	41.50 ± 2.26	64.51 ± 4.69	47.44 ± 5.25	17.07 ± 2.41
p value	0.61	0.85	0.42	0.06
N0 (n=14)	48.92 ± 2.16	56.12 ± 4.66	45.96 ± 4.21	10.16 ± 1.40
N^+ (n=25)	41.30 ± 1.72	63.59 ± 2.57	49.63 ± 2.42	13.96 ± 1.24
p value	**0.01**	0.14	0.42	0.06

Values mean percentage ± SEM
Unpaired student's t test or Mann-Whitney *U* test. Results in bold considered significant when $p<0.05$
$CD25^+$ is the sum of $CD25^{inter}$ and $CD25^{high}$

For the laryngeal cohort it was patients with early stage tumours that had the highest level of $CD4^+$ cells, furthermore early stage laryngeal patients had significantly higher levels than patients with early stage cancer of the oropharynx (Figure 4.4).

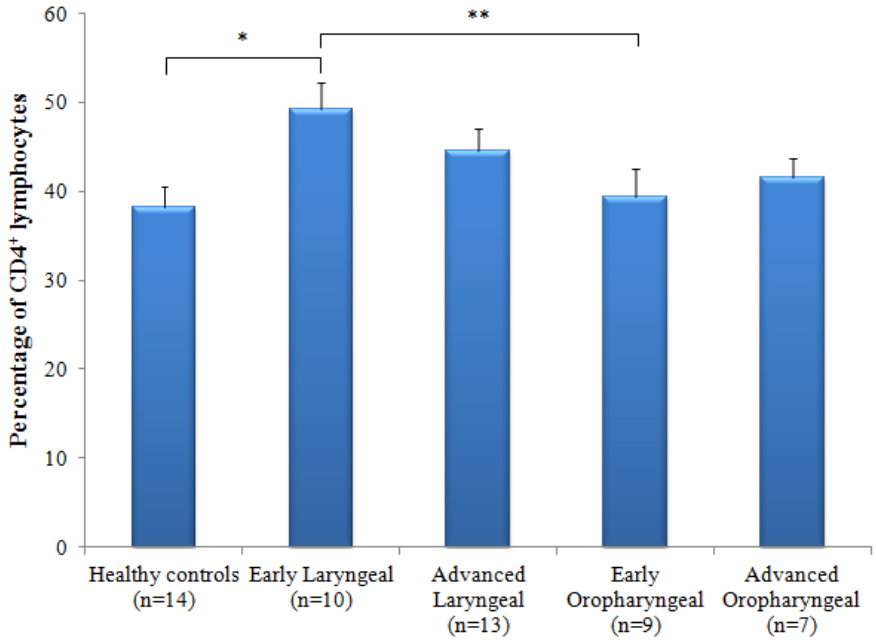


Figure 4.4 Percentage of lymphocytes expressing CD4 from healthy controls and HNSCC patients separated by tumour stage and subsite
Following labelling with CD4, CD25 and CD127 antibodies, the lymphocyte population was analysed by flow cytometry and following doublet discrimination the percentage of lymphocytes expressing CD4 was determined. Values are mean percentage ± SEM. Unpaired student's T test; only significant values are shown * p=0.008 ** p=0.03

4.3.1.2 Percentage of $CD4^+$ cells expressing CD25

The percentage of $CD4^+$ cells expressing CD25 ($CD4^+CD25^+$) and the subpopulation expressing the IL-2 receptor at intermediate levels ($CD4^+CD25^{inter}$) were slightly elevated in HNSCC patients, as a whole, in comparison with healthy controls however, this difference was not significant (Table 4.3). The proportion of $CD4^+$ cells expressing high levels of CD25 was similar between the whole patient cohort and healthy controls (Table 4.3). When comparing different head and neck subsites, although significance was not observed, patients with cancer of the oropharynx were seen to have a slightly increased level of $CD4^+CD25^+$ cells compared with laryngeal cancer patients

(Table 4.3). When HNSCC patients were separated with respect to nodal status and tumour stage, patients with nodal involvement and advanced stage tumours had increased levels of $CD4^{+}CD25^{high}$ cells in comparison with those without lymph node involvement and early stage tumours respectively, which approached significance (Table 4.3). Examining individual subsites revealed that the differences observed between tumour stages arose principally from patients with advanced stage oropharyngeal cancer who had elevated levels of $CD4^{+}CD25^{high}$ cells compared with the early stage oropharyngeal cohort, which tended towards significance. The same trend was seen to occur in the laryngeal cohort however, this difference did not reach significance (Table 4.3).

4.3.1.3 Percentage of Tregs expressing low levels of CD127

The percentage of $CD4^{+}CD127^{low/-}$ cells, for all levels of CD25 expression, was similar in the whole HNSCC patient cohort compared with the healthy controls (Table 4.4). On comparing tumour stages, patients with early stage tumours had a slightly lower but non-significant level of $CD4^{+}CD25^{+}CD127^{low/-}$ cells in comparison with patients having advanced stage tumours; this difference predominately arose from the laryngeal cohort where patients with advanced stage tumours had higher levels of $CD4^{+}CD25^{+}CD127^{low/-}$ cells in comparison with patients with early stage tumours, a different which approached significance (Table 4.4). Separating the $CD25^{+}CD127^{low/-}$ population by CD25 expression, showed that advanced stage patients had a significantly elevated level of $CD4^{+}CD25^{high}CD127^{low/-}$ cells compared with early stage patients. This significance arose from patients with advanced stage cancer of the larynx and oropharynx having a higher proportion of $CD4^{+}CD25^{high}CD127^{low/-}$ cells in comparison to patients with early stage laryngeal and oropharyngeal cancer however, these differences did not reach significance (Table 4.4). Examining nodal status, patients with tumours that had spread to the lymph nodes had significantly elevated levels of $CD25^{+}$ and $CD25^{high}CD127^{low/-}$ cells compared with patients who had no nodal involvement (Table 4.4).

Table 4.4 Percentage of $CD4^+$ cells expressing varying levels of CD25 and CD127 in healthy controls and HNSCC patients in relation to tumour stage, subsite and nodal status

	Percentage of $CD4^+$ cells expressing					
Cohort	$CD25^+CD127^{low/-}$	$CD25^{inter}CD127^{low/-}$	$CD25^{high}CD127^{low/-}$	$CD25^{-/+}CD127^{-/+}$	$CD25^-CD127^{-/+}$	$CD25^+CD127^+$
Healthy controls (n=14)	14.58 ± 1.27	8.77 ± 0.85	5.81 ± 0.66	85.15 ± 1.59	43.34 ± 4.31	41.81 ± 3.37
HNSCC patients (n=39)	15.25 ± 0.58	8.59 ± 0.41	6.67 ± 0.45	85.29 ± 0.55	39.21 ± 2.37	46.08 ± 2.13
p value	0.59	0.83	0.31	0.94	0.39	0.30
Early stage (n=19)	14.30 ± 0.78	8.61 ± 0.70	5.69 ± 0.48	86.36 ± 0.72	40.75 ± 3.56	45.61 ± 3.23
Advanced stage (n=20)	16.16 ± 0.82	8.57 ± 0.46	7.59 ± 0.69	84.28 ± 0.78	37.74 ± 3.20	46.54 ± 2.89
p value	0.10	0.97	**0.03**	0.06	0.53	0.83
Laryngeal (n=23)	14.87 ± 0.80	8.22 ± 0.44	6.65 ± 0.64	85.83 ± 0.76	41.12 ± 3.35	44.71 ± 3.02
Oropharyngeal (n=16)	15.81 ± 0.84	9.11 ± 0.76	6.69 ± 0.60	84.51 ± 0.76	36.45 ± 3.18	48.06 ± 2.91
p value	0.45	0.29	0.96	0.24	0.34	0.45
Early Laryngeal (n=10)	13.23 ± 1.06	7.83 ± 0.91	5.40 ± 0.58	87.48 ± 0.99	43.82 ± 5.43	43.66 ± 5.12
Advanced Laryngeal (n=13)	16.13 ± 1.05	8.52 ± 0.38	7.61 ± 0.98	84.57 ± 1.01	39.05 ± 4.31	45.52 ± 3.79
p value	0.06	0.20	0.09	**0.047**	0.49	0.77
Early Oropharyngeal (n=9)	15.49 ± 1.07	9.47 ± 1.06	6.02 ± 0.82	85.11 ± 0.92	37.34 ± 4.55	47.77 ± 3.96
Advanced Oropharyngeal (n=7)	16.21 ± 1.42	8.66 ± 1.16	7.56 ± 0.84	83.73 ± 1.27	35.30 ± 4.69	48.43 ± 4.64
p value	0.66	0.62	0.22	0.38	0.76	0.92
N0 (n=14)	13.25 ± 0.76	7.87 ± 0.58	5.37 ± 0.48	87.21 ± 0.72	44.08 ± 4.63	43.14 ± 4.38
N^+ (n=25)	16.35 ± 0.71	8.99 ± 0.54	7.39 ± 0.60	84.21 ± 0.68	36.48 ± 2.55	47.74 ± 2.27
p value	**0.01**	0.10	**0.03**	**0.01**	0.13	0.31

Values mean percentage ± SEM

Unpaired student's t test or Mann-Whitney *U* test. Results in bold considered significant when $p<0.05$

$CD25^+CD127^{low/-}$ is the sum of $CD25^{inter}$ $CD127^{low/-}$ and $CD25^{high}CD127^{low/-}$. $CD25^{-/+}CD127^{-/+}$ is the sum of $CD25^-CD127^{-/+}$ and $CD25^+CD127^+$

4.3.1.4 The percentage of Tregs in the peripheral circulation using different phenotypes

Lymphocytes were labelled with antibodies specific for the extracellular markers, CD4, CD25 and CD127, allowing Tregs to be identified and their level in the peripheral circulation of HNSCC patients assessed by various different commonly used phenotypes ($CD4^+CD25^+$, $CD4^+CD25^{high}$ and $CD4^+CD25^+CD127^{low/}$; Table 1.8) and also the $CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$ Treg populations examined by the current study for suppressive activity. Figure 4.5 shows how dramatically the levels of reported Tregs can vary depending upon the phenotype employed to investigate them. The $CD4^+CD25^+$ phenotype identifies the greatest level of Tregs in the peripheral circulation with a much lower percentage of Tregs identified by the $CD4^+CD25^{high}$ and $CD4^+CD25^+CD127^{low/-}$ phenotypes. The lowest frequency of circulating Tregs in HNSCC patients are observed by the most stringent $CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$ phenotypes.

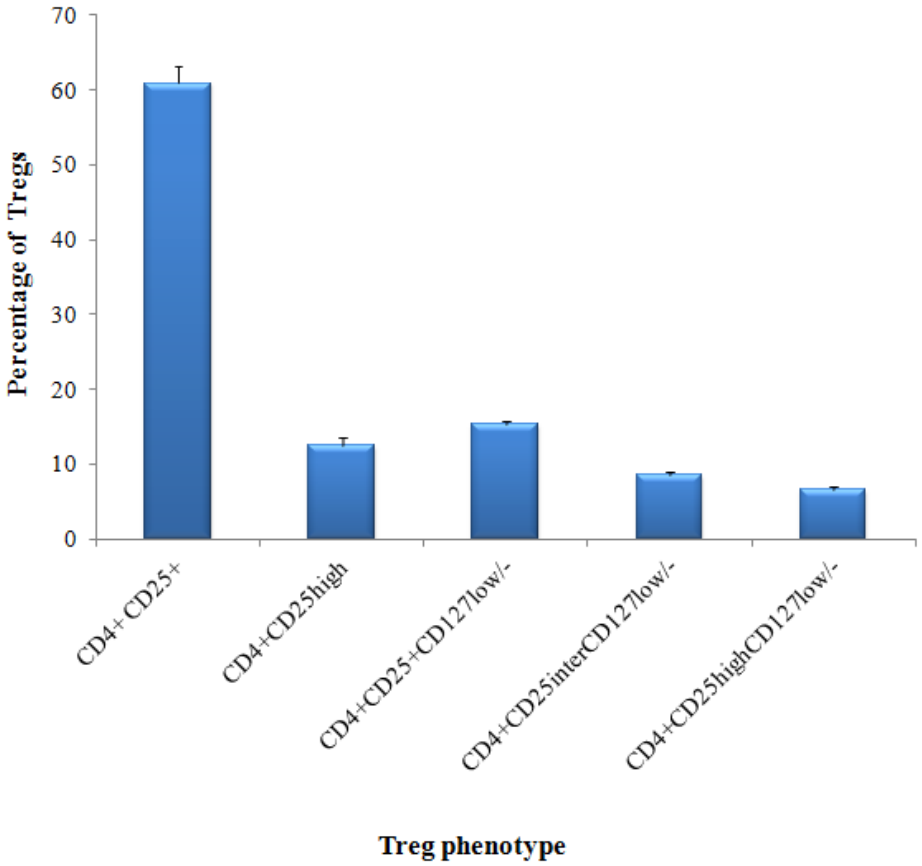


Figure 4.5 Percentages of Tregs in the peripheral circulation of HNSCC patients identified using different phenotypes
Following labelling with CD4, CD25 and CD127 antibodies, lymphocytes positive for the expression of CD4 were gated by their positive expression of CD25 to identify $CD4^{+}CD25^{+}$ Tregs. Within this population, the $CD4^{+}$ cells which expressed CD25 at levels exceeding that expressed by $CD4^{-}$ cells identified the $CD4^{+}CD25^{high}$ Tregs. Using these CD25 boundaries, $CD4^{+}$ cells expressing low levels of CD127 were gated on CD25 expression to identify $CD4^{+}CD25^{+}CD127^{low/-}$, $CD4^{+}CD25^{inter}CD127^{low/-}$ and $CD4^{+}CD25^{high}CD127^{low/-}$ Treg populations. Values are mean percentage ± SEM.

4.3.1.5 Percentage of $CD4^{+}$ effector T cell populations expressing varying levels of CD25 and CD127

The HNSCC patients, as a whole, showed similar levels of $CD4^{+}CD25^{-/+}CD127^{-/+}$ effector cells in comparison with healthy controls (Table 4.4). On comparing tumour stage, patients with early stage tumours had elevated levels of $CD4^{+}CD25^{-/+}CD127^{-/+}$ cells compared with patients with tumours of the advanced stage, a difference approaching significance (Table 4.4). This difference most probably arose due to the significant trend observed in the laryngeal cohort where patients with early stage laryngeal tumours had

increased levels of $CD4^+CD25^{-/+}CD127^{-/+}$ cells compared with patients with advanced stage cancer of the larynx (Table 4.4). On assessing nodal status, patients without nodal involvement had a significantly increased level of $CD4^+CD25^{-/+}CD127^{-/+}$ cells compared to patients with tumours that had spread to the lymph nodes. Dividing the $CD4^+CD25^{-/+}CD127^{-/+}$ effector T cell population into the two effector populations ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) highlighted no significant differences between the various cohorts. With the exception of the patient cohorts with early stage laryngeal cancer and without nodal involvement it was observed that each HNSCC group, regardless of subsite or tumour stage, had a greater proportion of $CD4^+CD25^+CD127^+$ effector T cells in comparison to the $CD4^+CD25^-CD127^{-/+}$ population. However, upon statistical analysis the difference only reached significance for the HNSCC patients with nodal involvement (p=0.03; Figure 4.6).

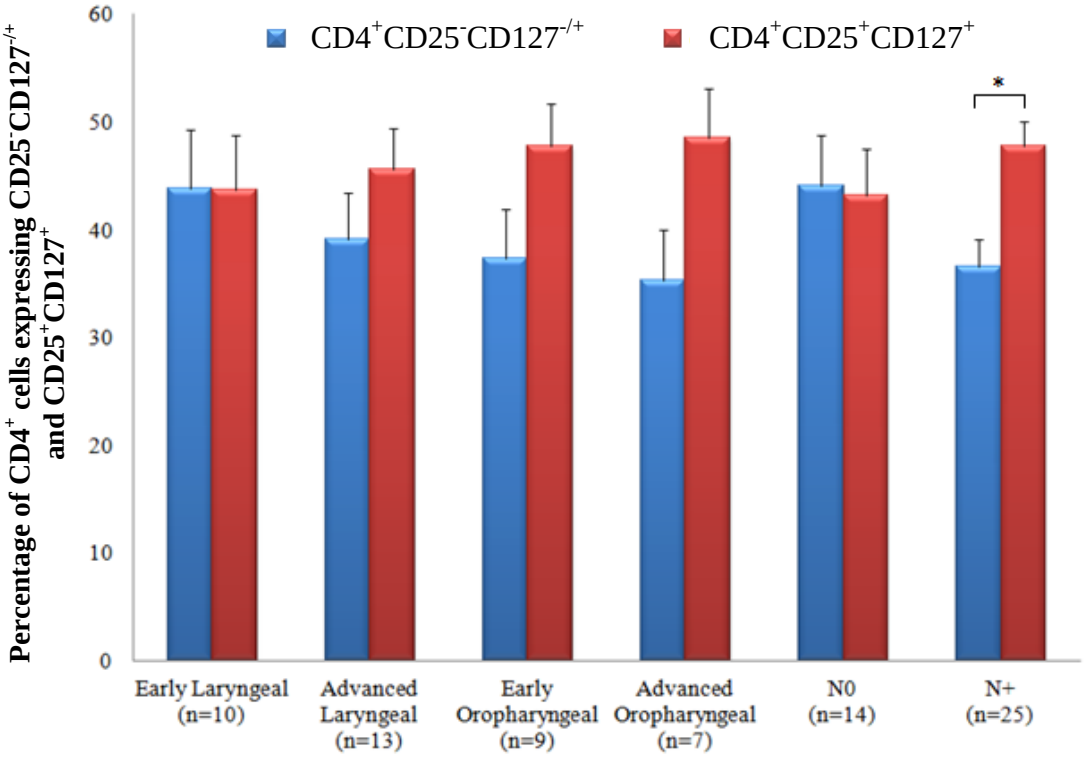


Figure 4.6 Percentage of $CD4^+$ cells from HNSCC patients expressing $CD25^-CD127^{-/+}$ and $CD25^+CD127^+$ separated by tumour stage and subsite, and nodal status
Following labelling with CD4, CD25 and CD127 antibodies, lymphocytes positive for the expression of CD4 were gated by their negative expression of CD25 to identify the $CD4^+CD25^-CD127^{-/+}$ effector T cells (blue) and their positive expression of CD25 and CD127 to identify the $CD4^+CD25^+CD127^+$ population (red). Values are mean percentage ± SEM. Paired student's t test, only significant values are shown, *p=0.03

4.3.2 Expression of Foxp3 by T cell subsets in HNSCC patients and healthy controls

A sample cohort of PBMC samples from HNSCC patients and healthy controls were phenotypically assessed using flow cytometry for the expression of Foxp3 in various T cell subsets. This examined the expression of the most specific Treg marker found to date in a number of different phenotypes used by research groups to identify the Treg population, including the $CD127^{low/-}$ phenotype. Due to small numbers statistical analysis was not performed.

4.3.2.1 Percentage of $CD4^+$ T cell subsets expressing Foxp3

The $CD4^+$ T cells positive for the expression of the IL-2 receptor ($CD4^+CD25^+$) and those expressing intermediate levels of CD25 ($CD4^+CD25^{inter}$) expressed Foxp3 at similar levels in HNSCC patients, as a whole, and healthy controls (Table 4.5). In contrast, the expression of the transcription factor by $CD4^+CD25^{high}$ cells and all three $CD127^{low/-}$ populations was greater in HNSCC patients compared with healthy controls (Table 4.5). The percentage of $CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$ effector T cells expressing Foxp3 was similar in the whole HNSCC patient cohort and healthy controls (Table 4.5).

Table 4.5 Mean percentage of T cell subsets expressing Foxp3 in HNSCC patients and healthy controls

T cell phenotypes		Cohort	
		Healthy controls (n=2)	HNSCC patients (n=4)
Percentage of T cells expressing Foxp3	$CD4^+CD25^+$	7.4	7.8
	$CD4^+CD25^{inter}$	6.1	4.0
	$CD4^+CD25^{high}$	13.6	34.0
	$CD4^+CD25^+CD127^{low/-}$	21.8	26.4
	$CD4^+CD25^{inter}CD127^{low/-}$	15.6	21.2
	$CD4^+CD25^{high}CD127^{low/-}$	59.1	72.7
	$CD4^+CD25^-CD127^{-/+}$	3.1	3.6
	$CD4^+CD25^+CD127^+$	5.2	2.9

For HNSCC patients, as a whole, the $CD4^{+}CD25^{high}CD127^{low/-}$ population had the highest percentage of Foxp3 positive cells, over 38% higher than the other Treg phenotypes, whilst the $CD4^{+}CD25^{+}$ phenotype has the lowest proportion of the transcription factor. Furthermore, irrespective of the Treg phenotype employed, it was shown that Tregs have a higher proportion of Foxp3 positive cells when compared to the effector T cell populations ($CD4^{+}CD25^{inter}$, $CD4^{+}CD25^{-}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$).

4.3.3 Suppressive activity of Tregs from HNSCC patients and healthy controls

The PBMC samples of 39 HNSCC patients and 14 healthy controls (Figure 4.1) were stained with a Treg sorting cocktail before being sorted by FACS to assess the suppressive activity of two Treg populations ($CD4^{+}CD25^{inter}CD127^{low/-}$ and $CD4^{+}CD25^{high}CD127^{low/-}$) on the two autologous effector T cell populations ($CD4^{+}CD25^{-}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$; Figure 2.11) using a CFSE assay. However, despite best efforts, due to insufficient cells isolated to set up the CFSE assay and the failure of the CFSE assay after the four day culture, the suppressive activity of Tregs from 11 HNSCC patients and 4 healthy controls were unable to be determined (Figure 4.1). For one of the healthy control samples analysed abnormally high percentages of suppression were observed when compared to the other 9 healthy controls which may have been due to some underlying illness. The percentage of suppression induced by the $CD4^{+}CD25^{high}CD127^{low/-}$ and $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs from this healthy control sample was over four fold and two fold higher, respectively, compared to the mean percentage calculated for the remaining 9 healthy controls; consequently these results were omitted from the analysis. The clinicopathological characteristics of the remaining 28 HNSCC patients and 9 healthy controls analysed for the suppressive activity of Tregs are shown in Table 4.6.

Table 4.6 Clinicopathological characteristics of HNSCC patients and healthy controls whose peripheral Tregs were analysed for suppressive activity

Characteristics	Number
HNSCC patient sex and age	
Male	25
Female	3
Total	28
Mean age (years)	60
Age range (years)	41-84
Healthy controls sex and age	
Male	7
Female	1
Total	9*
Mean age (years)	47
Age range (years)	23-63
Tumour stage	
Early (T1 and T2)	14
Advanced (T3 and T4)	14
Tumour site	
Larynx	14
Oropharynx	14
Tumour stage and site	
Early larynx	6
Advanced larynx	8
Early oropharynx	8
Advanced oropharynx	6
Nodal status	
Negative	8
Positive	20

* The sex and age of one healthy control was not recorded.

4.3.3.1 Suppressive activity of Tregs at different Treg : effector T cell ratios

In all samples a Treg:effector T cell ratio of 1:1 was established and where cell numbers allowed further ratios of 1:2, 1:5 and 1:10 were also investigated to demonstrate that the suppressive activity could be titrated. The varying ratios showed that as the proportion of Tregs was lowered the percentage of suppression also decreased, regardless

of Treg phenotype ($CD4^+CD25^{high}CD127^{low/-}$ and $CD4^+CD25^{inter}CD127^{low/-}$) and the effector T cell population ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) being suppressed (Figure 4.7 and Figure 4.8).

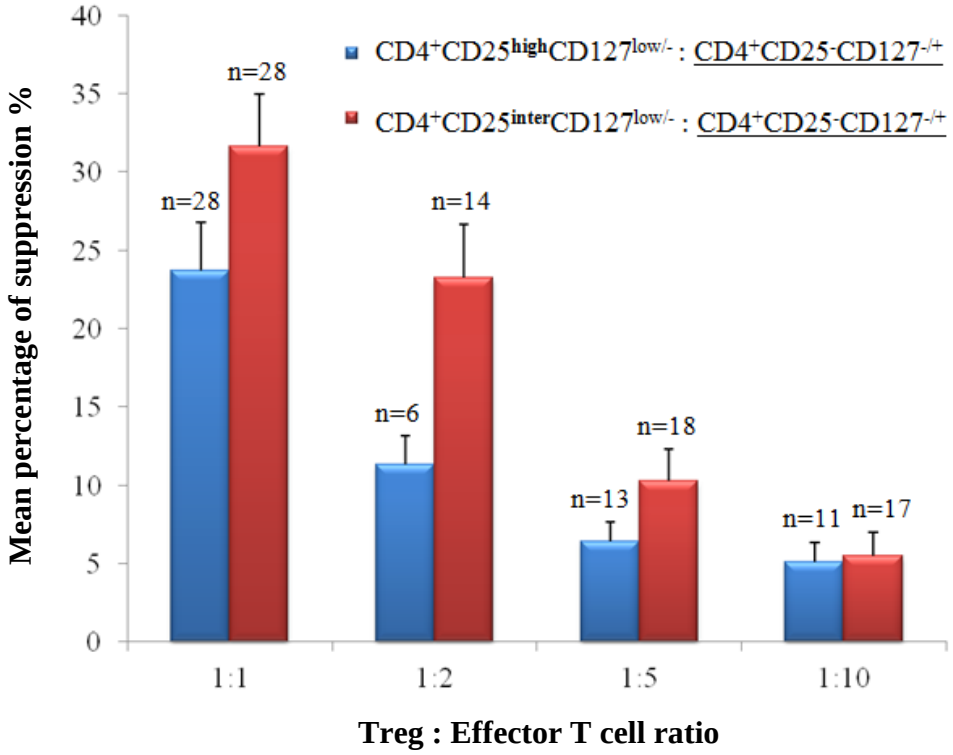


Figure 4.7 Mean percentage of suppression by $CD25^{high}CD127^{low/-}$ and $CD25^{inter}CD127^{low/-}$ Tregs on the proliferation of $CD25^-CD127^{-/+}$ effector T cells at varying ratios for HNSCC patients

FACS isolated $CD4^+CD25^{high}CD127^{low/-}$ and $CD4^+CD25^{inter}CD127^{low/-}$ Tregs were co-cultured with CFSE labelled $CD4^+CD25^-CD127^{-/+}$ effector T cells at ratios of 1:1, 1:2, 1:5 and 1:10 for four days before being acquired by flow cytometry and the data analysed by ModFit LT™ to determine the percentage of suppression induced by the Tregs. Values mean percentage ± SEM.

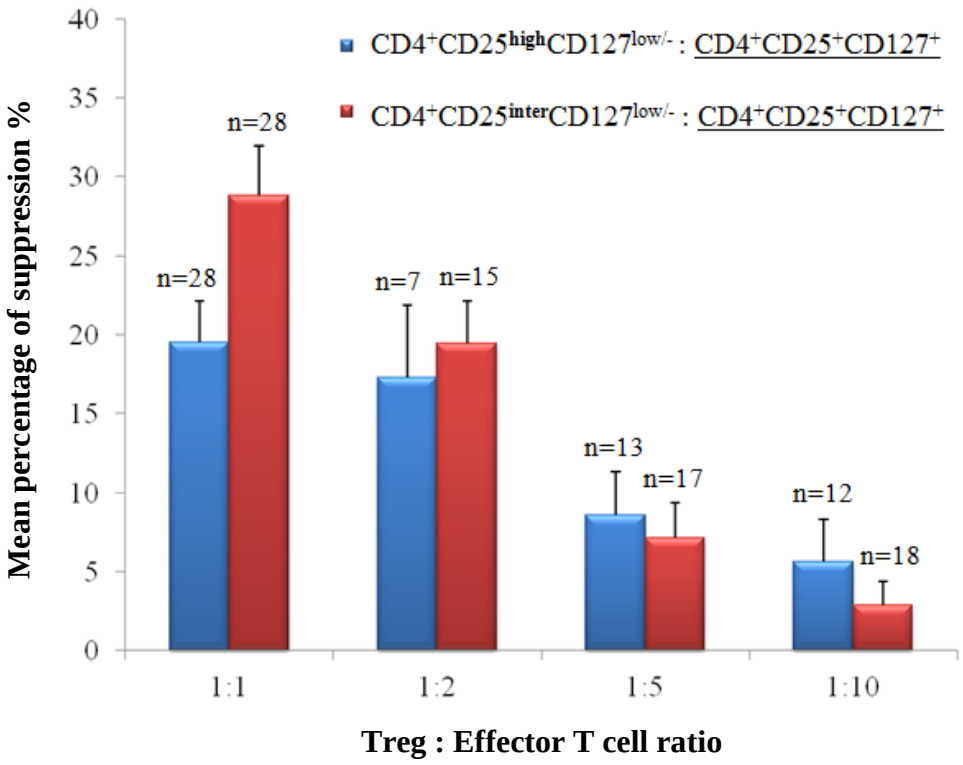


Figure 4.8 Mean percentage of suppression by $CD25^{high}CD127^{low/-}$ and $CD25^{inter}CD127^{low/-}$ Tregs on the proliferation of $\underline{CD25^{+}CD127^{+}}$ effector T cells at varying ratios for HNSCC patients

FACS isolated $CD4^{+}CD25^{high}CD127^{low/-}$ and $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs were co-cultured with CFSE labelled $CD4^{+}CD25^{+}CD127^{+}$ effector T cells at ratios of 1:1, 1:2, 1:5 and 1:10 for four days before being acquired by flow cytometry and the data analysed by ModFit LT^{TM} to determine the percentage of suppression induced by the Tregs. Values mean percentage ± SEM.

The observed dilution of Treg suppressive activity was in agreement with previous studies conducted on Tregs isolated from the peripheral circulation of patients with gastric cancer (Ichihara *et al.*, 2003; Shen *et al.*, 2009) and HNSCC (Mandapathil *et al.*, 2009).

Due to all samples being established at the 1:1 ratio, and the lower number of samples prepared at the ratios 1:2, 1:5 and 1:10, the analysis of subsequent results, to highlight any differences between the suppressive activity of Tregs in different HNSCC patient cohorts and healthy controls, focused on the results from the 1:1 ratio.

4.3.3.2 Suppressive activity of $CD4^+CD25^{inter/high}CD127^{low/-}$ Tregs on $CD4^+CD25^-CD127^{-/+}$ effector T cells

The HNSCC patients, as a whole cohort, had Tregs expressing both intermediate and high levels of CD25 suppressing the proliferation of the $CD25^-CD127^{-/+}$ effector population to a greater extent than those from healthy controls, and this difference reached significance for the $CD25^{high}$ Tregs (Table 4.7). The significance observed arose primarily from the oropharyngeal cohort, in particular those with early stage oropharyngeal cancer, where it was observed that both these patient cohorts significantly induced a greater level of suppression compared with healthy controls (p=0.04 and p=0.02, respectively). When both subsite and stage were taken into account, patients with advanced stage cancer of the larynx also had $CD25^{high}$ Tregs with significantly greater suppressive activity than the healthy controls (p=0.04). The suppressive activity observed between HNSCC subsites, tumour stages and nodal status were similar for both $CD25^{high}$ and $CD25^{inter}$ Tregs (Table 4.7). However, $CD25^{high}$ Tregs from patients with nodal involvement were significantly more suppressive compared with healthy controls (p=0.04).

Table 4.7 Percentage of suppression induced by Tregs on the proliferation of effector T cells at a 1:1 ratio for both healthy controls and HNSCC patients separated by tumour stage, subsite and nodal status

Cohort	Treg : Effector T cell			
	$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^+CD127^+$	$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^+CD127^+$
Healthy controls (n=9)	23.34 ± 7.09	11.43 ± 3.53	27.60 ± 5.97	12.34 ±4.31
HNSCC patients (n=28)	31.62 ± 3.33	23.73 ± 3.04	28.82 ± 3.16	19.49 ± 2.61
p value	0.25	**0.04**	0.85	0.18
Early stage (n=14)	30.11 ± 4.53	23.51 ± 4.07	27.99 ± 4.92	19.51 ± 4.31
Advanced stage (n=14)	33.13 ± 5.03	23.95 ± 4.67	29.64 ± 4.16	19.47 ± 3.12
p value	0.66	0.94	0.80	1
Laryngeal (n=14)	29.54 ± 4.04	22.05 ± 4.04	28.51 ± 4.77	19.65 ± 4.77
Oropharyngeal (n=14)	33.70 ± 4.65	25.41 ± 4.65	29.12 ± 4.33	19.33 ± 3.36
p value	0.54	0.59	0.93	0.95
Early Laryngeal (n=6)	21.20 ± 6.63	18.30 ± 6.95	20.42 ± 8.04	14.48 ± 8.16
Advanced Laryngeal (n=8)	35.80 ± 6.62	24.86 ± 4.96	34.59 ± 5.21	23.53 ± 3.83
p value	0.15	0.44	0.20	0.30
Early Oropharyngeal (n=8)	36.80 ± 4.79	27.43 ± 4.79	33.68 ± 5.75	23.28 ± 4.46
Advanced Oropharyngeal (n=6)	29.57 ± 8.20	22.73 ± 9.27	23.05 ± 6.27	14.07 ± 4.64
p value	0.46	0.64	0.24	0.19
N0 (n=8)	29.89 ± 5.95	22.28 ± 5.54	24.75 ± 5.32	11.21 ± 4.50
N^+ (n=20)	32.31 ± 4.10	24.30 ± 3.72	30.45 ± 3.91	22.80 ± 2.92
p value	0.75	0.77	0.43	**0.04**

Values mean percentage ± SEM

Unpaired student's t test or Mann-Whitney *U* test. Bold results considered significant when $p<0.05$

4.3.3.3 Suppressive activity of $CD4^+CD25^{inter/high}CD127^{low/-}$ Tregs on $CD4^+CD25^+CD127^+$ effector T cells

The mean percentage of suppression induced by $CD25^{high}$ Tregs from the whole HNSCC patient cohort on $CD25^+CD127^+$ effector T cells was slightly greater than that from healthy controls however, this difference did not reach significance (Table 4.7). A similar level of suppression for both HNSCC patients and healthy controls was induced by the $CD25^{inter}$ Tregs (Table 4.7). The suppressive activity of $CD25^{high}$ and $CD25^{inter}$ Tregs was similar between laryngeal and oropharyngeal HNSCC subsites and early and advanced tumour stages whereas $CD25^{high}$ Tregs from patients nodal involvement had significantly greater suppressive activity than patients without nodal involvement (Table 4.7).

4.3.3.4 Assessing the phenotype and suppressive activity of $CD25^{inter}$ and $CD25^{high}$ Tregs

During the identification and isolation of Treg and effector T cell populations for the assessment of Treg suppressive activity, it was observed that when the four T cell populations (2 Treg and 2 effector T cell; Figure 4.9a) were transposed on to a CD4 vs CD25 dot plot, the $CD4^+$ cells classified solely by their intermediate level of CD25 expression contained a substantial number of activated T cells ($CD4^+CD25^+CD127^+$, yellow dots) along with relatively few $CD4^+CD25^{inter}CD127^{low/-}$ Tregs (Figure 4.9b; red dots). Furthermore, this plot highlighted that the majority of activated T cells express intermediate levels of CD25 and that Tregs express a lower level of CD4 in comparison to the effector T cell populations (Figure 4.9b).

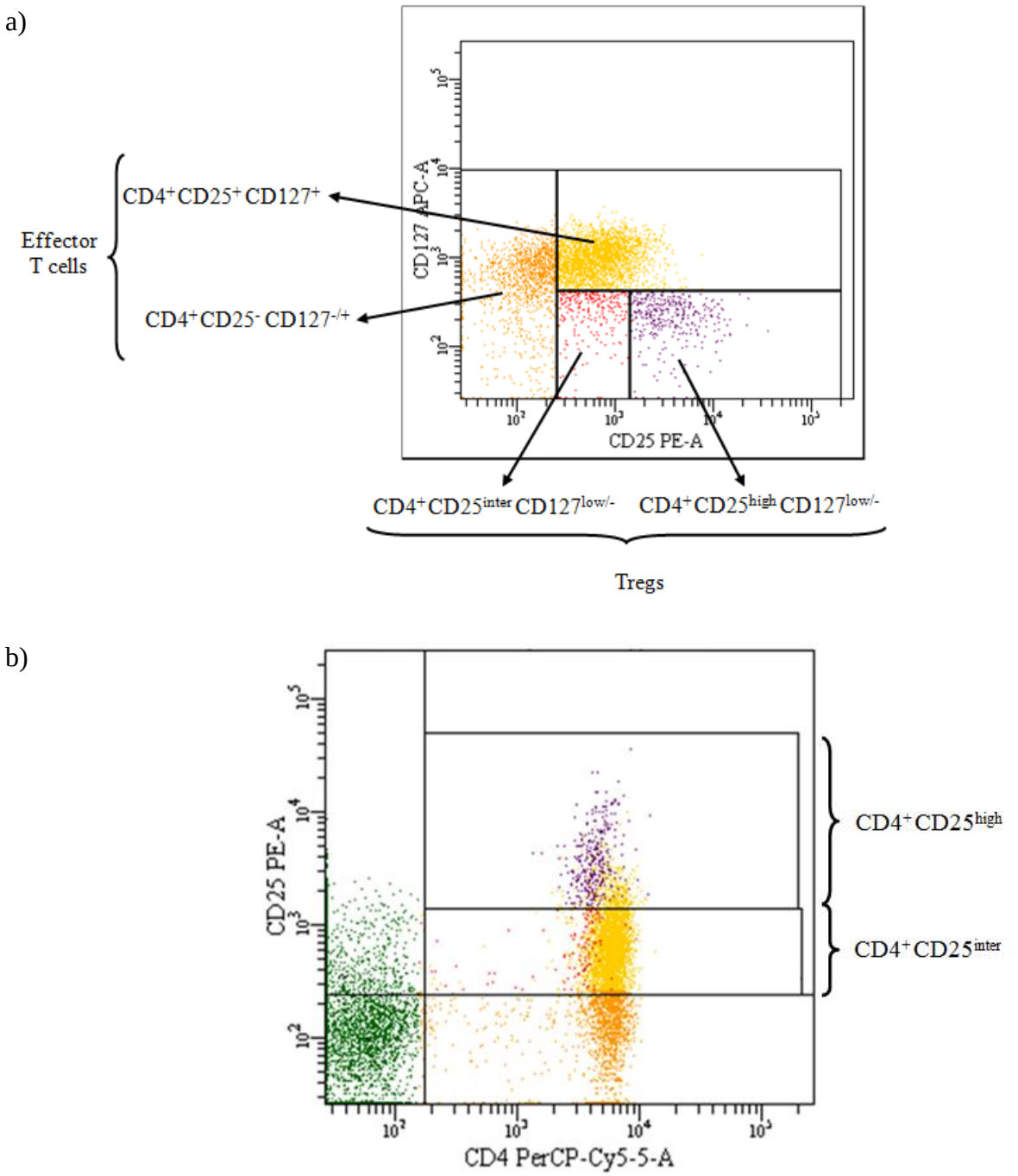


Figure 4.9 Distribution of isolated Treg and effector T cell populations based on the expression of CD4 and CD25

a) A PBMC sample was labelled with antibodies specific for CD4, CD25 and CD127, and following doublet discrimination the $CD4^+$ lymphocytes were gated to identify two Treg ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) and two effector T cell ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) populations based on the expression of CD25 and CD127. b) These four populations were plotted on a CD4 vs CD25 dot plot to show their distribution amongst the $CD4^+CD25^{high}$ and $CD4^+CD25^{inter}$ gates transposing the same colours from one plot to the other.

The $CD4^{+}CD25^{inter}CD127^{low/-}$ Treg population consistently induced a greater level of suppression compared with $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs at the 1:1 ratio (Table 4.7). This trend was also observed at the different Treg:effector T cell ratios however, with increased proportions of effector T cells the level of suppression induced by the two Treg populations became similar (Figure 4.7 and Figure 4.8).

Assessing the percentage of suppression induced by Tregs on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells at a 1:1 ratio, Tregs expressing an intermediate level of CD25 consistently induced a higher level of suppression compared with those with higher levels of the IL-2 receptor, regardless of tumour stage, site, nodal status and including healthy controls (Table 4.7). This reached significance for the whole HNSCC patient cohort, healthy controls (Figure 4.10) and patients with advanced stage laryngeal cancer (p=0.04).

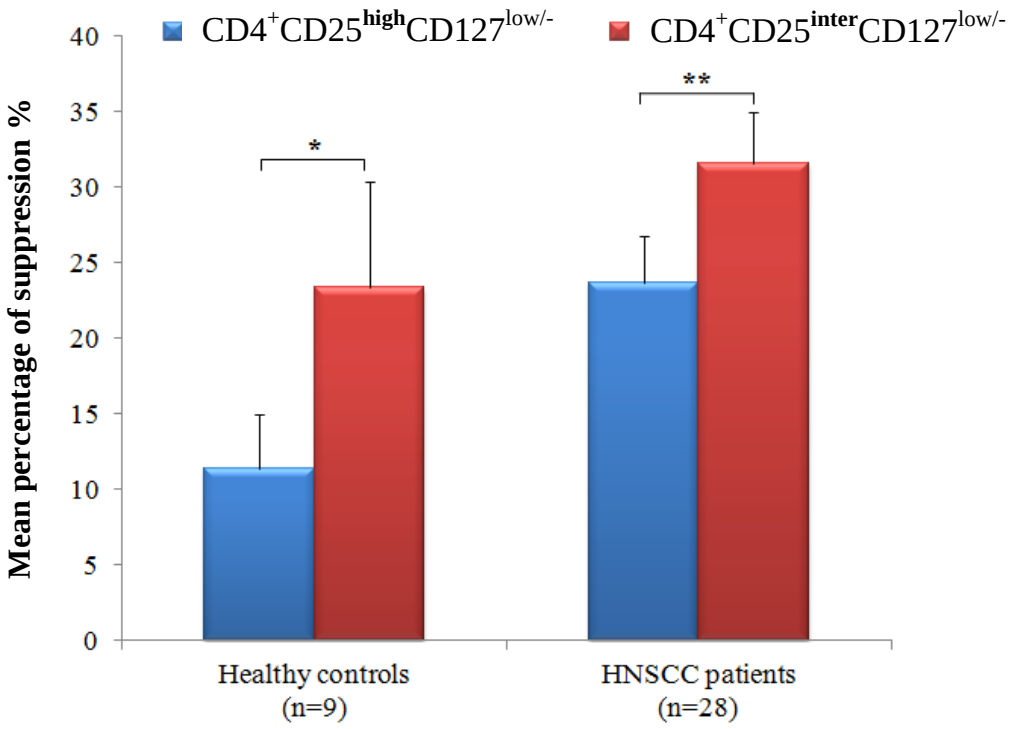


Figure 4.10 Mean percentage of suppression by $CD25^{high}$ and $CD25^{inter}$ Tregs on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells
FACS isolated $CD4^{+}CD25^{high}CD127^{low/-}$ and $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs were co-cultured with CFSE labelled $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells at a 1:1 ratio for four days before being acquired by flow cytometry and the data analysed by ModFit LT™ to determine the percentage of suppression induced by the Tregs. Values mean percentage ± SEM. Paired student's t test, *p=0.04 **p=0.02

This trend was also observed on the proliferation of $CD4^+CD25^+CD127^+$ effector T cells with the suppressive activity of $CD25^{inter}$ Tregs being significantly greater compared with $CD25^{high}$ Tregs for the majority of HNSCC patient cohorts, including advanced stage laryngeal cancer patients (p=0.02) and healthy controls (Figure 4.11).

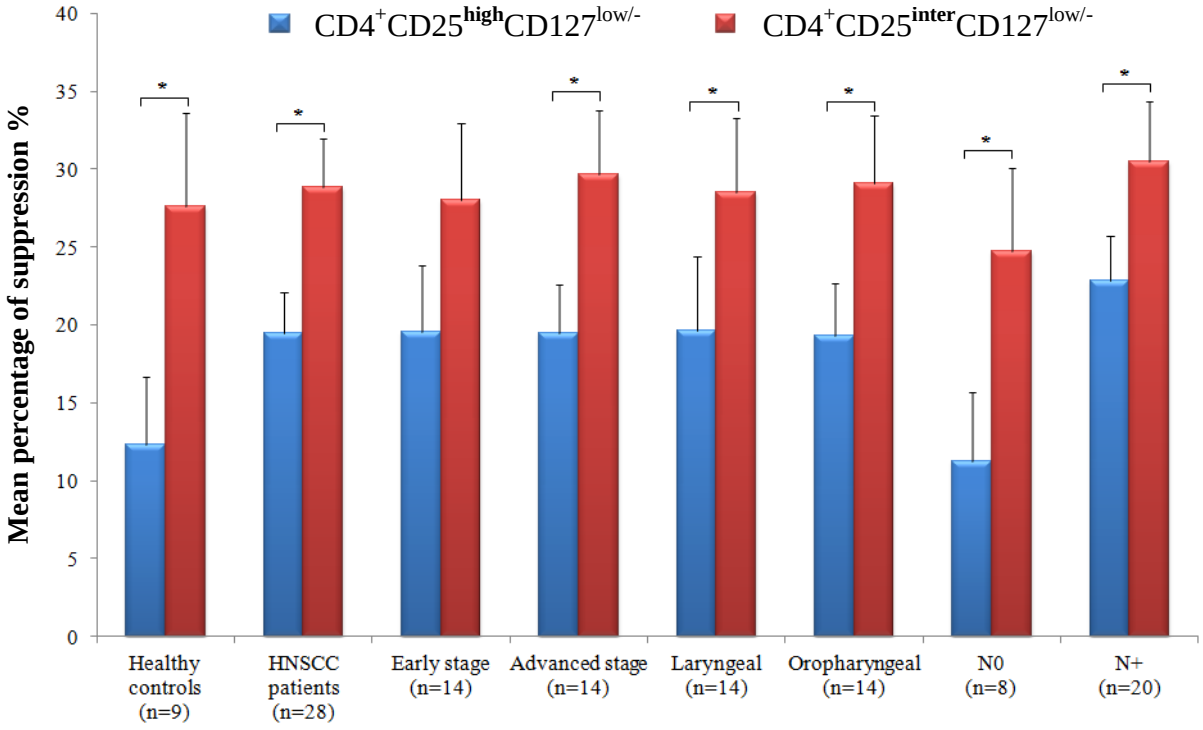


Figure 4.11 Mean percentage of suppression by $CD25^{high}$ and $CD25^{inter}$ Tregs on the proliferation of $CD4^+CD25^+CD127^+$ effector T cells
FACS isolated $CD4^+CD25^{high}CD127^{low/-}$ and $CD4^+CD25^{inter}CD127^{low/-}$ Tregs were co-cultured with CFSE labelled $CD4^+CD25^+CD127^+$ effector T cells at a 1:1 ratio for four days before being acquired by flow cytometry and the data analysed by ModFit LT™ to determine the percentage of suppression induced by the Tregs. Values mean percentage ± SEM. Paired student's t test, only significant values are shown *p=≤0.03

4.3.3.5 Comparison of the suppressive activity of Tregs on $CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$ effector T cells

It was observed that both the $CD25^{inter}$ and $CD25^{high}CD127^{low/-}$ Tregs induced a greater level of suppression on the proliferation of the $CD4^+CD25^-CD127^{-/+}$ effector T cells compared to the $CD4^+CD25^+CD127^+$ effector T cell population for all HNSCC patient cohorts (Table 4.7), however this only reached significance for the

$CD4^{+}CD25^{high}CD127^{low/-}$ Tregs isolated from patients with cancer of the oropharynx, p=0.03.

4.4 Discussion

Previous studies have reported that, in the peripheral circulation, cancer patients harbour increased levels of Tregs that have greater suppressive activity when compared with healthy controls (Table 1.8 and Table 4.1). However, the markers used to determine the phenotype of the Tregs varies between studies. The aim of the current study was to further understand the influence HNSCC has on the circulating Treg population by examining the different phenotypes, percentages and suppressive activity of Treg subsets in the peripheral circulation of HNSCC patients using the extracellular markers CD4, CD25 and CD127. To the author's knowledge this is the largest investigation assessing the level of Tregs using the CD127 phenotype in newly-presenting head and neck cancer patients and is the first study to examine the suppressive activity of $CD127^{low/-}$ Tregs in HNSCC patients, comparing between patients and healthy controls and several clinicopathological features.

4.4.1 Prevalence of $CD4^{+}$ cells in the peripheral circulation of healthy controls and HNSCC patients

A significantly increased percentage of lymphocytes expressing CD4 was observed in the HNSCC patient cohort in comparison with healthy controls, in particular in patients with laryngeal cancer, and more specifically early stage laryngeal cancer, and in patients with no nodal involvement. These results are surprising as it is well known that head and neck cancer patients along with the majority of other cancer patients are immunosuppressed due to the immune evasion strategies that are mediated by the tumour (Section 1.8; Duray *et al.*, 2010; Rabinovich *et al.*, 2007) and therefore it would perhaps be expected that the level of $CD4^{+}$ cells would be lower in HNSCC patients compared with healthy controls as reported by Chikamatsu *et al.* (2007) and Lau *et al.* (2007). Current findings are however

supported by Boucek *et al.* (2010) who also reported that HNSCC patients have a significantly higher percentage of circulating $CD4^+$ lymphocytes in comparison with healthy controls. In addition, Boucek and colleagues published similar percentages to that of the current study for the level of $CD4^+$ cells in HNSCC patients (45.3% vs. current study mean 44.03%) and healthy controls (38.1% vs. current study mean 38.14%). However, although a higher percentage of $CD4^+$ cells have been reported in HNSCC patients compared with healthy controls, their function may be compromised.

4.4.2 Prevalence of Tregs in the peripheral circulation of healthy controls and HNSCC patients

Tregs in the literature come in many different guises however, the frequency of Tregs in the peripheral circulation of HNSCC patients in the current study was similar to that in healthy controls, regardless of the phenotype used to investigated them ($CD4^+CD25^+$, $CD4^+CD25^{high}$, $CD4^+CD25^+CD127^{low/-}$, $CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$). The HNSCC patient cohort and healthy controls had a similar sex imbalance with a higher proportion of males recruited compared with females however, the mean age of the healthy controls was lower compared with the HNSCC patients. Thus there may be some changes related to ageing as reviewed by Dejaco *et al.* (2006). Although a similar frequency of Tregs between HNSCC patients and healthy controls is in contrast to the majority of results reported by other cancer publications and previous HNSCC studies (Table 1.8) not all cancer studies report an elevated trend. Similar levels of Tregs were also reported in the peripheral circulation of healthy controls and patients with oral SCC (Gasparoto *et al.,* 2010), biochemically progressive and localised prostate cancer (Yokokawa *et al.,* 2008), renal cell carcinoma (Liotta *et al.,* 2010), gastric cancer (Mizukami et al., 2008a) and metastatic melanoma (Viguier *et al.*, 2004).

4.4.2.1 Methodological issues with Treg identification

As there is no single definitive marker for Tregs it is difficult to compare the level of Tregs directly between different cancer studies. Over recent years three main Treg

phenotypes have evolved to identify this suppressive T cell population using the extracellular markers CD4, CD25 and more recently the lack of expression of CD127 ($CD4^+CD25^+$, $CD4^+CD25^{high}$ and $CD4^+CD25^+CD127^{low/-}$). It was demonstrated in the current study that by using these different phenotypes varying levels of Tregs in the peripheral circulation were identified, adding complexity to any comparative analysis. The mean frequency of Tregs identified in the peripheral circulation of HNSCC patients, as a percentage of $CD4^+$ cells, varies between publications from 1.2 to 21% as different phenotypes are employed (Gasparoto *et al.*, 2010; Mandapathil *et al.*, 2009; Schott *et al.*, 2010; Strauss *et al*., 2007a; Wild *et al*., 2010).

The level of Tregs detected in the peripheral circulation of HNSCC patients and healthy controls for the current study was much higher when compared with other cancer publications. For example the $CD4^+CD25^+$ phenotype observed a four-fold increase, and approximately a two-fold increase was seen for Tregs identified by $CD4^+CD25^{high}$ and $CD4^+CD25^+CD127^{low/-}$ phenotypes in HNSCC patients, in comparison to the majority of other cancer studies. These differences are most likely to be due to the use of different gating strategies, which can vary greatly between research groups, particularly concerning the expression of CD25 as described below. However, sample variation, the clone of monoclonal antibodies used, the intensity of the fluorochrome conjugated to the antibody and the flow cytometer employed may all contribute to variation in the levels of Tregs being reported.

For the current study the mean percentage of $CD4^+$ cells expressing CD25 by healthy controls was 56.82% and Yokokawa and colleagues (2008), using the same gating strategy, also reported a high percentage of $CD4^+$ cells expressing the IL-2 receptor by a healthy control sample (67.7%). However, it is frequently quoted that 10-15% of $CD4^+$ cells express CD25 (Baecher-Allan *et al.*, 2001; Levings *et al.*, 2001), although published reports do vary with a range of 6-40% of $CD4^+$ cells expressing the IL-2 receptor (Dieckmann *et al.*, 2001; Ng *et al.*, 2001; Wing *et al.*, 2002). These discrepancies are likely to be due to the human $CD25^+$ population not being clearly discernible thereby causing variation in the identification of the $CD25^+$ population (Baecher-Allan *et al.*, 2001). In addition, the definition of high levels of CD25 varies, along with how the different populations of CD25 are classified. For example, Strauss and colleagues use a method specific to their research group, identifying $CD25^{high}$ Tregs as $CD4^+$ cells expressing the

IL-2 receptor with a mean fluorescence ≥120. The reason for this being that they observed that only cells above this cut off point had suppressive activity (Strauss *et al.*, 2007b). The current study separated the $CD25^{+}$ population by negative, intermediate and high levels of expression (Figure 4.12a), determining the high level of CD25 expression based on the $CD4^{-}$ population. This strategy was the same as that of Chi *et al*. (2010) who defined their CD25 populations as negative, low and high (Figure 4.12b); with the low expression of CD25 equivalent to the current study's CD25 intermediate population. However, Baecher-Allan *et al*. (2001) and Wing *et al.* (2002) used the same terminology of negative, low and high or $CD25^{-}$, $CD25^{+}$ and $CD25^{++}$, on populations of CD25 cells which had been gated very differently (Figure 4.12c and d) using gates which left gaps in between them, consequently disregarding some $CD25^{+}$ cells, and not clearly stating how the $CD25^{high}$ gate was even identified.

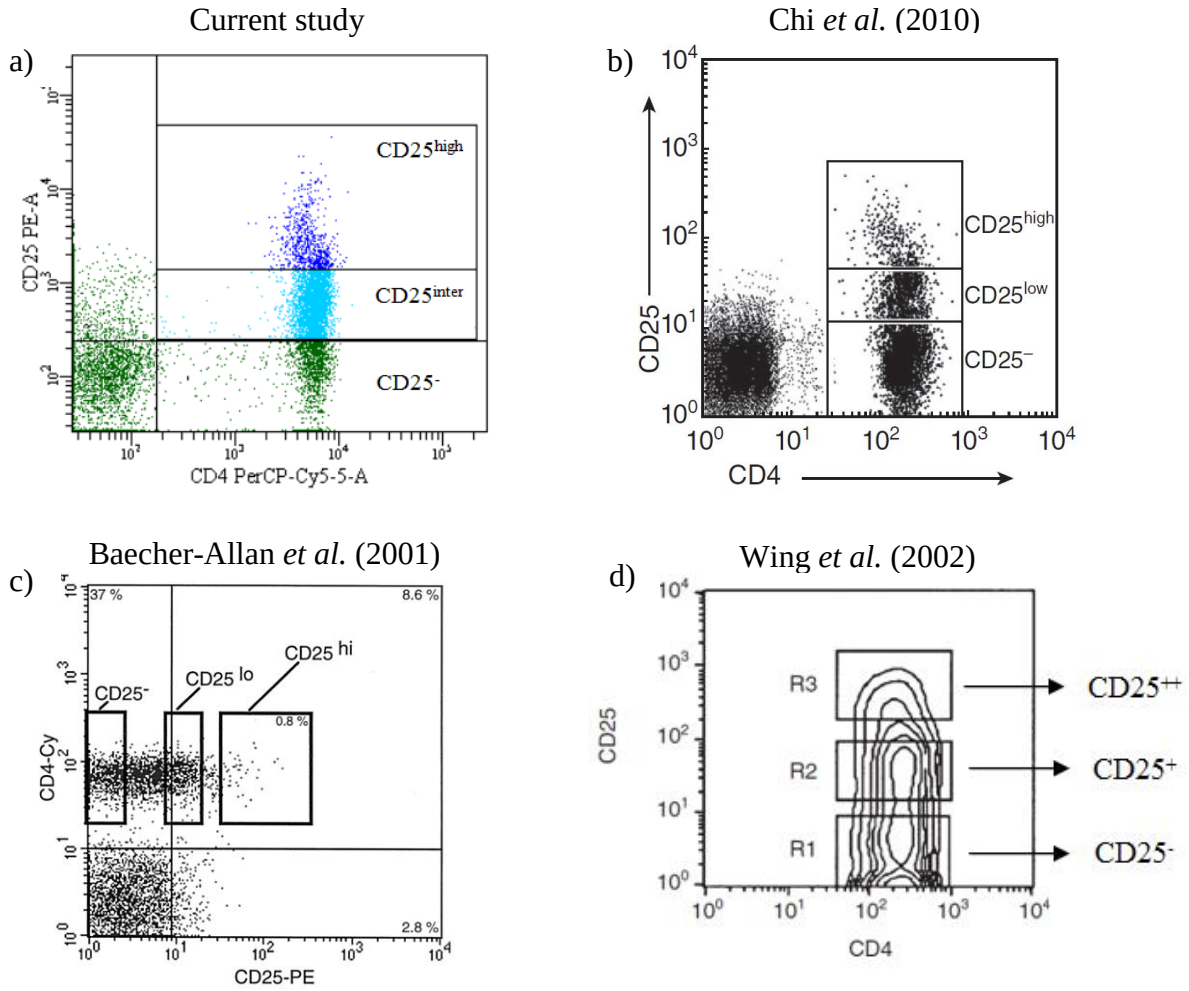


Figure 4.12 Different gating strategies used to identify $CD4^+$ T cells expressing varying levels of CD25

Following labelling of a PBMC sample with CD4 and CD25 antibodies and gating of the lymphocyte population, further gates were positioned to identify populations with different levels of CD25 (a) the current study applied a quadrant encompassing $CD4^-CD25^-$ cells in the lower left quadrant and identified $CD4^+CD25^{high}$ Tregs as $CD4^+$ cells expressing CD25 at levels exceeding that expressed by $CD4^-$ cells. Cells expressing intermediate levels of CD25 were above the $CD25^-$ quadrant marker but below the $CD25^{high}$ gate. (b) Chi *et al.* (2010) used the same gating strategy as the current study. (c) Baecher-Allan *et al.* (2001) identified the $CD4^+CD25^{negative}$, $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ populations using the indicated gates. (d) Wing *et al.* (2002) used a contour plot to identify $CD25^{++}$, $CD25^+$ and $CD25^-$ T cell populations.

Furthermore, although the lack of expression of CD127 has more recently become an identifier of Tregs, there is no agreed gating method for the identification of these suppressive T cells. Cells positive for CD127 provide a definitive population with many of the research groups using the same gating strategy (Shen *et al.*, 2009; Shenghui *et al.*, 2011;

Wild *et al*., 2010) however, by employing this method some $CD127^{low/-}$ Tregs have the same level of CD127 expression as the $CD127^{+}$ effector T cells (Figure 4.13; Shen *et al.*, 2009; Wild *et al.*, 2010). The gating strategy employed by the current study therefore used box gating to consistently distinguish the $CD127^{low/-}$ and $CD127^{+}$ populations (Figure 4.2c).

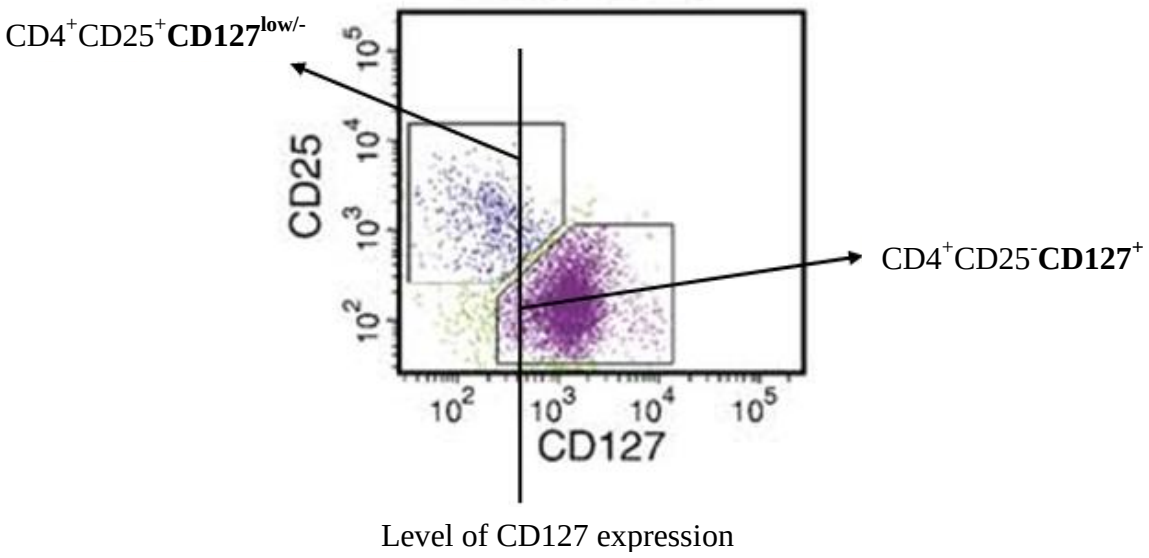


Figure 4.13 Identification and isolation of Tregs using the markers CD4, CD25 and CD127
Shen *et al.* (2009) have labelled a PBMC sample with CD3, CD4, CD25 and CD127 antibodies identifying a $CD4^{+}CD25^{+}CD127^{low/-}$ Treg population and a $CD4^{+}CD25^{-}CD127^{+}$ effector T cell population by flow cytometry. Some $CD127^{low/-}$ Tregs have the same level of CD127 expression as the $CD127^{+}$ effector T cells.

Regardless of the phenotype chosen to identify the Treg population, for some studies the gating strategy is not even shown or described (Boucek *et al.*, 2010; Liotta *et al.*, 2010; Schott *et al.*, 2010; Shen *et al.*, 2010), which makes it unclear how the Treg population was consistently identified between different PBMC samples. The comparison of Treg percentages in the peripheral circulation of cancer patients between studies, is complicated further by research groups reporting the level of Tregs as a percentage of different parameters, such as lymphocytes (Boucek *et al.*, 2010; Schaefer *et al.*, 2005), $CD4^{+}$ cells (Chi *et al.*, 2010; Gasparoto *et al*., 2010) or $CD4^{+}CD25^{+}$ cells (Schott *et al.*, 2010; Strauss *et al.*, 2007a). However, despite the methodological differences used to enumerate Tregs in different cancer types it is important that the role of Tregs in cancer and

the influence cancer has on the circulating Treg population is clarified, to determine new therapeutic targets and strategies to dampen the immunosuppressive environment created by the tumour and improve patient prognosis.

4.4.2.2 The frequency of Tregs with regard to clinicopathological characteristics

It is not surprising that comparison of results between studies looking at cancers arising from distinct locations in the body which differ in aetiology, prognosis and treatment strategies are not consistent as they are highly likely to vary in their influence on the Treg population. Such differences are exemplified well by Ichihara and colleagues (2003) who demonstrated that patients with cancer of the oesophagus had a significantly greater frequency of Tregs compared to patients with gastric cancer. Head and neck tumours arising from different subsites are frequently grouped together in research, but although united by location and histology the different primary sites vary in presentation, pathogenesis and survival, and may therefore also induce different effects on the Treg population. Few studies have compared the prevalence of Tregs between different HNSCC subsites, which may be due to the low number of patients recruited and/or the composition of the patient cohort; for example approximately 50% of the patients (n=18) recruited by Strauss *et al.* (2007a) had cancer of the oral cavity with the remaining patients having cancer arising from a further four different head and neck subsites, making reliable comparisons difficult. Chikamatsu and colleagues (2007) examined different HNSCC subsites, including cancer of the oral cavity (n=6), oropharynx (n=5), hypopharynx (n=11), larynx (n=17) and paranasal sinuses (n=3) and reported no association between the frequency of Tregs and tumour sites; the percentages for each subsite were not shown. However, a larger study (n=112) conducted by Boucek and colleagues (2010) did show that, although not significant, patients with oropharyngeal cancer from the tonsillar region (n=41) had slightly increased levels of $CD4^+CD25^+$ Tregs (16.2% of lymphocytes) in comparison to patients with cancer of the larynx (n=19; 15% of lymphocytes). This is in agreement with the current study which also observed a non-significant higher percentage of circulating Tregs ($CD4^+CD25^+$) in patients with oropharyngeal cancer in comparison to patients with malignancies arising in the larynx. Further studies with larger cohorts would

be needed to conclusively confirm whether tumours arising from different HNSCC primary locations can influence the levels of circulating Tregs.

In this work, the impact of the stage of the tumour on the prevalence of Tregs was also investigated and showed that HNSCC patients with advanced stage tumours had significantly increased levels of $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs in comparison to patients with early stage tumours; a trend shared by the $CD4^{+}CD25^{high}$ Treg phenotype with the difference approaching significance. Although investigated previously by studies of varying size (n=35-112), no significant differences have been reported by other head and neck studies for levels of Tregs in association with the stage of the tumour (Boucek *et al.,* 2010; Chikamatsu *et al.,* 2007; Strauss *et al.,* 2007a). However, patients with advanced stage tumours of other cancer types have been reported to harbour an increased frequency of circulating Tregs in comparison to patients with early stage tumours in gastric (Mizukami *et al.,* 2008b; Shen *et al.,* 2009), oesophageal (Kono *et al.,* 2006) and renal cancer (Liotta *et al.,* 2010). Patients with tumours that had spread to the lymph nodes had a significantly elevated level of $CD4^{+}CD25^{high}CD127^{low/-}$ and $CD4^{+}CD25^{+}CD127^{low/-}$ Tregs in comparison to patients with no nodal involvement and again the same trend was observed by $CD4^{+}CD25^{high}$ cells with the difference approaching significance; this is in agreement with results published for gastric cancer (Mizukami *et al.,* 2008b), colorectal cancer (Lin *et al*., 2012) and transitional cell carcinoma in the urinary system (Zhu *et al.,* 2009). In contrast, no significant difference was observed between the frequency of Tregs and nodal status in HNSCC (Lau *et al*., 2007; Strauss *et al.,* 2007a). The methodological differences highlighted previously may account for the observed differences however, the results of the current study suggest that increased tumour burden and metastatic spread may be associated with an increased frequency of Tregs in the peripheral circulation of HNSCC patients as the tumour continually promotes an immunosuppressive environment. It is unclear however, whether the presence of the regulatory population promotes the growth and spread of the tumour or whether these aspects cause an elevation in Treg frequency.

Head and neck cancer patients positive for the HPV, the majority of which arise from the oropharynx, are associated with better survival in comparison to patients negative for the expression of HPV (Section 1.5.2). A small recent study (n=12) observed no difference between the level of Tregs in the peripheral circulation of HNSCC patients positive or negative for the expression of HPV-16, however the number of patients in each

cohort was not reported (Heusinkveld *et al*., 2012). In contrast, patients with cervical dysplasia and a persistent HPV-16 infection (n=23) had a significantly increased frequency of circulating Tregs compared with patients negative for the expression of HPV-16 (n=24; (Molling *et al.,* 2007). HNSCC studies that have assessed the frequency of peripheral Tregs have not reported the HPV status of their patient cohort (Boucek *et al.,* 2010; Gasparoto *et al.,* 2010; Schott *et al.,* 2010; Strauss *et al.,* 2007a), and unfortunately the HPV status of the HNSCC patients recruited by the current study was not known so comparisons could not be made.

Comparing results amongst head and neck cancer studies is also difficult due to the patient recruitment strategies employed by different research groups. The current study only recruited newly-presenting patients that had received no previous diagnosis or treatment for cancer, with no active autoimmune or co-existing infectious disease; thereby enabling the direct influence of the head and neck tumour on the Treg population to be assessed. In contrast, several head and neck cancer studies have recruited patients that had received medical intervention (Strauss *et al.,* 2007b), had a recurrence (Schott *et al.,* 2010; Strauss *et al.,* 2007a) and/or were displaying no evidence of disease after curative therapies (Schaefer *et al.,* 2005; Schott *et al.,* 2010; Strauss *et al.,* 2007a), whilst for other studies it was difficult to determine the exact patient recruitment criteria (Gasparoto *et al.,* 2010). Strauss and colleagues (2007a) reported that HNSCC patients with no evidence of disease (NED; n=20) had a significantly higher percentage of Tregs in comparison to patients with active disease (AD; n=15). Further publications have also presented data illustrating the continued elevation of Tregs in some HNSCC patients after curative interventions (Schott *et al.,* 2010; Schuler *et al.,* 2011). Conversely, Schaefer *et al.* (2005) showed that patients with AD (n=8) and NED (n=11) had similar frequencies of Tregs, this may be due to the size of the study or that different curative therapies exert different long term effects on the Treg population. These results highlight the fact that, when comparing results with different patient recruitment criteria, it is important to consider possible effects of previous therapies as these may have a long term influence on the immune system (Lissoni *et al.,* 2009).

Although the current study observed no differences in the frequency of circulating Tregs between HNSCC patients and healthy controls or HNSCC subsites, the stage and nodal status of the head and neck tumour was shown to influence the level of the peripheral

Tregs. The mechanisms driving the expansion of Tregs in cancer patients are not fully understood, although the cause has been attributed to tumour secretions influencing recruitment of Tregs into the tumour, proliferation, and induction of Tregs from naïve precursors, as shown in both murine and human cancer studies (Colombo and Piconese, 2007; Curti *et al.,* 2007; Knutson *et al.,* 2007). In addition to dampening the action of tumour specific effector T cells, Tregs may also suppress tumour specific immunity by blocking the generation of immune responses where tumour antigens are present, for example in the lymph nodes. Elevated levels of Tregs in the tumour draining lymph nodes of cervical (Fattorossi *et al.,* 2004) and gastric cancer (Kawaida *et al.,* 2005) patients have been observed and it is suggested that an increase in the prevalence of Tregs in the peripheral circulation may be partially attributed to the convergence, and subsequent 'spillover', of lymphatics into blood vessels (Knutson *et al.,* 2007).

4.4.3 Prevalence of other T cell subsets in the peripheral circulation of healthy controls and HNSCC patients

For the HNSCC patient cohort, as a whole, the frequency of $CD4^+CD25^{-/+}CD127^{-/+}$ effector cells (combination of $CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$ effector T cell populations), was similar between healthy controls and HNSCC patients. However, patients without nodal involvement and early laryngeal tumours had a significantly higher level of these cells compared to those with nodal involvement and advanced stage tumours of the larynx, respectively. In addition, separating the $CD4^+CD25^{-/+}CD127^{-/+}$ cells in to the two effector T cell populations ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$), revealed that the HNSCC patient cohorts had a higher percentage of activated $CD4^+CD25^+CD127^+$ T cells compared with the $CD4^+CD25^-CD127^{-/+}$ effector cells, although, significance was only reached for the patient cohort with nodal involvement. Other studies have assessed the frequency of Tregs in the peripheral circulation, however, it appears that the level of specific effector T cell populations are not examined but instead more general lymphocyte subpopulations are analysed, such as $CD3^+$, $CD4^+$ and $CD8^+$ cells (Boucek *et al.,* 2010; Chikamatsu *et al.,* 2007; Lau *et al.,* 2007). Nevertheless, an increase in the frequency of effector T cells in HNSCC patient subgroups could suggest a T cell population attempting

to combat the growth of the tumour, although effectiveness would be determined by the strength of opposing immunosuppressive mechanisms employed by the tumour, including their susceptibility to the activity of Tregs, discussed below.

4.4.4 Assessing the expression of Foxp3 by T cell subsets in HNSCC patients and healthy controls

The forkheadbox transcription factor Foxp3 has been employed by many research groups to confirm and analyse the prevalence of Tregs (Table 1.8). Although the expression of Foxp3 is not solely restricted to the Treg population, the transcription factor is regarded to be one of the most specific Treg markers identified to date (Section 1.12).

The expression of Foxp3 by the Treg phenotypes, $CD4^{+}CD25^{+}$, $CD4^{+}CD25^{high}$, $CD4^{+}CD25^{+}CD127^{low/-}$, $CD4^{+}CD25^{inter}CD127^{low/-}$ and $CD4^{+}CD25^{high}CD127^{low/-}$, illustrated that in combination with CD25 expression, the $CD127^{low/-}$ Treg phenotypes expressed some of the highest levels of Foxp3, in particular $CD4^{+}CD25^{high}CD127^{low/-}$ cells; helping to validate the use of the markers CD4, CD25 and CD127 to identify, isolate and assess the suppressive activity of Tregs. Additionally, as expected, the effector T cell populations ($CD4^{+}CD25^{inter}$, $CD4^{+}CD25^{-}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$) expressed the lowest levels of the transcription factor. For each Treg phenotype the HNSCC patients consistently expressed a higher percentage of Foxp3 in comparison with healthy controls, which is in accordance with several head and neck cancer publications (Lau *et al.,* 2007; Mandapathil *et al.,* 2009; Schott *et al.,* 2010; Strauss *et al.,* 2007a) and non-small cell lung cancer (Ju *et al.,* 2009). In contrast, similar percentages of Foxp3 expression were observed in gastric cancer patients compared with healthy controls (Shen *et al.,* 2009).

The current study observed a positive association between the expression levels of CD25 and Foxp3 for both healthy controls and HNSCC patients with the expression of the transcription factor being over four-fold lower for cells positive for the expression of CD25 ($CD4^{+}CD25^{+}$, mean 7.8%) compared with the subset of cells expressing high levels of the IL-2 receptor ($CD4^{+}CD25^{high}$; mean 34%) in patients. This is in agreement with previous publications for gastric cancer patients (Shen *et al.,* 2009) and healthy controls (Liu *et al.,* 2006; Shen *et al.,* 2009; Yokokawa *et al.,* 2008) which all reported an increase in the

percentage of Foxp3 expression as the level of CD25, expressed by $CD4^+$ cells increased, although the percentage of cells expressing Foxp3 does vary between studies.

4.4.5 The suppressive activity of Tregs in the peripheral circulation of HNSCC patients and healthy controls

Studies reporting an elevation in the frequency of Tregs in the peripheral circulation of cancer patients would propose that this increase is partly responsible for the suppression of the host's anti-tumour response. Although this may well be the case, it is as important, if not more so, to assess the functional activity of these Tregs, as it is their suppressive capacity, not just their presence, that will determine how effective the host's anti-tumour attack is in combating the growth and progression of the tumour.

The functional activity of Tregs is commonly measured by the level of suppression induced on the proliferation of effector T cells at different Treg:effector T cell ratios. The current study assessed the level of suppression induced at the ratios 1:1. 1:2, 1:5 and 1:10 and in agreement with previous publications (Ichihara *et al.,* 2003; Mandapathil *et al.,* 2009; Shen *et al.,* 2010), the proliferation of effector T cells ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) was inhibited in the presence of Tregs ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) in a ratio dependent manner. Although the choice of ratios varies between studies the 1:1 ratio has always been employed, therefore in accordance with this, all suppression experiments in the current study were performed at the 1:1 ratio, and the results from these experiments were used for comparison.

Although the identification of Tregs by the phenotype $CD4^+CD25^+$ results in contamination with activated effector T cells, research groups continue to use this phenotype to isolate cells before assessing their functional activity (Table 4.1). $CD4^+CD25^+$ Tregs isolated from patients with renal cell carcinoma (Liotta *et al.,* 2010), hepatocellular carcinoma (Shen *et al.,* 2010), gastric cancer (Ichihara *et al.,* 2003) and colorectal cancer (Ling *et al*., 2007) have all been shown to have the capacity to suppress the proliferation of effector T cells, although the level of suppression was not compared with healthy controls. The current study identified and isolated Tregs using the more specific phenotype of low/negative CD127 expression, which is inversely correlated to the expression of Foxp3, as demonstrated by Liu *et al*. (2006) and Seddiki *et al*. (2006). To the

author's knowledge, this is the first investigation to assess the level of suppression induced by $CD127^{low/-}$ Tregs in newly-presenting HNSCC patients compared with healthy controls and also between tumour stage, subsite and nodal status. Two effector T cell populations ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) were identified, isolated and used to examine their susceptibility to Treg induced suppression. It has been reported that, in contrast to murine studies, human activated $CD4^+$ effector T cells can gain Foxp3 expression (Section 1.12). However, it was shown by this study that $CD4^+$ T cells isolated by the $CD127^{low/-}$ phenotype, regardless of CD25 expression, have the ability to suppress the proliferation of both effector T cell populations, confirming that sorting cells on the basis of low expression of CD127 does isolate a suppressive T cell population, from healthy controls and HNSCC patients.

It was observed that Tregs ($CD4^+CD25^{high}CD127^{low/-}$), isolated from HNSCC patients, induced a significantly greater level of suppression compared with healthy controls. This is in agreement with previous cancer studies, including head and neck cancer (Table 4.1), where it has been shown that $CD4^+CD25^+$ (n=9, Gasparoto *et al.,* 2010; n=5, Lau *et al*., 2007; n=3, Mandapathil *et al.*, 2009) and $CD4^+CD25^{high}$ Tregs (n=3, Mandapathil *et al*., 2009; n=20, Strauss *et al*., 2007a; n=10, Strauss *et al*., 2007b) isolated from HNSCC patients induced a significantly higher level of suppression compared with Tregs isolated from healthy controls. It could be suggested that the increased suppressive activity seen in Tregs from HNSCC patients may be due to their elevated percentage of expression of Foxp3 compared with that in Tregs from healthy controls as described earlier. The results however are in contrast to those reported by Chi *et al.* (2010) who reported similar percentages of suppression in bladder carcinoma patients (n=10) and healthy controls (n=10). In the current study, $CD4^+CD25^{inter}CD127^{low/-}$ Tregs from HNSCC patients and healthy controls also induced similar percentages of suppression on the proliferation of $CD4^+CD25^+CD127^+$ effector T cells, suggesting that it depends on the Treg and effector T cell populations being investigated.

Although a similar pattern in suppression is mediated by $CD127^{low/-}$ Tregs in this work, compared to other HNSCC studies the level of suppression observed was approximately three fold lower. For example, the percentage of suppression induced by $CD4^+CD25^{high}$ Tregs is reported at over 70% by other HNSCC publications (Mandapathil *et al.*, 2009; Strauss *et al.,* 2007a) whereas here it was determined to be 19-31%. In

contrast, comparing the mean percentage of suppression observed in healthy controls, suppression induced by $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs (11.43%) was similar to that reported by Strauss and colleagues (2007) by $CD4^{+}CD25^{high}$ Tregs (12%). In prostate cancer, for the same Treg ($CD4^{+}CD25^{high}$) and effector T cell ($CD4^{+}CD25^{-}$) phenotypes the percentage of suppression varied from 42 to 65% depending on the type of malignancy (biochemically progressive, localised or metastatic), and healthy controls induced a mean suppression of 31% (Yokokawa *et al.,* 2008). The difference in suppression levels between studies may again be attributed to different tumour sites investigated, but is also likely to be due methodological differences discussed previously, along with the methods employed to determine the percentage of suppression. The methods and analysis used to determine the percentage of suppression does vary greatly between studies, for example the level of proliferation of effector T cells can be determined either through the CFSE assay (Shen *et al.,* 2009; Shenghui *et al.,* 2011; Strauss *et al.,* 2007a) or [^{3}H] thymidine incorporation (Liotta *et al.,* 2010; Shen *et al.,* 2010; Yokokawa *et al.,* 2008). The length of Treg and effector T cell co-culture incubation varies from 3 (Shen *et al.,* 2010) to 7 days (Shen *et al.,* 2009) and some studies add IL-2 to the co-culture (Shen *et al.,* 2009; Strauss *et al.,* 2007a) whilst others do not (Shenghui *et al.,* 2011; Yokokawa *et al.,* 2008). In addition, although the majority of studies provide stimulation through the use of CD3 and CD28 antibodies these can either be plate bound (Chi *et al.,* 2010; Shen *et al.,* 2009) or soluble (Strauss *et al.,* 2007a). For analysis, some research groups, like the current study, calculate the percentage of suppression (Mandapathil *et al.,* 2009; Strauss *et al.,* 2007a; Yokokawa *et al.,* 2008) whilst others examine the levels of proliferation instead (Lau *et al.,* 2007; Shen *et al.,* 2009; Shen *et al.,* 2010).

4.4.5.1 The suppressive activity of Tregs with regard to clinicopathological characteristics

Few studies have examined the degree of suppression in relation to different clinicopathological parameters in the peripheral circulation of cancer patients; mainly due to their limited cohort size, n≤10 (Gasparoto *et al.,* 2010; Ichihara *et al.,* 2003; Lau *et al.,* 2007; Liotta *et al.,* 2010; Mandapathil *et al.,* 2009; Shenghui *et al.,* 2011; Strauss *et al.,* 2007b). The current study is one of the largest investigations to assess the suppressive

activity of Tregs in cancer patients (n=28), consequently, it was possible to examine the influence of tumour subsite, stage and nodal status on the suppressive activity of Tregs. No significant differences in the level of suppression between laryngeal and oropharyngeal HNSCC subsites or between early and advanced tumour stages for both $CD25^{inter}$ and $CD25^{high}$ Tregs were observed, irrespective of the effector T cell population being suppressed. Although no statistical differences were observed, trends were shown where patients with early stage oropharyngeal cancer may be more suppressive than those with advanced stage cancer of the oropharynx whereas the opposite was noted for the laryngeal cohort; the contradictory nature of these differences would nullify any HNSCC subsite and tumour stage effects. Supporting this idea, $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs isolated from patients with early stage oropharyngeal and advanced stage laryngeal cancer exerted significantly higher levels of suppression on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells compared with healthy controls. It may therefore be suggested that the role of Tregs is important at different stages of tumour development depending on the HNSCC subsite.

The results of the current study are in agreement with Strauss *et al.* (2007a) who also observed no significant association between the level of suppression and the stage of the head and neck tumour (n=20). In hepatocellular carcinoma Shen *et al.* (2010) also found no significant differences in the level of suppression between patients with early (n=8) or advanced (n=6) stage tumours. In contrast, Treg suppression was reported to be enhanced in advanced stage gastric cancer patients, but no statistics were presented and the number of patients assessed for suppressive Treg activity was unclear (Shen *et al.*, 2009). Yokokawa *et al.* (2008) showed similar results in prostate cancer.

A significant association between the level of suppression and nodal status was observed; $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs isolated from patients with tumours that had spread to the lymph nodes suppressed the proliferation of $CD4^{+}CD25^{+}CD127^{+}$ effector cells to a greater degree compared to patients without nodal involvement. It may therefore be suggested that an elevated level of suppression in the peripheral circulation of HNSCC patients is associated with metastatic spread and establishment of the tumour in the lymph nodes. It is unclear however, whether the presence of the regulatory population promotes the growth and spread of the tumour or whether nodal involvement causes an increase in Treg functional activity. It should be noted, that a large proportion of the HNSCC patient

cohort had tumours that had spread to the lymph nodes and increasing the number of patients without nodal involvement would help strengthen the association between nodal status and the level of suppression induced. These results are in contrast to the report by Strauss *et al.* (2007a) who showed no significant association between nodal status and the level of suppression in HNSCC, however the number and composition of the patient cohort analysed was unclear.

4.4.5.2 Comparison of the phenotype and suppressive activities of Tregs with intermediate and high expression of CD25

In attempts to refine the identification of a Treg population in humans it has been demonstrated that cells expressing high levels of the IL-2 receptor ($CD4^{+}CD25^{high}$) have the capacity to inhibit the proliferation of effector T cells, whereas cells expressing intermediate/low levels of CD25 did not possess this suppressive activity (Baecher-Allan *et al.*, 2001; Cesana *et al.*, 2006). It was therefore of interest whether the level of CD25 expression on more precisely-defined $CD127^{low/-}$ Tregs would influence the percentage of suppression induced by Tregs from healthy controls and HNSCC patients. To the author's knowledge assessing suppressive activity by level of CD25 expression on $CD127^{low/-}$ Tregs has not been previously examined in cancer patients.

Transposing, by colour, the T cells identified and isolated by the current study on to a CD4 vs CD25 dot plot, it became apparent that the cells classified solely by their intermediate level of expression of the IL-2 receptor have a substantial contamination of activated T cells ($CD4^{+}CD25^{+}CD127^{+}$) with relatively few $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs; therefore studies which have assessed $CD4^{+}CD25^{inter}$ cells for Treg function would be unlikely to have demonstrated suppressive activity (Baecher-Allan *et al.*, 2001; Cesana *et al.*, 2006), this has also been suggested by Seddiki *et al.* (2006). Furthermore, this plot highlighted that Tregs express a lower level of CD4 in comparison to the effector T cell populations, an observation also made by Hoffmann and colleagues (2006b).

The $CD4^{+}CD25^{inter}CD127^{low/-}$ Treg population consistently induced a greater level of suppression compared to $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs, reaching significance for healthy controls and a number of different HNSCC patient cohorts on both $CD4^{+}CD25^{-}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$ effector T cells at the 1:1 ratio. Although

not previously assessed in cancer patients, the level of suppression induced by $CD127^{low/-}$ Tregs separated by CD25 expression has been examined in healthy controls. Even though it was not stated how the levels of CD25 expression were determined and some $CD127^{low}$ T cells have been excluded due to gaps in the gating (Figure 4.14a), Seddiki *et al.* (2006) demonstrated that $CD127^{low}$ T cells expressing high and intermediate/low levels of CD25 both had the capacity to suppress the proliferation of effector T cells to a similar extent, as analysed by ^{3}H thymidine incorporation (Figure 4.14b). Although this is in contrast to the current study's finding, the trend reported by Seddiki and colleagues has also been supported in healthy controls by Lui *et al*. (2006; n=9) and Hartigan-O'Connor *et al.* (2007; n=4).

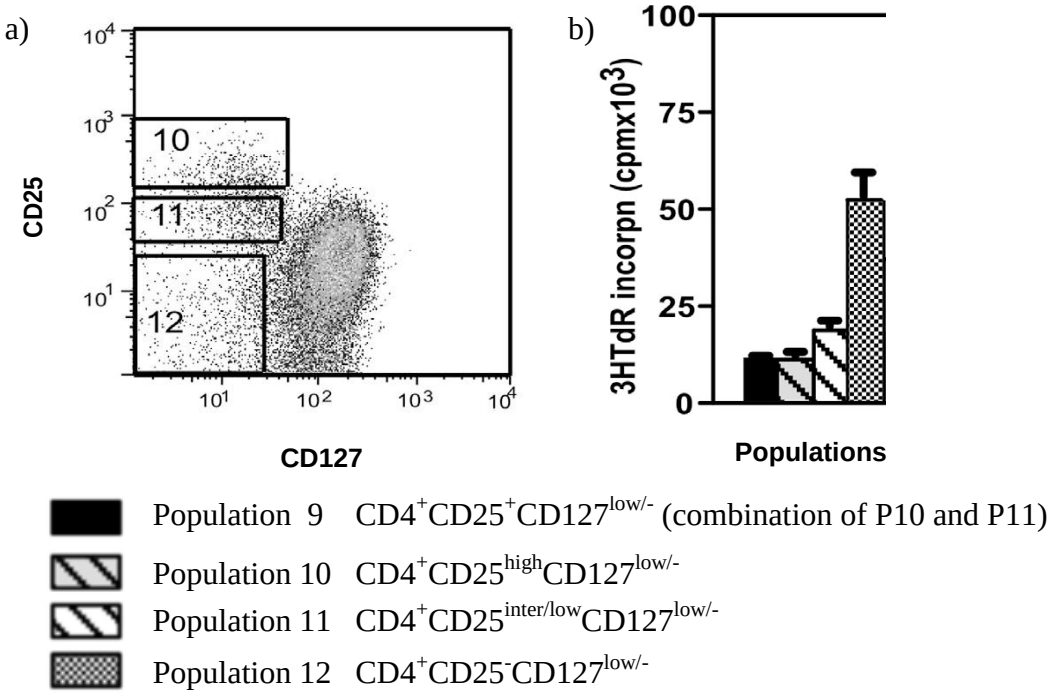


Figure 4.14 Level of suppression induced by $CD25^{high}$ and $CD25^{inter/low}$ $CD127^{low}$ Tregs on the proliferation of effector T cells
(a) Sorting strategy employed by Seddiki *et al.* (2006) to isolate different $CD4^{+}$ T cell subsets based on the expression of CD25 and $CD127^{low}$. (b) Following isolation of the populations and co-culture with effector T cells ($CD4^{+}CD25^{-}$) at a 1:1 ratio, ^{3}H-thymidine was added to the co-cultures before harvesting 16 hours later to determine ^{3}H-thymidine incorporation.

The current study has highlighted how distinct populations of cells, identified on the basis of expression levels of surface markers, show significantly different biological

effects, however, these cell populations should not be considered as static entities. For instance Hartigan-O'Connor and colleagues (2007) suggested that the $CD25^{inter}CD127^{low/-}$ cells may contain precursors to fully activated $CD25^{high}CD127^{low/-}$ Tregs, and demonstrated during 64 hours of stimulation that the $CD25^{inter}CD127^{low/-}$ cells up regulated the expression of CD25 and Foxp3, coupled with down regulation of CD127; unfortunately the same procedure was not conducted on the $CD25^{high}CD127^{low/-}$ population. Thus it is conceivable that the Treg populations could develop during assay incubation periods and acquire or lose functional capabilities.

The percentage of Foxp3 expression by $CD25^{inter}CD127^{low/-}$ Tregs was found to be lower than in the $CD25^{high}CD127^{low/-}$ population, however, it would be of interest to determine whether over the 96 hours of co-culture, during the CFSE assay, the $CD25^{inter}CD127^{low/-}$ Tregs up regulate their expression of CD25 and Foxp3 and whether the level of Foxp3 expression exceeds that in the $CD25^{high}CD127^{low/-}$ cells, thereby correlating to the higher level of suppression observed. Unfortunately time restraints did not allow for these experiments to be conducted. If the $CD4^{+}CD25^{inter}CD127^{low/-}$ phenotype identifies a population of cells with the potential to become highly suppressive in HNSCC patients, the prevalence of this population would provide a useful prognostic marker. A higher frequency of $CD25^{inter}CD127^{low/-}$ Tregs could lead to elevated suppression of the host's anti tumour response and would allow continued growth and progression of the tumour. In contrast increased levels of Tregs have been associated with increased survival for some cancer types (Section 1.13); conducting a retrospective study would indicate whether the $CD4^{+}CD25^{inter}CD127^{low/-}$ Treg population has the potential to become a prognostic marker in predicting the chance of recurrence and overall survival in HNSCC.

4.4.5.3 Comparison of effector T cells susceptibility to Treg induced suppression

The level of suppression induced by Tregs on the two different effector T cell populations isolated was investigated. In the HNSCC patient cohorts, $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells were found to be more susceptible to the suppressive activity of both $CD25^{inter}$ and $CD25^{high}$ Tregs in comparison to the $CD4^{+}CD25^{+}CD127^{+}$ population, however this only reached significance for patients with oropharyngeal cancer. It would therefore be interesting to assess, in particular for oropharyngeal cancer patients, whether a

higher proportion of activated $CD4^{+}CD25^{+}CD127^{+}$ cells, that are attempting to combat the growth of the tumour and are less susceptible to the suppressive actions of Tregs, are associated with improved patient prognosis.

4.4.6 Conclusion

The difficulties in directly comparing the frequency and level of suppression induced by Tregs between cancer studies due to methodological differences have been highlighted. However, despite these differences, it is still important that within studies comparisons are made between cancer patients and healthy controls and different clinicopathological features to clarify the role of Tregs in specific cancer types. This will help to find new therapeutic targets and establish whether circulating Tregs can be used to determine patient prognosis. The current study has investigated and compared the differences surrounding the frequency and suppressive function of Tregs in the peripheral circulation of healthy controls and HNSCC patients and the influence of tumour stage, subsite and nodal status.

The level of Tregs was similar between HNSCC patients and healthy controls however, $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs from HNSCC patients exerted a significantly greater level of suppression in comparison with the healthy controls. In contrast, tumour stage was not observed to influence the suppressive activity of Tregs however, a higher level of $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs was demonstrated in patients with advanced stage tumours; this suggests that although similar in functional capacity, a greater level of suppression may have been induced in patients with advanced stage tumours compared with early stage tumours due to the increased frequency of the suppressive T cell population. No significant differences were observed for frequency or levels of suppression between tumour subsites, whilst patients with nodal involvement were shown to harbour an increased prevalence of $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs that also had greater suppressive activity, compared to patients with no nodal involvement. The current study has therefore highlighted the importance of assessing both the frequency and functional capacity of Tregs in HNSCC patients which need to be precisely defined in terms of their clinicopathology. At present, either or both of these parameters could be used to provide an indication of patient prognosis.

Chapter 5.

Phenotype, frequency and suppressive activity of T cell subsets in the tumour microenvironment of HNSCC patients

5.1 Introduction

Whilst investigations in to the peripheral circulation of cancer patients offer an indication of the effects the tumour is exerting upon the host, direct examination of the tumour microenvironment provides a detailed insight into the localised anti-tumour immune responses mediated by the host and the immune evasion strategies influenced by the tumour. The tumour microenvironment consists of a complex network of epithelial cells, stroma, chemical secretions, vascular and lymphatic vessels, and a plethora of infiltrating immune cells (Fridman *et al.*, 2012), whose actions range from promotion to inhibition of tumour growth and progression. It is thought to be the balance of the types of immune cells present which contribute to the overall immune response and thus patient outcome (Simonson and Allison, 2011). To further understand the head and neck tumour microenvironment, this study has investigated the prevalence of different $CD4^+$ T cell populations and the suppressive activity of Tregs infiltrating HNSCC in relation to the peripheral circulation and tumour subsite.

To suppress tumour growth and development the host's anti-tumour attack is driven predominantly by two T lymphocyte subsets, IFN-γ producing $CD4^+$ helper cells and cytotoxic $CD8^+$ T cells, with the role of inhibiting and eradicating tumour cells thus promoting tumour reduction (Zamarron and Chen, 2011). Publications have reported the presence of tumour specific T cells in several cancer types (Andersen *et al.*, 2005; Junker *et al.*, 2012; Nagorsen *et al.*, 2005; Novellino *et al.*, 2005), particularly in melanoma patients where research is focusing on their use in adoptive T cell therapy (Besser *et al.*, 2010; Dudley *et al.*, 2008; Restifo *et al.*, 2012). However, these anti-tumour lymphocyte subsets are challenged with numerous evasion strategies thought to be orchestrated at least in part by the tumour itself. For example in HNSCC both circulating and tumour infiltrating T cells have been shown to be functionally impaired and more susceptible to apoptosis, consequently compromising the host's anti-tumour response (Hoffmann *et al*., 2002; Kim *et al*., 2004; Reichert *et al.*, 2002).

$CD4^+$ effector T cells secreting IFN-γ and tumour necrosis factor (TNF) are regarded as T-helper 1 cells (Th1) that favour the host by promoting the actions of cell-mediated immunity [$CD8^+$ T cells, NK cells, antigen presenting cells] and consequently tumour destruction. However, depending upon the conditions, $CD4^+$ effector T cells can

also differentiate into T-helper 2 cells (Th2) which favour a more humoral antibody mediated immune response and are characterised by the secretion of IL-4, IL-6 and IL-10. These cytokines have been associated with immune suppression and inhibition of tumour cell apoptosis (Pries and Wollenberg, 2006; Simonson and Allison, 2011) and a partial bias towards a Th2 cytokine profile has been reported in the plasma of HNSCC patients (Lathers and Young, 2004), which would logically favour tumour growth. In addition, the infiltration of $CD4^+$ Tregs into the tumour is also thought to promote an immunosuppressive environment, with the ability to suppress T cell activation, proliferation and function (Shevach, 2009). In numerous cancer types, including HNSCC, the proportion of Tregs in the infiltrating lymphocyte population has been shown to be increased compared with non-malignant tissue and the peripheral circulation of patients (see below), however their relationship to patient prognosis is not entirely clear. A further $CD4^+$ effector T cell subset secreting the cytokine IL-17 has recently been discovered, T-helper 17 cells (Th17). However, their role in cancer is currently not fully understood as Th17 cells have been shown to reduce tumour cell proliferation but also up regulate angiogenesis-promoting factors in HNSCC (Kesselring *et al.*, 2010); which effect will predominate is unclear.

T lymphocytes are not the only immune cells that infiltrate the tumour microenvironment, NK cells also aid the anti-tumour immune response by targeting tumour cells that have down regulated the expression of MHC class I molecules in attempts to prevent host immune recognition (Simonson and Allison, 2011). Macrophages too are recruited to the cancerous mass by the secretion of a number of chemokines by tumour cells. Similar to the $CD4^+$ Th1/Th2 division, macrophages can also be divided by their functional characteristics with M1 macrophages assisting the host's anti-tumour response and M2 macrophages promoting tumour growth and the creation of an immunosuppressive environment (Duray *et al.*, 2010).

The tumour is also thought to suppress anti-tumour immune responses by directly secreting immunosuppressive factors such as TGF-β and IL-10, which act to inhibit anti-tumour immune actions, encourage tumour cell survival and assist in the generation of Tregs (Zamarron and Chen, 2011). The secretion of vascular endothelial growth factor (VEGF) and TGF-β can also stimulate MDSC which possess a wide range of actions that promote tumour cell invasion and angiogenesis as well as influencing the actions of

immune cells by driving $CD4^+$ Th2 and Treg differentiation, suppressing T cell proliferation, function and inducing T cell apoptosis (Tong *et al.*, 2012). It is therefore the balance of all these immune cells and their secretions, immune attack and suppression, that influence the success or failure of an anti-tumour response.

The infiltration of lymphocytes into the tumour microenvironment has been assessed by many research groups in several different cancer types, including HNSCC, in attempts to predict patient prognosis (Distel *et al.*, 2009; Grabenbauer *et al.*, 2006; Pretscher *et al.*, 2009; Sato *et al.*, 2005; Zingg *et al.*, 2010), however there are conflicting reports over whether the presence of tumour infiltrating lymphocytes (TIL) are associated with positive or negative prognosis, or of no relevance at all. Additionally, dividing and assessing the infiltrating lymphocyte population by subset still results in conflicting reports for different cancer types, with the presence of Tregs found to be both positively and negatively prognostic in various cancer types (Table 1.9).

The majority of studies assessing the infiltration of Tregs into the tumour use immunohistochemical detection of the most specific Treg marker found to date, Foxp3 (Kobayashi *et al.*, 2007; Liu *et al.*, 2012; Salama *et al.*, 2009). The same method was used to confirm the presence of infiltrating Tregs in the HNSCC samples investigated in the current study. However, as discussed in Chapter 4, identification of Tregs confirms the presence of a suppressive T cell subset but does not assess their functional activity.

Studies identifying Tregs in isolated TIL have shown that the proportion of Tregs in the lymphocyte population is elevated compared with non-malignant tissue (Table 5.1) and the peripheral circulation (Table 5.2).

Table 5.1 Representative studies demonstrating increased prevalence of Tregs infiltrating malignant tissue compared with non-malignant tissue from cancer patients

Cancer Type	Treg phenotype	Mean percentage of Tregs in malignant tissue	Mean percentage of Tregs in non-malignant tissue	Reference
Colorectal	$CD4^+Foxp3^+$	10.4	4.7	Lin *et al.* (2012)
Oesophageal	$CD4^+CD25^+Foxp3^+$	24.6	7.1	Maruyama *et al.* (2010a)
Hepatocellular	$CD4^+CD25^{high}$	8.5	5.5	Shen *et al.* (2010)
Hepatocellular	$CD4^+CD25^+Foxp3^+$	14.2	5.3	Zhou *et al.* (2009)
Gastric	$CD4^+CD25^+Foxp3^+$	12.4	4.1	Mizukami *et al.* (2008a)
Colorectal	$CD4^+CD25^+$	15.2	4.8	Ling *et al.* (2007)

Table 5.2 Representative studies demonstrating increased prevalence of Tregs infiltrating malignant tissue compared with the peripheral circulation of cancer patients

Cancer Type	Treg phenotype	Mean percentage of Tregs in malignant tissue	Mean percentage of Tregs in the peripheral circulation	Reference
Hepatocellular	$CD4^+CD25^{high}Foxp3^+$	2.6	2.0	Feng *et al.* (2011)
Bladder	$CD4^+CD25^{high}$	15.4	8.7	Chi *et al.* (2010)
Renal	$CD4^+CD25^+Foxp3^+$	6.5	3.5	Liotta *et al.* (2010)
Renal	$CD4^+CD25^{high}Foxp3^+$	2.6	0.4	Attig *et al.* (2009)
Breast	$CD4^+CD25^{high}$	27.9	6.12	Gobert *et al.* (2009)
Gastric	$CD4^+CD25^+CD127^{low/-}$	21.08	7.77	Shen *et al.* (2009)
HNSCC	$CD4^+CD25^+$	13	3	Strauss *et al.* (2007b)
HNSCC	$CD4^+CD25^{high}$	3	1	Strauss *et al.* (2007b)

For the studies that have proceeded to isolate the infiltrating regulatory population, it has been reported that these Tregs are functionally suppressive in HNSCC, (Strauss *et al.*, 2007b), bladder (Chi *et al.*, 2010), ovarian (Curiel *et al.*, 2004), breast (Gobert *et al*., 2009), colon (Kryczek *et al*., 2009), renal (Liotta *et al.*, 2010), lung (Woo *et al.*, 2002), colorectal (Ling *et al*., 2007) and hepatocellular (Zhou *et al*., 2009) cancer. However, there are only limited investigations which have determined whether the presence of tumour both elevates the percentage of Tregs infiltrating the cancerous mass and influences Treg suppressive capacity, by comparing the suppressive activity of the circulating Treg population and that of the infiltrating population (Chi *et al.*, 2010; Strauss *et al.*, 2007b). In addition, when comparisons have been made between the peripheral circulation and tumour microenvironment, for either phenotype or functional activity of Tregs, the two sample cohorts have not always consisted of the same patients (Gobert *et al.*, 2009; Strauss *et al.*, 2007b; Woo *et al*., 2001). Therefore, to further elucidate the role of tumour Tregs in head and neck cancer, the current investigation first identified the presence of Tregs within HNSCC from different tumour subsites using Foxp3 immunohistochemistry. The frequency and suppressive activity of $CD127^{low/-}$ Tregs from the tumour microenvironment were subsequently examined and compared with Tregs from the patient's corresponding PBMC samples; ensuring that clinicopathological parameters that could influence the Treg population, such as tumour stage, subsite and nodal status, remained constant. However, it was considered that the microenvironment of the tumour may influence the infiltrating effector T cells susceptibility to be suppressed *in vitro* due to having already been suppressed by Tregs or numerous other immunosuppressive mechanisms *in vivo*, consequently masking the full functional capacity of tumour infiltrating Tregs. To assess this, tumour infiltrating Tregs were co-cultured with autologous effector T cells from both the tumour and peripheral circulation.

To the author's knowledge, tumour infiltrating Tregs with the $CD127^{low/-}$ phenotype have not previously been identified, isolated or assessed for functional activity in newly-presenting HNSCC patients. It was hypothesised that there would be a higher percentage of Tregs in the tumour compared with the peripheral circulation, and that the infiltrating Treg population would possess greater functional capacity due to the local influence of the tumour microenvironment and the tumour's requirement to continually promote an immunosuppressive environment.

5.2 Materials and methods

5.2.1 HNSCC patients

Following ethical (Yorkshire and the Humber research ethics committee; REC reference 10/H1304/7 and 10/H1304/8) and NHS Trust R and D (R0988 and R0986) approval, and obtaining written informed consent, newly-presenting HNSCC patients were recruited on to the study. HNSCC tumour sections (n=67) were used to examine the infiltration of $Foxp3^+$ cells by immunohistochemistry, whilst a separate cohort of HNSCC patients provided 38 tumour specimens. Of these, 6 samples were used for the optimisation of TIL isolation. Due to the low number of cells isolated from dissociation and cell sorting, and poor staining of TIL by flow cytometry, 17 tumour samples were assessed for the suppressive activity of infiltrating Tregs (Figure 5.1).

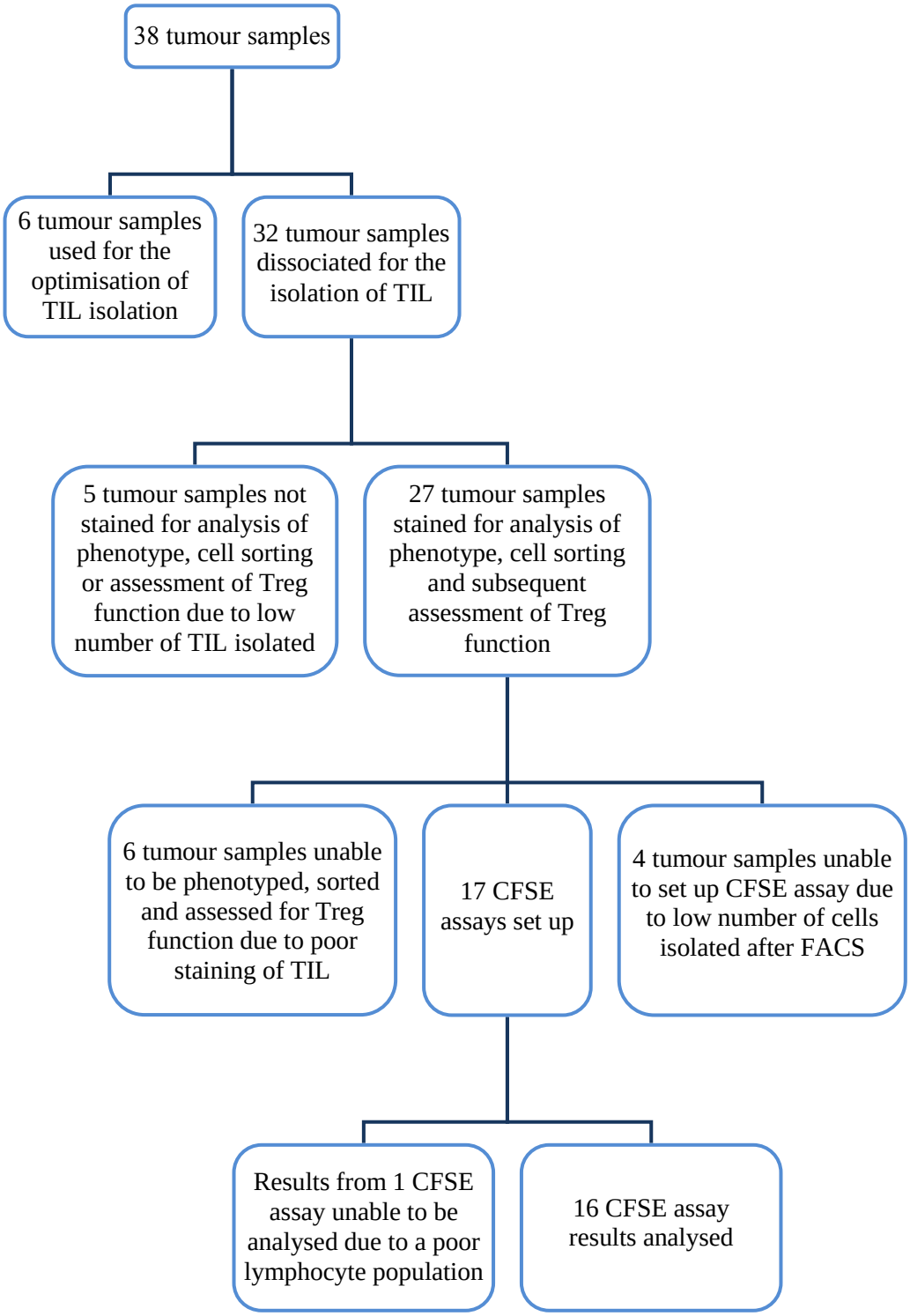


Figure 5.1 CONSORT diagram to illustrate the tumour samples that were analysed for phenotype and assessed for Treg function

Tumour samples (n=38) were obtained to analyse the phenotype of specific T cell subsets and assess Treg function in the tumour microenvironment. However, due to optimisation, the number of TIL isolated by dissociation and cell sorting, poor staining of the TIL and the success of the CFSE assay not all tumour samples could be examined for these parameters.

5.2.2 Staining of HNSCC sections for infiltrating Foxp3$^+$ cells by immunohistochemistry

Formalin fixed, paraffin embedded HNSCC specimens were immunostained with a mouse monoclonal Foxp3 primary antibody (1:200 dilution; Clone 236A/E7, Abcam, Cambridge, UK) using the method described in detail in Section 2.11. Following staining, the sections were mounted with a coverslip using Histomount (National Diagnostics) and prepared for analysis to assess the presence of Foxp3$^+$ cells within the head and neck tumour microenvironment.

5.2.3 Quantification of HNSCC sections stained for Foxp3$^+$ cells

As outlined in Section 2.11.1, positive immunostaining was identified and photographed in 5 high powered fields from both the tumour-associated stroma and the tumour nests of HNSCC specimens using a light microscope (400x magnification) and Image J software. An overall mean score from two independent assessors for each compartment and HNSCC specimen was determined.

5.2.4 Isolation of TIL from HNSCC specimens

Infiltrating tumour lymphocytes were isolated from HNSCC specimens as outlined in Section 2.14. Briefly following removal of fat, blood or necrotic areas, freshly isolated tumour specimens were weighed, cut into small pieces using scalpels and following an antibiotic wash were dissociated for 2-4 hours at 37^{o}C in medium containing 0.02% (w/v) DNase (Roche Diagnostics) and 0.02% (w/v) collagenase (Sigma) whilst under constant rotation. Dissociated tumour fragments were subsequently washed in medium and plated overnight to enable the majority of fibroblasts, epithelial cells and monocytes to adhere to the plastic (Section 2.14). The medium, containing TIL that had not adhered overnight, was collected the following morning and cells were isolated by centrifugation for immediate use in FACS to isolate and assess the suppressive function of Tregs, or frozen down for use at a later date (Section 2.14.1).

5.2.5 Assessing the phenotype and frequency of T cell subsets in TIL and the peripheral circulation of HNSCC patients

Thawed or freshly isolated TIL (Section 2.14) and thawed PBMC samples (Section 2.13), donated by HNSCC patients, were labelled with a Treg sorting cocktail (anti-CD4, anti-CD25 and anti-CD127; BD Biosciences) to enable the isolation of regulatory and effector T cell populations by FACS for use in subsequent assessment of Treg function (Section 2.16). As outlined in Chapter 4 (Section 4.2.2), the gating strategy used by FACS made it possible to examine the prevalence of $CD4^+$ cells (Figure 4.2a), $CD4^+CD25^+$, $CD4^+CD25^{inter}$ and $CD4^+CD25^{high}$ cells (Figure 4.2b), Tregs and effector T cells (Figure 4.2c) in the TIL and where available the patient's corresponding PBMC sample.

5.2.6 Assessment of the suppressive activity of Tregs isolated from the tumour microenvironment and peripheral circulation of HNSCC patients using a CFSE assay

Immediately following isolation by FACS (Section 2.16) Tregs from 17 tumour specimens were examined for suppressive activity on the proliferation of autologous tumour effector T cells via a CFSE assay (Section 2.18). Of these, 15 tumour samples had corresponding PBMC samples and the suppressive activity of Tregs from the periphery and tumour were simultaneously assessed for suppressive function on the proliferation of autologous effector T cells via various co-culture combinations (Figure 5.2).

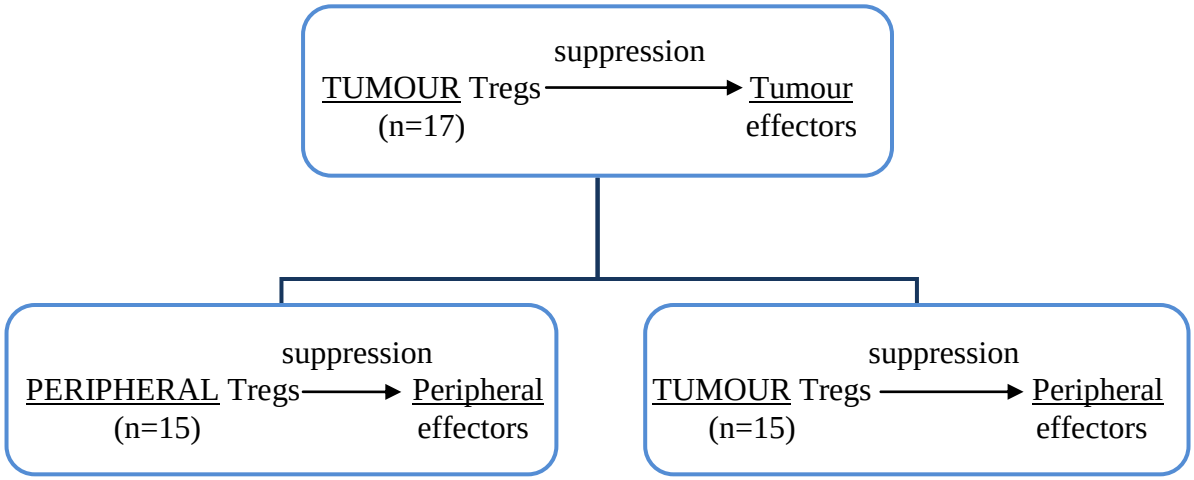


Figure 5.2 Diagram to illustrate the co-culture combinations of tumour infiltrating and peripheral Tregs and effector T cells
FACS isolated $CD4^{+}CD25^{inter}CD127^{low/-}$ and $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs from the tumour or peripheral circulation of HNSCC patients were co-cultured with CFSE labelled autologous effector T cells ($CD4^{+}CD25^{-}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$) from either the periphery or tumour microenvironment at a 1:1 ratio for four days before being acquired by flow cytometry and the data analysed by ModFit LT™ to determine the percentage of suppression induced by the Tregs.

The concentration and cell viability of each isolated population was determined using trypan blue exclusion (Section 2.4) to establish whether sufficient cells had been obtained to set up the CFSE assays. In the majority of cases the CFSE assay was run with 5×10^4 effector T cells cultured in each well of a 96 well plate (Section 2.18). However, when insufficient cells were isolated the number of effector cells plated was successfully scaled down to 1.5×10^4/well. The CFSE assay was set up and analysed as outlined in Section 4.2.4 with the 1:1 ratio always prepared and the remaining ratios (Treg:effector T cell, 1:2, 1:5 and 1:10) set up where possible. Percentages of suppression were calculated based on the proliferation indexes determined by ModFit LT™ software (Verity Software House, Section 2.19).

5.2.7 Statistical analysis

Statistical analysis was performed using SPSS version 19; the normality of the data was assessed by the Shapiro-Wilk test. Statistical tests were performed as outlined in

Section 4.2.5, depending on the distribution of data and whether independent or dependent data sets were being analysed. Values were considered significant when $p<0.05$.

5.3 Results

5.3.1 Identification of Foxp3$^+$ cells infiltrating the tumours of head and neck cancer patients

Paraffin embedded sections of HNSCC specimens (n=67; Table 5.3) were immunostained for Foxp3 to confirm the infiltration Tregs in both the tumour-associated stroma and the tumour nests. The infiltration of Foxp3$^+$ cells, in both compartments, was assessed and related to tumour stage, subsite and nodal status.

Table 5.3 Clinicopathological features of HNSCC patients whose tumour specimens were assessed for Foxp3$^+$ cell infiltration

Characteristics	Number
HNSCC patients sex and age	
Male	56
Female	11
Total	67
Mean age (years)	63
Age range (years)	30-92
Tumour stage	
Early (T1 and T2)	36*
Advanced (T3 and T4)	30*
Tumour site	
Laryngeal	42
Oropharyngeal	25
Tumour stage and site	
Early laryngeal	21
Advanced laryngeal	21
Early oropharyngeal	15*
Advanced oropharyngeal	9*
Nodal status	
Negative (N0)	33
Positive (N$^+$)	34

* One oropharyngeal tumour was of unknown T stage

In the whole HNSCC patient cohort, significantly more Foxp3 staining was observed in the tumour-associated stroma compared with that in the tumour nests ($p<0.001$; Figure 5.3); dividing the patient group by tumour stage, subsite and nodal status maintained this significant trend ($p\leq0.001$).

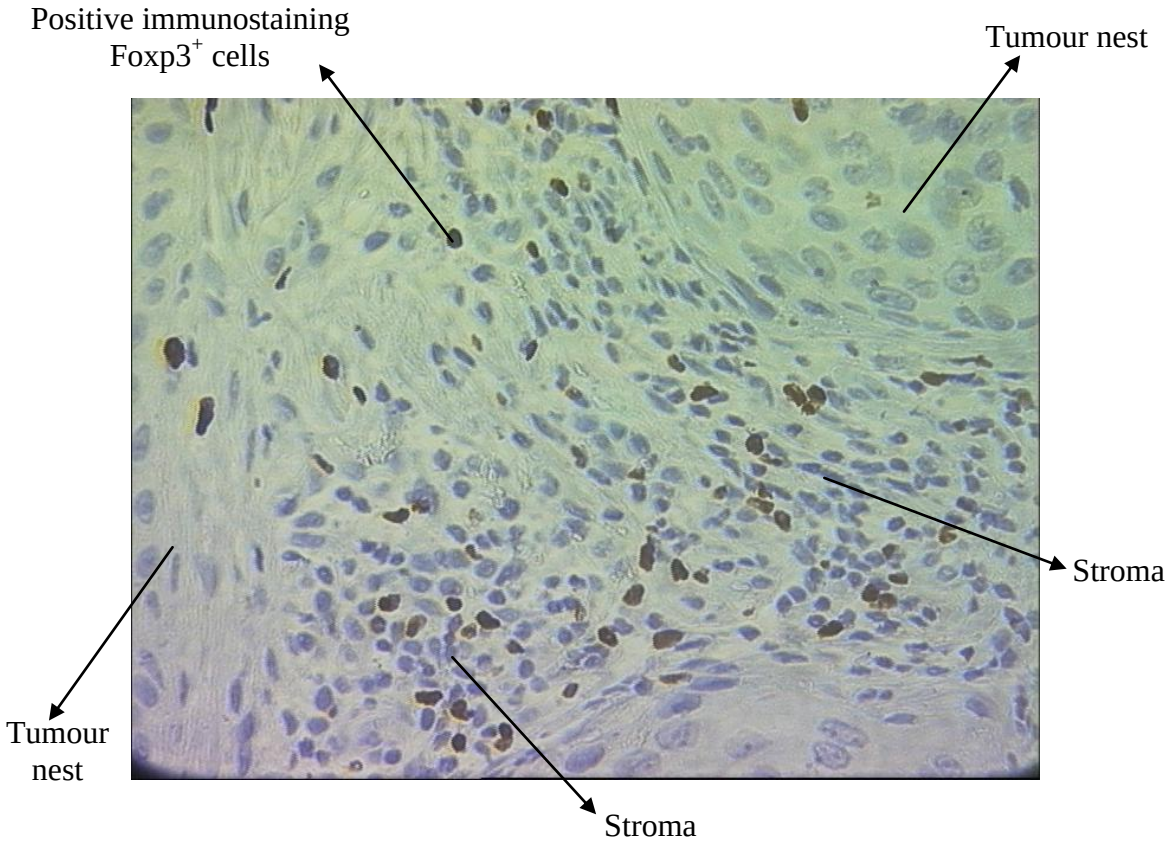


Figure 5.3 Immunohistochemical staining for Foxp3 in HNSCC specimens
Formalin fixed and paraffin embedded sections of HNSCC specimens were immunostained with a monoclonal mouse anti-human Foxp3 antibody (clone 236A/E7) at a 1:200 dilution and the infiltration of $Foxp3^+$ cells was assessed in five fields of stroma and five fields of tumour nests. Brown staining indicates cells positive for Foxp3 and the blue is haematoxylin counterstain (x400 magnification).

The infiltration of $Foxp3^+$ cells into both the stroma and tumour was similar between tumour stages and nodal involvement (Table 5.4). However, when comparing different head and neck subsites, patients with cancer of the oropharynx had a significantly greater infiltration of $Foxp3^+$ cells compared to patients with laryngeal cancer in both the stroma and tumour (Table 5.4).

Table 5.4 Mean score of Foxp3$^+$ cells infiltrating the stroma and tumour of HNSCC sections in relation to tumour stage, subsite and nodal status

	Foxp3 positive staining	
Cohort	Stroma	Tumour
HNSCC patients (n=67)	55.27 ± 5.17	5.58 ± 0.95
Early stage (n=36)	59.25 ± 7.43	5.43 ± 1.24
Advanced stage (n=30)	51.12 ± 7.36	5.64 ± 1.53
p value	0.48	0.47
Laryngeal (n=42)	43.80 ± 6.15	3.25 ± 0.59
Oropharyngeal (n=25)	74.52 ± 7.99	9.51 ± 2.16
p value	**0.002**	**0.002**
Early laryngeal (n=21)	48.23 ± 9.74	3.07 ± 0.75
Advanced laryngeal (n=21)	39.38 ± 7.62	3.42 ± 0.93
p value	0.82	0.95
Early oropharyngeal (n=15)*	74.68 ± 10.61	8.73 ± 2.61
Advanced oropharyngeal (n=9)*	78.51 ± 13.48	10.80 ± 4.31
p value	0.39	0.88
N0 (n=33)	49.31 ± 7.03	5.30 ± 1.56
N$^+$ (n=34)	61.04 ± 7.54	5.86 ± 1.13
p value	0.30	0.40

Values mean score ± SEM
Mann-Whitney *U* test. Results in bold considered significant when $p<0.05$
* One oropharyngeal tumour was of unknown T stage

5.3.2 Optimising the isolation of TIL from HNSCC

To assess the frequency and function of Tregs infiltrating the tumours of head and neck cancer patients the method of isolating TIL from the tumour specimens firstly had to be established.

Work had previously been carried out on HNSCC tissue by another PhD student in the research group assessing the expression of integrins on tumour cells in relation to metastasis, which required the disaggregation of head and neck tumour specimens (Woods *et al.*, 2011). The optimal concentration of enzymes required to dissociate tumour samples and preserve integrin expression had been determined, including using the enzymes collagenase type IV (0.02%; w/v) and DNase type I (0.02%; w/v); these concentrations

were used in order to preserve the expression of lymphocyte markers, CD4, CD25 and CD127.

The limiting factor in assessing the suppressive activity of Tregs infiltrating the tumour microenvironment was cell number, therefore it was important to do everything possible to maximise this. It was shown that trying to identify the lymphocyte population by flow cytometry immediately following dissociation was very difficult due to the presence of numerous cell types including epithelial cells and fibroblasts (Figure 5.4a). Therefore to provide a more defined lymphocyte population, the dissociated tumour fragments were cultured overnight to enable the epithelial cells, fibroblasts and monocytes to adhere to the culture flask leaving non-adherent cells, including TIL, remaining in suspension. As illustrated by Figure 5.4b this provided a more clearly defined lymphocyte population. Although the comparison between immediate use and overnight culture was assessed for only one HNSCC specimen, employing the overnight method consistently generated a population of TIL that could be more clearly identified throughout the study (Figure 5.4c).

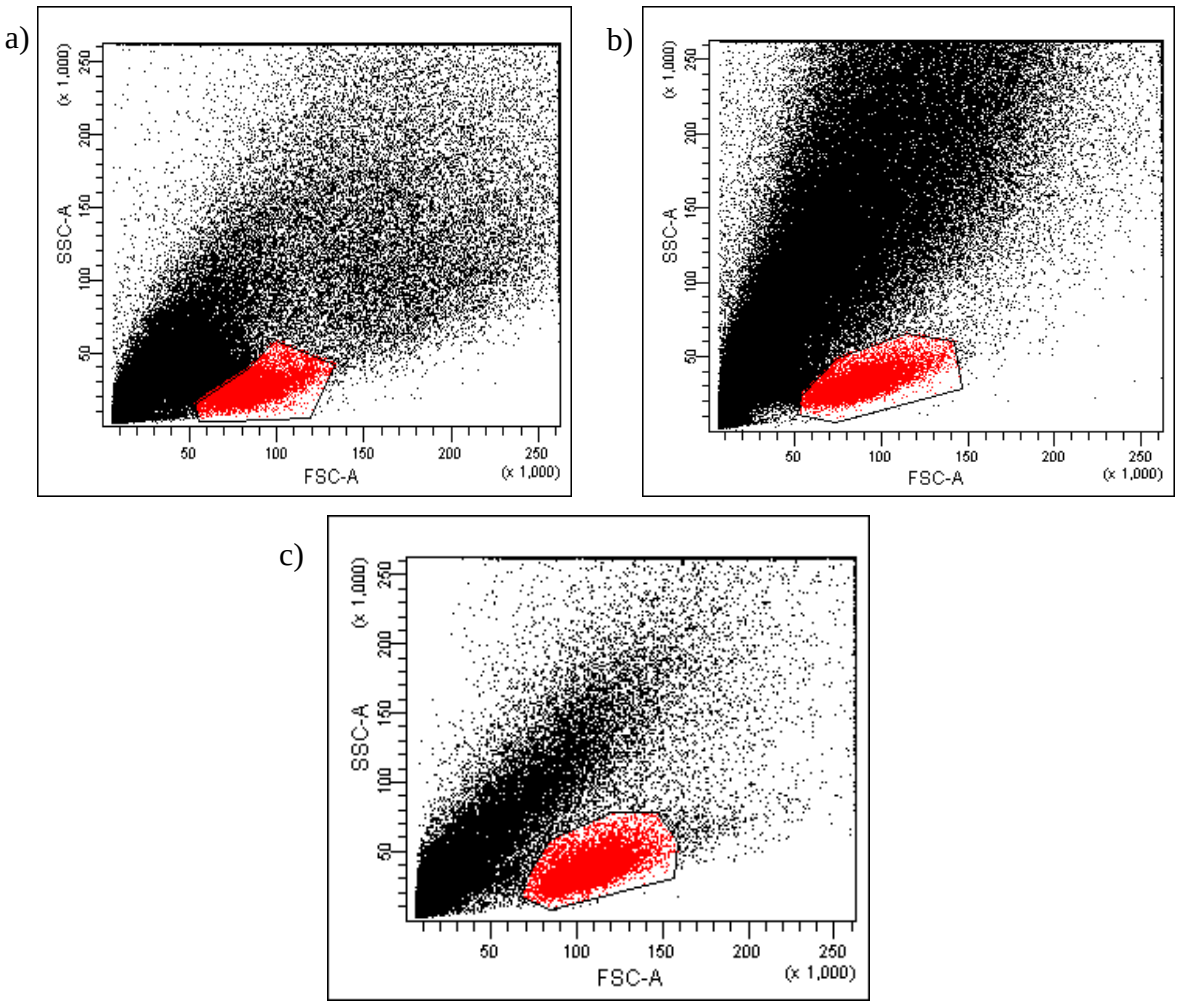


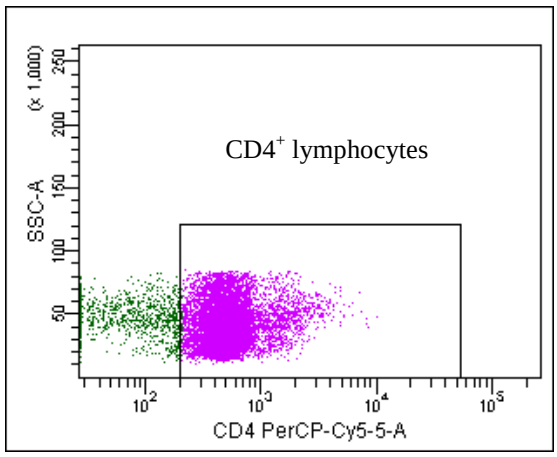
Figure 5.4 Identifying the TIL population by flow cytometry
Immediately following dissociation of a HNSCC specimen 1 x 10^6 cells were acquired using the FACSAria II to identify the TIL population (a). The remaining cell suspension was cultured overnight and the following day the medium, containing TIL, was collected and 1 x 10^6 cells were acquired again by flow cytometry to identify a clearer lymphocyte population (b). The same overnight procedure was used for subsequent specimens throughout the study, a representative of which is shown in the last plot (c).

To maximise the number of TIL that could be isolated, tumour specimens (primary tumour site; n=1, tumour infiltrated lymph node samples; n=3) were initially dissociated overnight, as this length of time visibly reduced the size of the tumour fragments, indicating continued disaggregation of the tissue samples. However, although overnight dissociation isolated a good number of TIL, it also resulted in the reduced expression of the lymphocyte markers CD4, CD25 and CD127, which are required to assess the prevalence of specific T cell populations and for the isolation of Tregs and effector T cells by FACS.

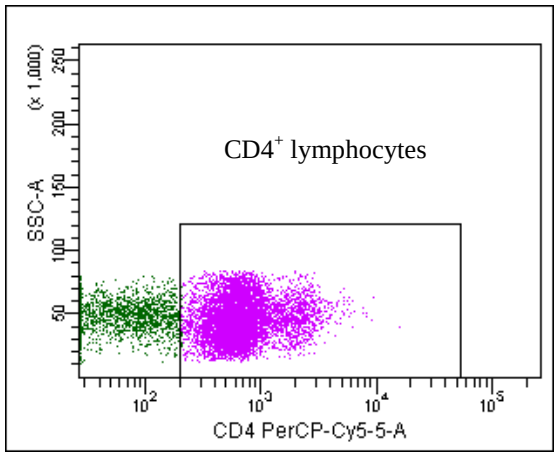
It was therefore essential to establish a length of dissociation that would isolate a good number of TIL whilst also maintaining the expression of lymphocyte markers. The dissociation time was determined by dividing one HNSCC specimen into three and disaggregating for two hours, four hours and overnight before phenotypically analysing for the expression of lymphocyte markers by labelling the cells with the Treg sorting cocktail. Using both two and four hours of dissociation (Figure 5.5a and b, respectively) the level of CD4 expression was similar, both clearly showing a population of lymphocytes that could be identified by their positive expression of CD4. However, following the overnight dissociation there was a loss in CD4 expression that made it very difficult to distinguish a $CD4^+$ lymphocyte population (Figure 5.5c); this experiment demonstrated that an appropriate length of dissociation for a HNSCC specimen was 2-4 hours. Although the time course experiment was only performed on one HNSCC sample, dissociating the head and neck tumour samples for 2-4 hours consistently produced TIL populations that could be gated by CD4, CD25 and CD127 expression.

Length of dissociation

a) 2 hours



b) 4 hours



c) overnight

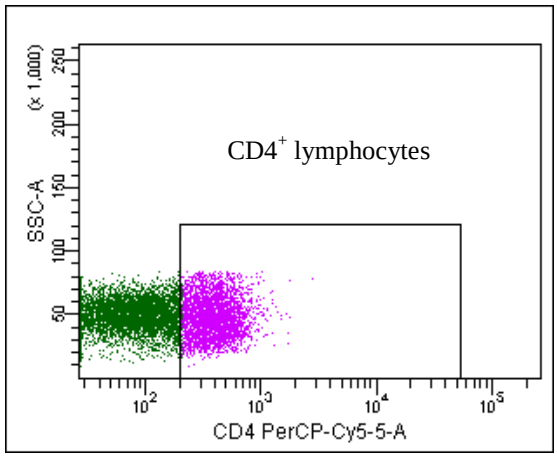


Figure 5.5 Dot plots showing an appropriate length of dissociation for HNSCC specimens in terms of CD4 expression

An HNSCC specimen was divided into three and dissociated for 2 hours (a), 4 hours (b) and overnight (c) before being labelled with an anti-CD4 PerCP-Cy5.5 antibody. The isolated TIL populations were gated and following doublet discrimination the resulting single cell lymphocyte populations from the three different dissociation time points were assessed for the expression of CD4.

During the optimisation of the dissociation protocol it was observed that, although dissociating head and neck tumour samples for 2-4 hours generated a clear lymphocyte population positive for the expression of CD4, the level of expression varied more in TIL (Figure 5.6a) compared with that in the lymphocyte population isolated from the peripheral circulation (Figure 5.6b). Additionally the level of expression of CD4 by the peripheral lymphocyte population tended to be much higher.

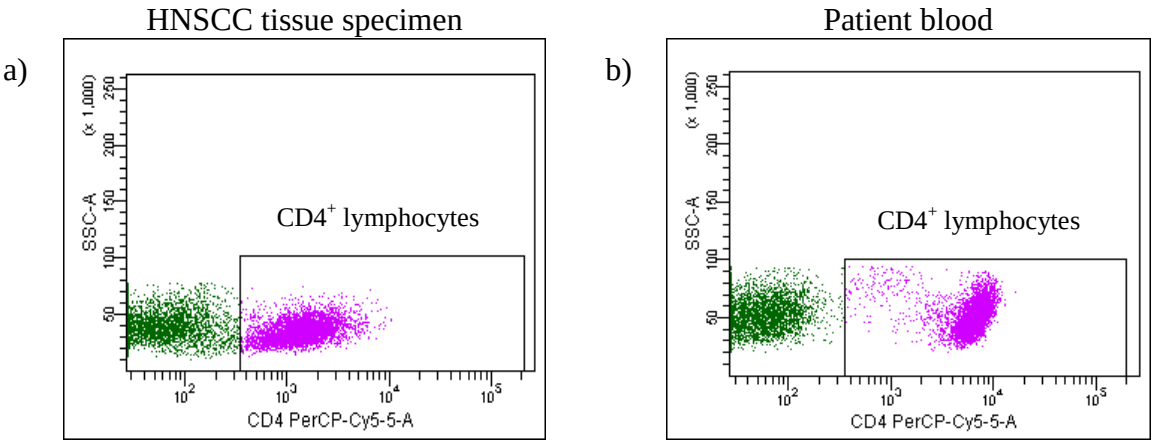


Figure 5.6 Expression of CD4 by TIL isolated from HNSCC tissue and HNSCC patient peripheral blood
(a) TIL and (b) PBMC samples from the same HNSCC patient were labelled with an anti-CD4-PerCP.Cy5.5 antibody and following gating of the lymphocyte population and doublet discrimination the resulting single cell lymphocyte populations were assessed for CD4 expression.

5.3.3 Assessing the phenotype and frequency of T cell subsets in the tumour microenvironment

Lymphocytes isolated from 27 tumour samples were labelled with a Treg sorting cocktail to be characterised phenotypically using flow cytometry (Figure 5.1). However, due to the poor staining of the lymphocyte population in some instances 6 samples were unable to be analysed and upon subsequent examination of clinical information it was found that 2 of the patients recruited on to the study had previously received therapy for HNSCC, these patients were removed from the analysis as they did not meet the recruitment criteria (Section 2.12). The remaining 19 tumour specimens consisted of 4 samples from the primary tumour site and 15 nodal samples that had SCC involvement

(Table 5.5), due to this the samples were initially analysed as two separate groups (primary tumour and tumour infiltrated nodes) to account for the influences the different microenvironments may have had on the frequency of various T cell subsets.

Table 5.5 Clinicopathological characteristics of HNSCC patients whose TIL were phenotypically characterised from primary tumours and tumour infiltrated nodes

Characteristics	Primary tumour samples	Tumour infiltrated lymph node samples
HNSCC patient sex and age		
Male	4	12
Female	0	3
Total	4	15
Mean age (years)	58.5	61
Age range (years)	41-78	41-84
Tumour stage		
Early (T1 and T2)	0	4
Advanced (T3 and T4)	4	8
Unknown	0	3
Tumour site		
Larynx	3	6
Oropharynx	0	6
Hypopharynx	1	1
Unknown	0	2
Tumour stage and site		
Early larynx	0	0*
Advanced larynx	3	5*
Early oropharynx	0	4
Advanced oropharynx	0	2
Other/Unknown	1	4
Nodal status		
Negative	0	0
Positive	4	15

* The tumour stage of one of the laryngeal samples was not recorded

5.3.3.1 Assessing the phenotype and frequency of T cell subsets in the tumour infiltrated lymph nodes and primary tumour microenvironment

The frequency of T cell subsets in the lymphocyte population isolated from tumour infiltrated lymph nodes was analysed with regard to tumour subsite (larynx and oropharynx). Lymphocytes isolated from resected lymph nodes taken from patients with cancer of the larynx had a slightly higher proportion of lymphocytes expressing CD4 and $CD4^+$ cells expressing $CD25^+CD127^{low/-}$, together with a lower frequency of $CD4^+CD25^{-/+}CD127^{-/+}$ effector T cells, compared to patients with oropharyngeal cancer, however these differences did not reach significance (Table 5.6).

Table 5.6 Percentage of cells expressing specific T cell markers in tumour infiltrated lymph nodes in relation to tumour subsite

		Cohort		
T cell phenotypes		Laryngeal (n=6)	Oropharyngeal (n=6)	*p* value
	Percentage of lymphocytes expressing CD4	46.55 ± 5.35	40.47 ± 4.07	0.39
Percentage of $CD4^+$ cells expressing	$CD25^+$	66.95 ± 4.45	64.2 ± 3.12	0.62
	$CD25^{inter}$	46.58 ± 6.45	45.78 ± 5.600	0.93
	$CD25^{high}$	20.37 ± 3.83	18.42 ± 2.81	0.69
	$CD25^+CD127^{low/-}$	38.00 ± 2.62	31.75 ± 1.63	0.07
	$CD25^{inter}CD127^{low/-}$	19.22 ± 2.08	15.25 ± 1.68	0.17
	$CD25^{high}CD127^{low/-}$	18.78 ± 3.73	16.50 ± 2.36	0.62
	$CD25^{-/+}CD127^{-/+}$	62.37 ± 2.49	68.43 ± 1.87	0.08
	$CD25^-CD127^{-/+}$	33.47 ± 4.48	34.62 ± 2.73	0.83
	$CD25^+CD127^+$	28.90 ± 5.79	33.82 ± 4.21	0.51

Values mean percentage ± SEM
Unpaired student's t test
$CD25^+$ is the sum of $CD25^{inter}$ and $CD25^{high}$
$CD25^+CD127^{low/-}$ is the sum of $CD25^{inter}$ $CD127^{low/-}$ and $CD25^{high}CD127^{low/-}$
$CD25^{-/+}CD127^{-/+}$ is the sum of $CD25^-CD127^{-/+}$ and $CD25^+CD127^+$

} exemplified in Figure 4.2

The majority of the primary tumour specimens in this small cohort were from patients with advanced stage cancer that had arisen from the larynx (Table 5.5) and therefore tumour subsite and stage comparisons within the primary tumour cohort could not

be made. However, the frequency of T cell subsets within primary tumour and tumour infiltrated lymph nodes, were compared. The percentage of $CD4^+$ lymphocytes was slightly higher in the lymph nodes compared with the primary tumours however, this difference was again not significant and the prevalence of the other T cell subsets were similar between the two sample groups (Table 5.7).

Table 5.7 Percentage of cells expressing specific T cell markers in primary tumour and tumour infiltrated lymph node samples

T cell phenotypes		Cohort: Primary tumour samples (n=4)	Cohort: Lymph node samples (n=15)	*p* value
	Percentage of lymphocytes expressing CD4	36.30 ± 7.31	42.51 ± 3.05	0.38
Percentage of $CD4^+$ cells expressing	$CD25^+$	64.33 ± 8.65	65.21 ± 2.17	0.48
	$CD25^{inter}$	41.40 ± 6.61	44.29 ± 3.59	0.71
	$CD25^{high}$	22.93 ± 6.78	20.93 ± 2.11	0.76
	$CD25^+CD127^{low/-}$	39.80 ± 7.84	36.07 ± 6.52	0.75
	$CD25^{inter}CD127^{low/-}$	18.65 ± 1.68	16.93 ± 1.19	0.97
	$CD25^{high}CD127^{low/-}$	21.15 ± 7.07	19.14 ± 1.98	0.65
	$CD25^{-/+}CD127^{-/+}$	61.30 ± 8.20	64.27 ± 1.69	0.67
	$CD25^-CD127^{-/+}$	34.83 ± 8.59	34.58 ± 2.10	0.50
	$CD25^+CD127^+$	26.48 ± 6.67	29.69 ± 3.10	0.80

Values mean percentage ± SEM
Unpaired student's t test or Mann-Whitney *U* test.
$CD25^+$ is the sum of $CD25^{inter}$ and $CD25^{high}$
$CD25^+CD127^{low/-}$ is the sum of $CD25^{inter}$ $CD127^{low/-}$ and $CD25^{high}CD127^{low/-}$
$CD25^{-/+}CD127^{-/+}$ is the sum of $CD25^-CD127^{-/+}$ and $CD25^+CD127^+$

} exemplified in Figure 4.2

5.3.3.2 Comparison of the phenotype and frequency of T cell subsets in the tumour microenvironment and peripheral circulation of HNSCC patients

As there were no statistically significant differences for the frequency of different T cell subsets between the primary tumour and tumour infiltrated lymph node samples, the two cohorts were combined to compare the prevalence of T cell subsets between the tumour microenvironment and peripheral circulation. The entire primary tumour cohort (n=4) and 11 of the tumour infiltrated lymph node specimens had samples from both the tissue and

the peripheral circulation available for analysis. The T cell subsets from different locations were also compared with regard to tumour subsite, larynx (n=8) and oropharynx (n=5); due to the low number of tumour samples from patients with cancer of the hypopharynx (n=2) this subsite was not statistically assessed.

The percentage of lymphocytes expressing CD4 and the frequency of $CD4^+$ cells expressing CD25 was similar for the tumour and PBMC cohorts, as a whole, and when divided by tumour subsite (Table 5.8). However, the frequency of $CD4^+$ cells expressing intermediate levels of the IL-2 receptor ($CD25^{inter}$) in the peripheral circulation was significantly elevated compared with that in the tumour microenvironment, this trend was also observed in both the laryngeal and oropharyngeal sample cohort however, these differences did not reach significance. In contrast, the prevalence of $CD4^+CD25^{high}$ cells was significantly increased in the tumour microenvironment compared with the peripheral circulation; this trend was also shown in individual tumour subsites however, significance was again not met (Table 5.8).

The frequency of $CD127^{low/-}$ Tregs, irrespective of the level of CD25 expression, was significantly elevated in the tumour microenvironment compared with the PBMC samples (Table 5.9). This significant trend was also observed in the laryngeal cohort when the HNSCC patients were divided by subsite. For patients with cancer of the oropharynx the same trend was shown but only reached significance for $CD25^+CD127^{low/-}$ and $CD25^{high}CD127^{low/-}$ Tregs (Table 5.9).

The frequency of $CD4^+CD25^{-/+}CD127^{-/+}$ effector T cells was found to be significantly elevated in the peripheral circulation, as a whole, compared with the tumour microenvironment, irrespective of tumour subsite. Dividing the $CD4^+CD25^{-/+}CD127^{-/+}$ population into the two effector populations revealed that it was the $CD4^+CD25^+CD127^+$ cells which were significantly increased in the peripheral circulation compared with the tumour microenvironment. This was observed for both tumour subsites analysed but only reached significance for the patients whose primary tumour arose from the larynx (Table 5.9).

Table 5.8 Percentage of cells expressing specific T cell markers in the tumour microenvironment and corresponding PBMC samples in relation to tumour subsite

		Percentage of $CD4^+$ cells expressing		
Cohort	Percentage of lymphocytes expressing CD4	$CD25^+$	$CD25^{inter}$	$CD25^{high}$
Tumour samples (n=15)	42.13 ± 3.18	66.09 ± 2.88	47.29 ± 3.16	18.80 ± 2.13
PBMC samples (n=15)	43.77 ± 1.86	69.56 ± 3.41	58.43 ± 3.06	11.13 ± 0.87
p value	0.59	0.45	**0.03**	**0.002**
Laryngeal tumour (n=8)	42.49 ± 5.32	66.14 ± 5.01	48.35 ± 4.45	17.79 ± 2.95
Laryngeal PBMC (n=8)	45.65 ± 2.09	70.90 ± 5.18	59.98 ± 4.42	10.93 ± 0.93
p value	0.50	0.49	0.11	0.07
Oropharyngeal tumour (n=5)	42.1 ± 4.57	64.46 ± 3.81	47.20 ± 6.63	17.26 ± 3.14
Oropharyngeal PBMC (n=5)	38.04 ± 3.14	70.32 ± 6.37	59.44 ± 5.88	10.88 ± 2.28
p value	0.35	0.53	0.31	0.09

Values mean percentage ± SEM
Paired student's t test or Wilcoxon Signed Rank test.
Results in bold considered significant when $p<0.05$
$CD25^+$ is the sum of $CD25^{inter}$ and $CD25^{high}$

Table 5.9 Percentage of $CD4^+$ cells expressing varying levels of CD25 and CD127 in the tumour microenvironment and corresponding PBMC samples in relation to tumour subsite

	Percentage of $CD4^+$ cells expressing					
Cohort	$CD25^+CD127^{low/-}$	$CD25^{inter}CD127^{low/-}$	$CD25^{high}CD127^{low/-}$	$CD25^{-/+}CD127^{-/+}$	$CD25^-CD127^{-/+}$	$CD25^+CD127^+$
Tumour samples (n=15)	35.13 ± 2.28	18.00 ± 1.12	17.13 ± 2.10	65.31 ± 2.36	33.29 ± 2.75	32.02 ± 2.94
PBMC samples (n=15)	16.51 ± 0.56	9.71 ± 0.55	6.80 ± 0.39	84.27 ± 0.55	31.57 ± 3.36	52.69 ± 3.29
p value	**<0.001**	**<0.001**	**<0.001**	**0.001**	0.69	**<0.001**
Laryngeal tumour (n=8)	35.58 ± 2.99	19.43 ± 1.35	16.11 ± 3.01	65.04 ± 3.16	33.56 ± 4.90	31.48 ± 4.35
Laryngeal PBMC (n=8)	15.21 ± 0.48	9.13 ± 0.56	6.09 ± 0.42	85.50 ± 0.32	30.56 ± 5.10	54.94 ± 5.05
p value	**<0.001**	**<0.001**	**0.01**	**<0.001**	0.65	**0.01**
Oropharyngeal tumour (n=5)	30.76 ± 1.58	15.44 ± 2.04	15.32 ± 2.51	69.26 ± 2.05	33.94 ± 3.24	35.32 ± 4.81
Oropharyngeal PBMC (n=5)	18.94 ± 0.62	11.56 ± 0.89	7.50 ± 0.79	81.72 ± 0.70	30.42 ± 6.32	51.30 ± 6.05
p value	**0.001**	0.13	**0.04**	**0.004**	0.68	0.14

Values mean percentage ± SEM
Paired student's t test or Wilcoxon Signed Rank test.
Results in bold considered significant when $p<0.05$
$CD25^+CD127^{low/-}$ is the sum of $CD25^{inter}CD127^{low/-}$ and $CD25^{high}CD127^{low/-}$
$CD25^{-/+}CD127^{-/+}$ is the sum of $CD25^-CD127^{-/+}$ and $CD25^+CD127^+$

5.3.4 Suppressive activity of Tregs in the tumour microenvironment of HNSCC patients

Lymphocytes isolated from 21 tumour specimens, including primary tumour and tumour infiltrated lymph nodes, were successfully labelled with a Treg sorting cocktail (Figure 5.1) to allow the suppressive activity of isolated Tregs ($CD4^{+}CD25^{inter}CD127^{low/-}$ and $CD4^{+}CD25^{high}CD127^{low/-}$) on autologous effector T cell populations ($CD4^{+}CD25^{-}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$) to be assessed by a CFSE assay. However, due to the low number of cells isolated by this method and limitations of the CFSE assay 5 tumour specimens were unable to be assessed for the suppressive activity of infiltrating Tregs (Figure 5.1). In addition, as mentioned previously, results from 2 tumour specimens were removed from the study due to the realisation that the patients had received treatment for their HNSCC prior to sampling. The clinicopathological characteristics of the remaining 14 HNSCC patients from which tumour specimens were obtained and successfully assessed for Treg function are shown in Table 5.10; these included 2 samples from the primary tumour site and 12 samples from associated tumour infiltrated lymph nodes.

Table 5.10 Clinicopathological characteristics of HNSCC patients whose primary tumour or nodal infiltrating Tregs were assessed for suppressive activity

Characteristics	Primary tumour samples	Tumour infiltrated lymph node samples
HNSCC patient sex and age		
Male	2	9
Female	0	3
Total	2	12
Mean age (years)	48.5	59
Age range (years)	41-56	41-78
Tumour stage		
Early (T1 and T2)	0	3
Advanced (T3 and T4)	2	8
Unknown	0	1
Tumour site		
Larynx	1	6
Oropharynx	0	5
Hypopharynx	1	1
Unknown	0	0
Tumour stage and site		
Early larynx	0	0
Advanced larynx	1	5
Early oropharynx	0	3
Advanced oropharynx	0	2
Other/Unknown	1	2
Nodal status		
Negative	0	0
Positive	2	12

5.3.4.1 Suppressive activity of tumour infiltrating Tregs at different Treg : effector T cell ratios

As previously mentioned in Chapter 4, all samples were cultured at a Treg:effector T cell ratio of 1:1 and where cell numbers allowed further ratios were established to show that the level of suppression induced by isolated Tregs could be titrated (Section 4.3.3.1). For the 2 primary tumour specimens there were only sufficient cells to prepare a 1:1 ratio however, with the tumour infiltrated nodal samples it has been demonstrated that,

regardless of Treg or effector T cell phenotype, as the proportion of Tregs ($CD4^+CD25^{high}CD127^{low/-}$ and $CD4^+CD25^{inter}CD127^{low/-}$) is reduced the percentage of suppression observed on the proliferation of effector T cells ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) also decreases (Figure 5.7 and Figure 5.8 respectively).

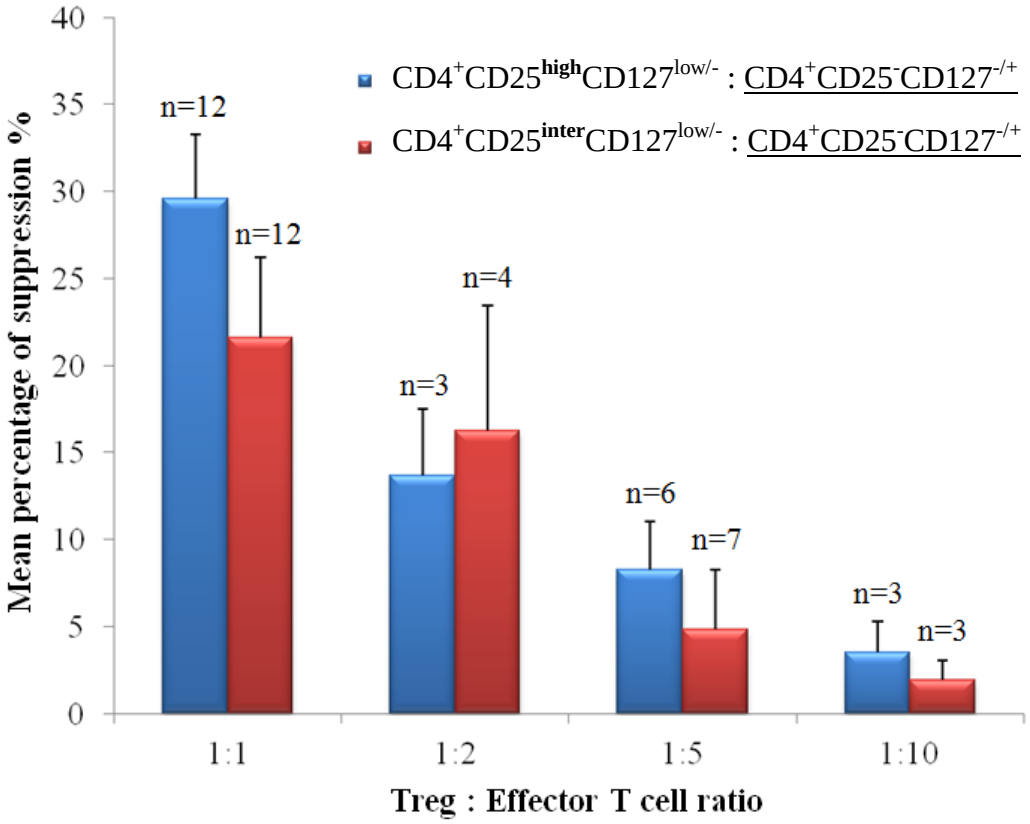


Figure 5.7 Mean percentage of suppression by $CD25^{high}$ and $CD25^{inter}CD127^{low/-}$ Tregs on the proliferation of $CD25^-CD127^{-/+}$ effector T cells at varying ratios for tumour infiltrated lymph node samples

FACS isolated $CD4^+CD25^{high}CD127^{low/-}$ and $CD4^+CD25^{inter}CD127^{low/-}$ Tregs were co-cultured with CFSE labelled $CD4^+CD25^-CD127^{-/+}$ effector T cells at ratios of 1:1, 1:2, 1:5 and 1:10 for four days before being acquired by flow cytometry and the data analysed by ModFit LT™ to determine the percentage of suppression induced by the Tregs. Values are mean percentage ± SEM.

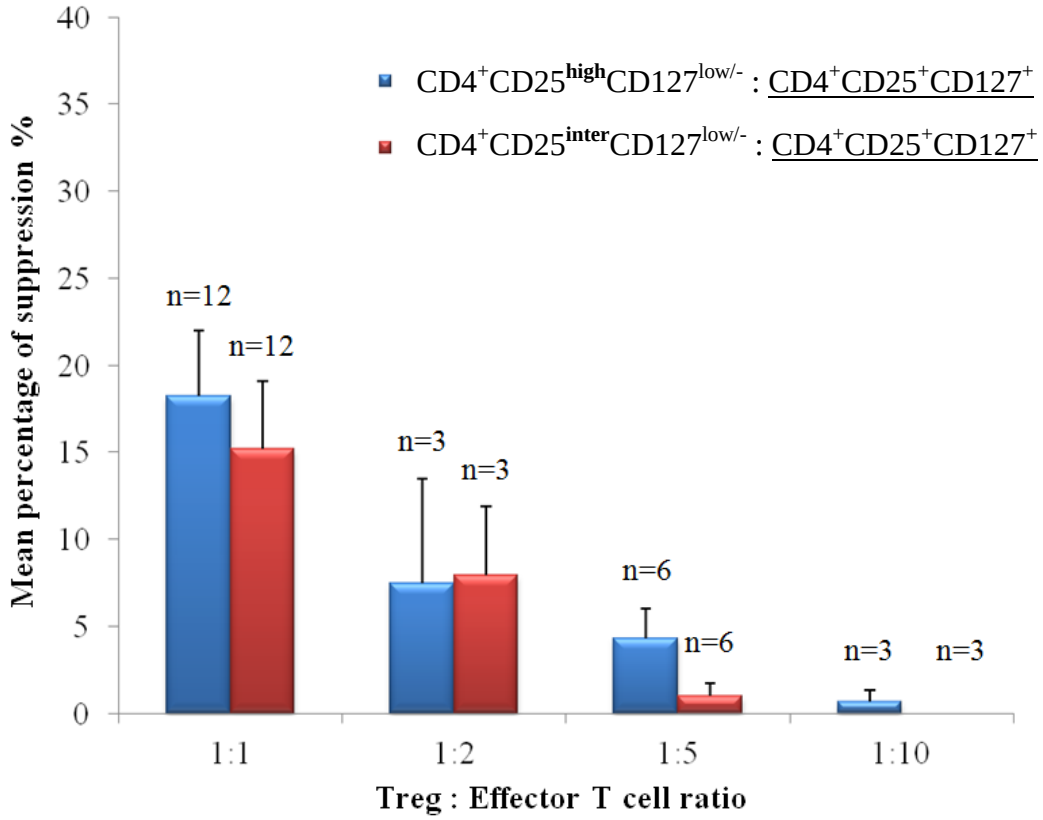


Figure 5.8 Mean percentage of suppression by $CD25^{high}$ and $CD25^{inter}CD127^{low/-}$ Tregs on the proliferation of $\underline{CD25^{+}CD127^{+}}$ effector T cells at varying ratios for tumour infiltrated lymph node samples

FACS isolated $CD4^{+}CD25^{high}CD127^{low/-}$ and $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs were co-cultured with CFSE labelled $CD4^{+}CD25^{+}CD127^{+}$ effector T cells at ratios of 1:1, 1:2, 1:5 and 1:10 for four days before being acquired by flow cytometry and the data analysed by ModFit LT™ to determine the percentage of suppression induced by the Tregs. Values are mean percentage ± SEM.

As shown, the mean percentages of suppression induced by both Treg populations ($CD25^{inter}$ and $CD25^{high}CD127^{low/-}$) was greater on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells (Figure 5.7) compared with that observed on the $CD4^{+}CD25^{+}CD127^{+}$ effector T cell population (Figure 5.8), regardless of the Treg:effector T cell ratio examined.

The observed dilution of Treg suppressive activity was in agreement with previous studies conducted on Tregs isolated from tumour infiltrated lymph nodes (Kawaida *et al.*, 2005; Liyanage *et al.*, 2002; Viguier *et al.*, 2004) and the primary tumour site (Chi *et al.*, 2010; Gobert *et al.*, 2009; Ling *et al.*, 2007; Zhou *et al.*, 2009) of various cancer types. However, due to the primary tumour samples only being established at the 1:1 ratio and the

low number of tumour infiltrated nodal samples prepared at the remaining ratios, the analysis of subsequent results for the suppressive activity of tumour isolated Tregs focused on the results from the 1:1 ratio.

5.3.4.2 Suppressive activity of Tregs on effector T cells in tumour infiltrated lymph nodes and the primary tumour microenvironment

As mentioned previously, at the 1:1 ratio both $CD127^{low/-}$ Treg populations isolated from tumour infiltrated lymph nodes induced a higher level of suppression on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells compared with the $CD4^{+}CD25^{+}CD127^{+}$ effector T cells (Figure 5.7 and Figure 5.8). This reached significance for the $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs (p=0.01), however significance was not met for the $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs (p=0.20). Additionally, although not reaching significance (p=0.19), it was observed that the $CD25^{high}CD127^{low/-}$ Tregs induced a slightly higher percentage of suppression on the proliferation of the $CD4^{+}CD25^{-}CD127^{-/+}$ effector population compared with $CD25^{inter}CD127^{low/-}$ Tregs (Figure 5.7). However, similar levels of suppression were observed on the $CD4^{+}CD25^{+}CD127^{+}$ effector T cell population (p=0.33; Figure 5.8).

When assessing the suppressive activity of Tregs isolated from tumour infiltrated lymph nodes with regard to tumour subsite, no significant differences in the level of suppression were observed between the laryngeal and oropharyngeal cohorts, irrespective of the Treg population being analysed ($CD4^{+}CD25^{inter}CD127^{low/-}$ and $CD4^{+}CD25^{high}CD127^{low/-}$) or the effector T cell population being suppressed ($CD4^{+}CD25^{-}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$; Table 5.11). At the different HNSCC subsites there were no statistically significant differences between the suppressive activities of $CD25^{inter}$ and $CD25^{high}$ Tregs (data not shown) however, Tregs expressing high levels of the IL-2 receptor tended to induce a slightly higher level of suppression on the $CD4^{+}CD25^{-}CD127^{-/+}$ effector population in the laryngeal cohort compared with $CD25^{inter}CD127^{low/-}$ Tregs (Table 5.11). In addition, a greater level of suppression was observed on the $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells compared with the $CD4^{+}CD25^{+}CD127^{+}$ effector population, regardless of the Treg phenotype, for both the laryngeal and oropharyngeal cohorts.

Table 5.11 Percentage of suppression induced by Tregs on the proliferation of effector T cells for tumour infiltrated lymph nodes separated by tumour subsite

	Cohort		
Treg : Effector T cell	Laryngeal (n=6)	Oropharyngeal (n=5)	*p* value
$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	18.60 ± 3.62	20.08 ± 9.19	0.88
$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	30.40 ± 4.09	24.34 ± 6.20	0.42
$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^+CD127^+$	11.56 ± 4.39	14.42 ± 5.91	0.53
$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^+CD127^+$	15.23 ± 5.51	20.14 ± 6.15	0.57

All conducted at a 1:1 ratio
Values mean percentage ± SEM
Unpaired student's t test or Mann Whitney *U* test.

The primary tumour specimens consisted of a single patient with cancer of the hypopharynx and one with laryngeal cancer (Table 5.10), therefore no statistical analysis was possible. The percentages of suppression induced by Tregs from the tumour infiltrating lymph node samples and those from the primary tumour cohort were compared but no significant differences were observed (data not shown). The patients from which the two primary tumour samples were obtained both simultaneously underwent a neck dissection, therefore the suppressive activity of Tregs from the primary tumour microenvironment were directly compared to those isolated from their corresponding tumour infiltrated lymph nodes. For the hypopharyngeal patient greater levels of suppression were observed in the tumour infiltrating lymph nodes compared with the primary tumour microenvironment. However, for the laryngeal patient the opposite trend was observed with the primary tumour microenvironment demonstrating similar or greater percentages of suppression irrespective of the effector population being suppressed (Figure 5.9; results shown for the $CD4^+CD25^-CD127^{-/+}$ effector T cell population only).

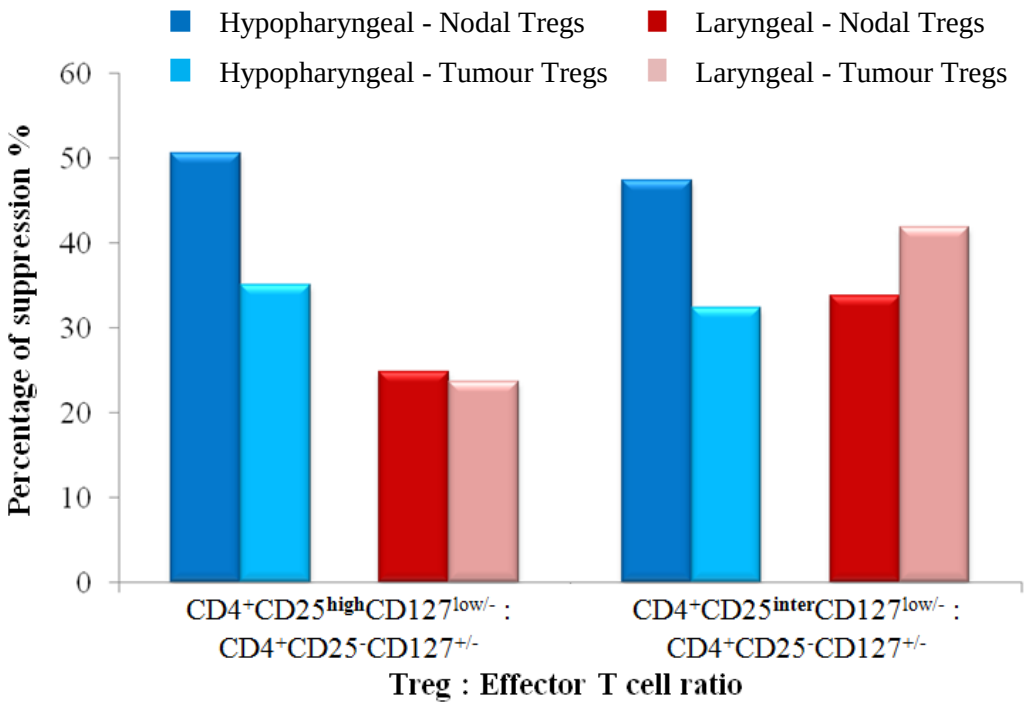


Figure 5.9 Percentages of suppression induced by $CD25^{high}$ and $CD25^{inter}CD127^{low/-}$ Tregs on the proliferation of $CD25^{-}CD127^{-/+}$ effector T cells from tumour infiltrated lymph nodes and corresponding primary tumour samples
FACS isolated $CD4^{+}CD25^{high}CD127^{low/-}$ and $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs from either tumour infiltrated lymph nodes or the primary tumour microenvironment were co-cultured with CFSE labelled autologous $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells at a ratio of 1:1 for four days before being acquired by flow cytometry and the data analysed by ModFit LT^{TM} to determine the percentage of suppression induced by the Tregs.

5.3.4.3 Suppressive activity of Tregs on effector T cells in the tumour microenvironment and peripheral circulation of HNSCC patients

Due to the primary tumour and tumour infiltrated lymph node specimens showing different trends the samples were not combined when the levels of suppressive activity induced in the tumour microenvironment and peripheral circulation were compared.

Of the Tregs from tumour infiltrating lymph nodes that were assessed for suppressive activity ten of the specimens had corresponding samples from the peripheral circulation that were also successfully analysed for Treg suppressive function. Both of the primary tumour specimens had corresponding PBMC samples and the mean percentages have been presented, however, due to low numbers, statistical analysis was not performed but the data are presented (Table 5.12).

Table 5.12 Percentage of suppression induced by Tregs on the proliferation of effector T cells in the tumour microenvironment and peripheral circulation of HNSCC patients separated by tumour subsite

Cohort	Treg : Effector T cell			
	$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^+CD127^+$	$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^+CD127^+$
Nodal samples (n=10)	24.34 ± 4.99	27.08 ± 4.04	16.10 ± 4.34	19.26 ± 3.95
Corresponding PBMC samples (n=10)	26.79 ± 5.77	18.77 ± 4.50	23.26 ± 5.76	18.06 ± 2.62
p value	0.67	0.29	0.26	0.84
Laryngeal nodal (n=5)	19.12 ± 4.38	27.52 ± 3.56	14.45 ± 4.26	18.28 ± 5.62
Laryngeal PBMC (n=5)	23.52 ± 10.33	19.66 ± 8.34	19.63 ± 10.09	19.74 ± 4.08
p value	0.65	0.51	0.65	0.88
Oropharyngeal nodal (n=4)	25.10 ± 9.93	20.68 ± 6.45	12.40 ± 7.17	18.55 ± 7.68
Oropharyngeal PBMC (n=4)	28.80 ±7.77	20.08 ± 5.25	22.75 ± 8.38	16.13 ± 4.63
p value	0.71	0.96	0.36	0.83
Primary tumour samples (n= 2)	37.15 ± 4.75	29.40 ± 5.70	34.40 ± 11.30	19.00 ± 1.00
Corresponding PBMC samples (n=2)	41.25 ± 6.15	27.10 ± 18.00	44.40 ± 4.60	25.80 ± 8.40
p value	*	*	*	*

All conducted at a 1:1 ratio
Values mean percentage ± SEM
Paired student's t test comparing between tissue infiltrating Tregs and peripheral Tregs.
* Primary tumour cohort not statistically assessed due to low sample numbers

The data in Table 5.12 show that, regardless of the Treg population ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) examined or the effector T cell population suppressed ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$), Tregs isolated from the tumour infiltrated lymph nodes and peripheral circulation induce similar levels of suppression on their autologous effector T cells.

5.3.4.4 Assessing the suppressive activity of tumour Tregs on tumour infiltrating and peripheral effector T cells

To determine whether the tumour influences the effector T cell's susceptibility to Treg induced suppression, Tregs isolated from tumour infiltrated lymph nodes were co-cultured with autologous effector T cells isolated from the tumour infiltrated node and peripheral effector T cells to compare the levels of suppression. The same experiments were performed for the primary tumour samples, however due to the sample number being low the results were not statistically compared.

Irrespective of the Treg population analysed ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) or the effector T cell population being suppressed ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$), the co-culture of nodal Tregs with nodal effector T cells observed higher percentages of suppression compared with the incubation of nodal Tregs with peripheral effectors, however these differences did not reach significance (Table 5.13). The cohorts were also divided by tumour subsite but no statistical differences were observed between patients with cancer of the larynx or oropharynx (data not shown). A similar trend was observed for the level of suppression shown by primary tumour Tregs on primary tumour effectors which was greater than that observed for the co-culture of primary tumour Tregs and peripheral effectors from the same patient (Table 5.13).

Table 5.13 Percentage of suppression induced by nodal and primary tumour infiltrating Tregs on the proliferation of autologous effector T cells

	Treg : Effector T cell			
Cohort	$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^+CD127^+$	$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^+CD127^+$
Nodal Tregs and peripheral effectors (n=10)	17.19 ± 3.96	14.08 ± 4.55	10.57 ± 3.96	10.53 ± 2.45
Nodal Tregs and nodal effectors (n=10)	24.34 ± 4.99	27.08 ± 4.04	16.10 ± 4.34	19.26 ± 3.95
p value	0.15	0.09	0.16	0.09
Primary tumour Tregs and peripheral effectors (n= 2)	24.30 ± 12.8	19.85 ± 10.75	29.45 ± 2.95	17.35 ± 0.65
Primary tumour Tregs and primary tumour effectors (n=2)	37.15 ± 4.75	29.40 ± 5.70	34.40 ± 11.30	19.00 ± 1.00
p value	*	*	*	*

All conducted at a 1:1 ratio
Values mean percentage ± SEM
Paired student's t test or Wilcoxon Signed Rank test.
* Primary tumour cohort not statistically assessed due to low sample numbers

5.3.4.5 Assessing the suppressive activity of tumour and peripheral Tregs on peripheral effector T cells

To directly analyse the suppressive function of Tregs isolated from tumour infiltrated lymph nodes and the peripheral circulation the levels of suppression were compared when the Tregs from the two different environments were both co-cultured with autologous peripheral effector T cells. It was demonstrated that, regardless of CD25 expression by the Tregs, the percentages of suppression induced by peripheral Tregs was always greater than nodal Tregs, irrespective of the effector population being suppressed. This difference reached significance for all of the Treg populations with the exception of those expressing high levels of CD25 when co-cultured with the $CD4^{+}CD25^{-}CD127^{-/+}$ effectors; the same trend was observed for the primary tumour cohort (Table 5.14). Unfortunately there were insufficient cells to assess the suppressive function of nodal and peripheral Tregs on the proliferation of tumour infiltrated lymph node effector T cells.

Table 5.14 Percentage of suppression induced by nodal, primary tumour and peripheral Tregs on the proliferation of autologous peripheral effector T cells

	Treg : Effector T cell			
Cohort	$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^-CD127^{-/+}$	$CD4^+CD25^{inter}CD127^{low/-}$: $CD4^+CD25^+CD127^+$	$CD4^+CD25^{high}CD127^{low/-}$: $CD4^+CD25^+CD127^+$
Nodal Tregs and peripheral effectors (n=10)	17.19 ± 3.96	14.08 ± 4.55	10.57 ± 3.96	10.53 ± 2.45
Peripheral Tregs and peripheral effectors (n=10)	26.79 ± 5.77	18.77 ± 4.50	23.26 ± 5.76	18.06 ± 2.62
p value	**0.01**	0.25	**0.02**	**0.01**
Primary tumour Tregs and peripheral effectors (n= 2)	24.30 ± 12.8	19.85 ± 10.75	29.45 ± 2.95	17.35 ± 0.65
Peripheral Tregs and peripheral effectors (n=2)	41.25 ± 6.15	27.10 ± 18.00	44.40 ± 4.60	25.80 ± 8.40
p value	*	*	*	*

All conducted at a 1:1 ratio
Values mean percentage ± SEM
Paired student's t test or Wilcoxon Signed Rank test.
Results in bold considered significant when $p<0.05$
* Primary tumour cohort not statistically assessed due to low sample numbers

5.4 Discussion

The composition of the head and neck tumour sample cohort collected throughout this study was influenced by the treatment strategies employed by the Hull ENT department for HNSCC patients. With the improvement of chemotherapy regimens the excision of primary tumours has decreased year on year. In addition, radiotherapy and endoscopic laser surgery are preferred treatment strategies particularly in the management of early stage laryngeal cancer where there is a risk that conventional surgery may affect the function of the larynx (Section 1.7). In oropharyngeal cancer the location of the tumour can also be hard to access by surgery, however undergoing a neck dissection is a favoured method for the treatment of metastatic lymph nodes due to the complication rate being low and larger lymph nodes being unsuccessfully treated by chemo/radiotherapy (Scott-Brown *et al.*, 2008). This resulted in a larger number of lymph node metastases being investigated rather than primary tumour samples in the current study.

Tumour infiltrating Tregs have been identified in numerous cancer types however, their role in tumour immunity and their use as a prognostic determinant remains to be clarified (Table 1.9). The aim of the current study was to further understand the Treg population infiltrating the head and neck tumour microenvironment by assessing their phenotype, frequency and suppressive activity. In addition, the influence of the tumour microenvironment on $CD4^+$ effector T cell populations was also examined. To the authors knowledge this is the first study to examine the frequency and function of $CD127^{low/-}$ Tregs infiltrating the tumour microenvironment of newly-presenting HNSCC patients. In addition the suppressive activity of Tregs of any phenotype from tumour associated draining lymph nodes has not previously been assessed in HNSCC.

5.4.1 $Foxp3^+$ cells infiltrating the tumours of head and neck cancer patients

The infiltration of $Foxp3^+$ cells (Tregs) was assessed by immunohistochemistry in the tumour associated stroma and tumour nests of HNSCC specimens. In the whole patient cohort there was a significantly greater number of $Foxp3^+$ cells present in the stroma

compared with the tumour, and this significant trend was maintained as the patient group was divided by several clinicopathological features. The preferential accumulation of $Foxp3^+$ cells in stromal tissue/surrounding the tumour sites instead of infiltrating into the tumour cells has also been observed in colon, breast, oral SCC and cervical cancer (Lee *et al.,* 2010b; Liu *et al.,* 2011; Watanabe *et al.,* 2010; Wu *et al.,* 2011). From these studies it could be suggested that Tregs primarily accumulate and exert their suppressive activity upon the effector T cell population from within the stromal region.

In both the stroma and tumour nests, a significantly higher number of infiltrating $Foxp3^+$ cells were found in oropharyngeal tumours compared with the microenvironment of laryngeal tumours. Treg infiltration in HNSCC patients tends not to be assessed with regard to tumour subsite despite the differences in aetiology, clinical presentation and rates of survival, however, Sun *et al.,* (2012) reported no significant association between head and neck primary tumour site and the number of infiltrating $Foxp3^+$ Tregs. Although these results are in contrast to those reported by the current study, Sun and colleagues did not have any oropharyngeal cancer patients within their recruited cohort. An increasing proportion of oropharyngeal tumours are now reported to be HPV positive. The HPV status of the tumour specimens in the current study are unknown, however, it has been reported by Nasman and colleagues (2012) that patients with tonsillar SCC positive for HPV had a significantly higher number of infiltrating $Foxp3^+$ cells compared with HPV negative tumours; the HPV status of the tumour specimens may therefore influence subsite specific differences for the infiltration of $Foxp3^+$ cells.

No significant differences were observed in the stroma or tumour when the patient cohort was divided by tumour stage or nodal status. The lack of correlation between tumour stage and/or nodal involvement is in agreement with some results published for breast, gastric, colorectal, head and neck and nasopharyngeal cancer patients (Gobert *et al.,* 2009; Mizukami *et al.,* 2008b; Salama *et al.,* 2009; Sun *et al.,* 2012; Zhang *et al.,* 2010). However, other studies have reported a significant association between tumour stage and $Foxp3^+$ Treg infiltration; in colorectal cancer and nasopharyngeal carcinoma early stage tumours were significantly correlated with a higher number/density of infiltrating $Foxp3^+$ Tregs compared with tumours of the advanced stage (Frey *et al.,* 2010; Salama *et al.,* 2009; Zhang *et al.,* 2010). In contrast patients with advanced stage endometrial cancer had a significantly higher number of $Foxp3^+$ cells compared with early stage tumours (de Jong *et*

al., 2009). In addition, breast and cervical cancer patients with lymph node metastases were demonstrated to have a higher percentage/number of infiltrating Foxp3^{+} Tregs compared to patients without nodal involvement (Bates *et al.,* 2006; Wu *et al.,* 2011), whereas the opposite trend was reported in colorectal cancer patients (Frey *et al.,* 2010). The reasons for these discrepancies may be down to the different types of tumour investigated or the different methods of assessment of the immunostaining.

The main purpose of the immunostaining in this study was to confirm the presence of the tumour infiltrating Tregs so that the study could then go on to isolate and investigate the Tregs further and assess their prevalence and functional activity in HNSCC.

5.4.2 Optimising the isolation of TIL from HNSCC specimens

To assess the frequency and suppressive activity of Tregs infiltrating the tumours of HNSCC patients it was necessary to establish a method to isolate TIL from head and neck tumour specimens.

Previous work in the author's laboratory had determined the optimal concentration of enzymes required to dissociate head and neck tumour samples and preserve integrin expression (Woods *et al.,* 2011). Strauss and colleagues (2007b) had also used the same enzymes to isolate TIL from the head and neck tumour microenvironment. The concentration of DNase employed by the current study matched that used by Strauss and colleagues however, a lower concentration of collagenase was used (0.05%; w/v) in order to preserve the expression of the lymphocyte markers for the isolation of Treg and effector T cell populations by FACS.

The isolation of the maximum number of TIL from HNSCC specimens was imperative as this would be the limiting factor in assessing the functional activity of tumour infiltrating Tregs. Although previous cancer studies have identified Tregs by flow cytometry immediately following dissociation, without the removal of tumour cells (Gobert *et al.*, 2009; Lau *et al.,* 2007; Viguier *et al.,* 2004), it was demonstrated in the current investigation that by using this method the lymphocyte population was very difficult to identify by flow cytometry due to the presence of numerous cell types including epithelial cells and fibroblasts. It was subsequently shown that by culturing the dissociated tumour fragments overnight, to remove adherent epithelial cells, fibroblasts and monocytes, a more

defined lymphocyte population was obtained. This method is slightly different to that used by many other research groups, including Strauss *et al.* (2007b) in HNSCC, which have used a discontinuous Ficoll-Paque gradient to separate lymphocytes from dissociated tissue before proceeding to identify Tregs (Chi *et al.*, 2010; Feng *et al.*, 2011; Lin *et al.*, 2012; Shen *et al*., 2009). Although a few of these studies went on to isolate and assess the functional activity of the identified Tregs low sample numbers were used ($n \leq 5$; Liotta *et al.*, 2010; Woo *et al.*, 2002; Zhou *et al.*, 2009) possibly due to the low number of Tregs isolated. However, Chi and colleagues (2010) and Strauss *et al.* (2007b) did examine the suppressive activity of Tregs isolated from ten specimens of bladder carcinoma and HNSCC, respectively. The reason a discontinuous gradient was not used in the current study was to try and minimise any loss of the lymphocytes during isolation.

It has been demonstrated previously that proteolytic enzymes, such as collagenase and DNase, can reduce the expression of T lymphocyte markers from tonsil samples and peripheral blood (Mulder *et al.*, 1994; Van Damme *et al.*, 2000). In agreement, the current study found that although an increased length of dissociation resulted in improved tissue disaggregation, this had to be weighed up against the detrimental effect that collagenase and DNase had on the expression of lymphocyte markers; with overnight disaggregation resulting in reduced expression of CD4, CD25 and CD127. An appropriate length of dissociation for a HNSCC specimen was determined to be 2-4 hours, which consistently provided a TIL population that could be gated on the basis of CD4, CD25 and CD127 expression to identify and isolate Treg and effector T cell populations.

The intensity of CD4 expression varied more in TIL compared with the same patient's peripheral $CD4^+$ population. The 'spread' of CD4 expression in malignant tissue compared with the peripheral circulation has also been published by Shen *et al.* (2010) in hepatocellular carcinoma. In this instance, the $CD4^+$ population in malignant hepatocellular tissue was not as clearly distinguished as in HNSCC. The reason for the variation of CD4 expression in malignant tissue is unknown but it could be suggested that the tumour microenvironment may influence the expression of CD4 on the infiltrating lymphocyte population.

5.4.3 Phenotype and frequency of T cell subsets in the tumour microenvironment of HNSCC patients

The phenotype and frequency of various T cell subsets in both the tumour infiltrated lymph nodes and primary tumour microenvironment of HNSCC patients were assessed by flow cytometry. This study was designed to develop our understanding of the tumour infiltrated nodal environment and how it might contribute to the poorer prognosis in those patients with nodal involvement (Section 1.2.3). In the resected lymph nodes, although patients with cancer of the larynx were observed to harbour a slightly higher frequency of $CD4^+$ lymphocytes and $CD4^+CD25^+CD127^{low/-}$ Tregs and a lower prevalence of $CD4^+CD25^{-/+}CD127^{-/+}$ effector T cells compared with oropharyngeal cancer patients these differences did not reach significance and the prevalence of the remaining T cell subsets were similar between the two head and neck subsites. These findings are in contrast with the results observed during the immunohistochemical staining of HNSCC primary tumour specimens where oropharyngeal tumours had a greater infiltration of $Foxp3^+$ Tregs compared with laryngeal tumours. These differences may be attributed to the different microenvironments assessed, the tumour infiltrated lymph nodes and primary tumour site, or the method of Treg identification. By immunohistochemistry the identification of Tregs relied upon the expression of the single marker Foxp3, whereas in the dissociated tumour samples the regulatory population was identified by flow cytometry using a much more stringent phenotype utilising the markers CD4, CD25 and the lack of CD127 expression. Furthermore, the patients studied by immunohistochemistry were different compared to those used in the dissociated tumour experiments and the HPV status of the patient specimens was unknown. Tumours positive for HPV differ by aetiology and prognosis (Section 1.5.2) and it has been reported that HPV positive tumours had a significantly higher number of infiltrating $Foxp3^+$ T cells compared with HPV negative tumours for patients with tonsillar SCC (Nasman *et al.*, 2012), therefore this could influence the results observed.

The infiltration and prevalence of Tregs into tumour draining lymph nodes has been assessed in several other cancer types, including gastric (Kawaida *et al.*, 2005; Maruyama *et al.*, 2010b; Mizukami *et al*., 2008b; Shen *et al.*, 2009), melanoma (Viguier *et al.*, 2004), pancreatic, breast (Liyanage *et al*., 2002) and ovarian (Curiel *et al.*, 2004) cancer. These

studies have predominately focused on the differences between tumour infiltrated and tumour free lymph nodes, demonstrating in the majority of cases that metastatic lymph nodes accumulate a significantly higher frequency of Tregs compared with tumour free lymph nodes isolated from the same patient (Kawaida *et al*., 2005; Maruyama *et al*., 2010b; Mizukami *et al.*, 2008b; Viguier *et al.*, 2004). In contrast, Curiel *et al.* (2004) reported in ovarian cancer that tumour draining lymph nodes had a lower prevalence of $CD4^+CD25^+$ Tregs compared with lymph nodes from individuals without cancer. These differences could be attributed to the types of cancer analysed, the phenotype employed to identify the Treg population or the source of tumour draining and control lymph nodes. The aim of the current work was to evaluate the prevalence of different $CD4^+$ T cell subsets in HNSCC with regard to tumour subsite and therefore ethical approval was not sought to collect tumour free lymph nodes.

Another study to use the $CD127^{low/-}$ phenotype to identify Tregs in tumour draining lymph nodes was Shen and colleagues (2009). In their entire cohort (n=57) the mean percentage of Tregs identified was 18.36% which was almost two fold lower than that observed by the current study (36.07%) however, as discussed in Chapter 4, this could be due to a number of methodological variations including different cancer types and the gating strategies employed to isolate the Tregs.

The HNSCC specimens obtained from primary tumour sites were few in number and consisted mainly of advanced stage cancers (T3 and T4) that had arisen from the larynx, therefore effects of tumour stage and subsite were unable to be determined. Other studies investigating the prevalence of T cell subsets infiltrating head and neck tumours by flow cytometry also had limited patient cohorts and comparisons between tumour subsites and stages were also not assessed (Gasparoto *et al.*, 2010; Strauss *et al*., 2007b). For other cancer types it has been reported that tumours of the advanced stage had a significantly elevated frequency of infiltrating Tregs compared with early stage tumours; hepatocellular (Shen *et al.*, 2010), renal (Liotta *et al.*, 2010), gastric (Ichihara *et al*., 2003; Shen *et al.*, 2009) and ovarian (Curiel *et al.*, 2004) carcinoma. However, in oesophageal cancer there was no significant difference observed in Treg frequency between early and advanced stage tumours (Maruyama *et al.*, 2010a).

On comparison of T cell susbets in the tumour infiltrated lymph nodes and primary tumour microenvironment, the lymph nodes were observed to have a higher proportion of

$CD4^+$ lymphocytes compared with the primary tumour microenvironment, although this difference was not significant and the prevalence of the remaining T cell subsets was similar between the two locations. Pretscher *et al.* (2009) have also reported similar frequencies of $Foxp3^+$ Tregs infiltrating the primary tumour and metastatic lymph nodes of HNSCC patients using immunohistochemistry, implying that the metastasised tumour recruits T cells in a similar manner to the primary tumour.

5.4.3.1 Phenotype and frequency of T cell subsets in the tumour microenvironment compared with the peripheral circulation of HNSCC patients

To assess the differences between the infiltrating and circulating T cell subsets, proportions of various $CD4^+$ populations were compared between the tumour microenvironment and peripheral circulation of head and neck cancer patients.

When examining Treg phenotypes, no significant differences were observed for the prevalence of $CD4^+CD25^+$ cells between the tumour and periphery. This is in agreement with Woo and colleagues (2002) who also reported no significant differences in the prevalence of these cells in the tumour microenvironment and peripheral circulation of patients with non-small cell lung cancer. In the current study, there was a significantly higher frequency of $CD4^+$ cells expressing high levels of the IL-2 receptor in the tumour microenvironment compared with the peripheral circulation; although this trend was still evident when the cohort was divided by tumour subsite, significance was not maintained.

In contrast, $CD127^{low/-}$ Tregs, regardless of the level of CD25 expression, were significantly elevated in the tumour microenvironment compared with the peripheral circulation and this trend was maintained in the laryngeal cohort alone but only for the $CD25^+CD127^{low/-}$ and $CD25^{high}CD127^{low/-}$ populations in the oropharyngeal cohort. This is in agreement with Shen *et al.* (2009) who also found significantly elevated proportions of $CD4^+CD25^+CD127^{low/-}$ Tregs in TIL (mean 21.08%) compared with the peripheral circulation (mean 7.77%) of gastric cancer patients; again these percentages are approximately two fold lower than those observed in the current study, TIL (mean 35.13%) and peripheral circulation (mean 16.51%), however, as discussed previously this is likely to be due to the different gating strategies employed to identify $CD127^{low/-}$ Tregs and the different cancer types being assessed.

In head and neck cancer Strauss *et al.* (2007b) reported a significantly higher level of $CD4^{+}CD25^{high}$ and $CD4^{+}CD25^{+}$ Tregs in the tumour microenvironment (n=15) compared with the peripheral circulation (n=14) of patients. Although the results published for the $CD4^{+}CD25^{+}$ phenotype differ from those observed by the current study, the majority of patients whose tumour infiltrating Tregs were assessed by Strauss and colleagues had oral cancer whilst the patient cohort from the current study consisted mainly of patients with cancer of the larynx and oropharynx, which is likely to contribute to the different findings. Furthermore, in contrast with the work conducted for this thesis, Strauss *et al.* did not use matched patient samples for the tumour and peripheral cohorts with only five of the fifteen patients providing both tumour and PBMC samples. The remaining nine peripheral blood samples were provided by HNSCC patients with active disease, however, their clinicopathological characteristics were unknown. It was deemed important for the current study that the two sample cohorts originated from the same patients to ensure tumour subsite and stage remained constant and therefore the prevalence of specific T cell subsets, including Tregs, in the tumour microenvironment and peripheral circulation were under the influence of the same clinicopathological features.

The significant elevation of Tregs in TIL, identified using various phenotypic markers, compared with the peripheral circulation has also been reported in several different cancer types (Table 5.2) and could suggest that the tumour is somehow recruiting or expanding the suppressive Treg population. In ovarian, oesophageal and gastric cancer it has been demonstrated that CCL17 and CCL22 have the ability to induce the migration of tumour infiltrating Tregs and it is therefore suggested that these chemokines are involved in the trafficking of the Treg population (Curiel *et al.*, 2004; Maruyama *et al.*, 2010a; Mizukami *et al.*, 2008b). In support of this a significant correlation between the degree of CCL22 and/or CCL17 positive cells and the frequency of Tregs in the tumour microenvironment of breast, oesophageal and gastric cancer patients has been reported (Gobert *et al.*, 2009; Maruyama *et al.*, 2010a; Mizukami *et al.*, 2008b). The mechanisms employed to promote the expansion of Tregs are not fully understood. In both murine and human studies it has been shown that the Treg population can expand either through the proliferation of recruited Tregs or by the induction of Tregs from naïve precursors (Colombo and Piconese, 2007; Curti *et al.*, 2007; Gobert *et al.*, 2009; Knutson *et al.*, 2007). In particular for tumour infiltrated lymph nodes the elevated frequency of Tregs could arise

from the expansion of the regulatory population already residing in the lymph nodes (Viguier *et al.*, 2004).

The prevalence of lymphocytes positive for the expression of CD4 was similar between the tumour microenvironment and periphery. However, in the peripheral circulation a significantly higher frequency of effector T cells, $CD4^{+}CD25^{inter}$, $CD4^{+}CD25^{-/+}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$, were observed compared with the tumour microenvironment. This trend was maintained when the cohort was divided by tumour subsite and reached significance for both the laryngeal and oropharyngeal cohorts when the frequency of $CD4^{+}CD25^{-/+}CD127^{-/+}$ cells were examined however, significance was only reached for the patients with cancer of the larynx when the prevalence of activated $CD4^{+}CD25^{+}CD127^{+}$ effector T cells were assessed. This suggests a reciprocal balance between the effector T cell and Treg populations in the tumour microenvironment and peripheral circulation of HNSCC patients.

Previous studies assessing the prevalence of Tregs infiltrating the tumour microenvironment focus mainly on the regulatory population and do not additionally analyse the frequency of effector populations that can be influenced by the suppressive T cells; however it is interesting to observe that in the peripheral circulation a significantly higher prevalence of activated effector T cells were observed. Nevertheless, the frequency of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells was similar between the periphery and tumour microenvironment. The reduced prevalence of activated effector T cells in the tumour microenvironment is likely to be due to the actions implemented by the tumour to prevent the initiation of the host's anti-tumour attack; this includes the suppressive influence of the elevated level of Tregs (Shevach, 2009) as well as the impairment of antigen presentation and the inhibitory effect of the enzyme indoleamine 2,3-dioxygenase (Section 1.8).

5.4.4 The suppressive activity of Tregs isolated from the tumour microenvironment of HNSCC patients

An elevated level of Tregs in the tumour microenvironment of HNSCC patients has been demonstrated but it is their suppressive activity which will also influence how

effective the host's anti-tumour response is in combating the growth and progression of the tumour.

In keeping with the work performed in the peripheral circulation (Chapter 4) the level of suppression induced by tumour infiltrating lymph node Tregs at the ratios 1:1, 1:2. 1:5 and 1:10 was assessed and in agreement with previous studies (Kawaida *et al*., 2005; Liyanage *et al.*, 2002; Viguier *et al.*, 2004) Tregs ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) inhibited the proliferation of effector T cells ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) in a ratio dependent manner; as the proportion of Tregs increased so did the level of suppression. Tregs isolated from the primary tumour of various cancer types have also been shown to suppress the proliferation of autologous effector T cells in a ratio dependent manner (Chi *et al*., 2010; Gobert *et al.*, 2009; Ling *et al.*, 2007; Zhou *et al.*, 2009), however, this was not possible in the current study due to the low number of Tregs isolated from the head and neck primary tumours. In contrast, Strauss *et al.* (2007b) demonstrated that Tregs isolated from the TIL of head and neck cancer patients induced similar levels of suppression at both a 1:2 and 1:5 ratio (Treg:effector T cell). With the majority of studies using the 1:1 ratio and to maintain consistency, all suppression experiments conducted by the current study were established at the 1:1 ratio and the results from these experiments were used for comparison.

$CD127^{low/-}$ Tregs, with both intermediate and high levels of CD25 expression, isolated from either tumour infiltrated lymph nodes or the primary tumour site of HNSCC patients had the ability to suppress the proliferation of autologous effector T cells ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$).

Tregs, regardless of their CD25 expression, isolated from tumour infiltrated lymph nodes induced a greater level of suppression on the proliferation of $CD4^+CD25^-CD127^{-/+}$ effector T cells compared with the $CD4^+CD25^+CD127^+$ effector T cells, and this trend was maintained in both the laryngeal and oropharyngeal sample cohorts. This is in agreement with the observations made in the peripheral circulation (Section 4.3.3.5).

For the entire HNSCC patient cohort and for patients with laryngeal cancer Tregs expressing high levels of the IL-2 receptor induced a slightly higher level of suppression on the proliferation of $CD4^+CD25^-CD127^{-/+}$ effector T cells compared with $CD4^+CD25^{inter}CD127^{low/-}$ Tregs, however, no statistical differences were observed. This is in contrast to the results shown in the peripheral circulation where $CD25^{inter}$ Tregs

consistently suppressed the proliferation of both effector T cell populations to a greater degree compared with $CD25^{high}$ Tregs, reaching significance for a number of HNSCC patient cohorts and healthy controls (Section 4.3.3.4). This difference between the tumour infiltrating and peripheral Tregs could be attributed to the immunosuppressive conditions of the tumour microenvironment influencing the functional ability of both the $CD25^{inter}CD127^{low/-}$ and $CD25^{high}CD127^{low/-}$ Tregs. To establish whether the level of CD25 expression does influence the suppressive capacity of $CD127^{low/-}$ Tregs infiltrating head and neck tumours a larger cohort of patients would need to be assessed.

As previously mentioned, suppressive $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{high}$ Tregs have been isolated from a range of different primary tumour sites (Section 5.1) as well as the tumour infiltrated lymph nodes of patients with pancreatic cancer (Liyanage *et al*., 2002), gastric carcinoma (Kawaida *et al*., 2005) and melanoma (Viguier *et al.*, 2004). However, although these studies have shown the presence of a functionally suppressive infiltrating T cell population the level of suppressive activity has not been assessed with regard to clinicopathological features which might be attributed to the low number of patients investigated ($n \leq 5$; Curiel *et al.,* 2004; Gobert *et al.,* 2009; Kryczek *et al.,* 2009; Ling *et al.,* 2007; Liotta *et al.,* 2010; Liyange *et al.,* 2002; Woo *et al.,* 2002; Zhou *et al.,* 2009).

In the tumour infiltrated lymph nodes similar levels of suppression, regardless of the Treg phenotype analysed ($CD4^{+}CD25^{inter}CD127^{low/-}$ and $CD4^{+}CD25^{high}CD127^{low/-}$) or the effector T cell population being suppressed ($CD4^{+}CD25^{-}CD127^{-/+}$ and $CD4^{+}CD25^{+}CD127^{+}$), were observed for the laryngeal and oropharyngeal cohorts. Due to the low number of primary tumour samples that were successfully assessed for suppressive activity, differences in the level of suppression between tumour subsites or stages could not be assessed. Strauss *et al.* (2007b) have also examined the suppressive activity of Tregs infiltrating the tumours of HNSCC patients (n=10) however, due to the majority of their patients having cancer of the oral cavity comparisons between tumour subsites could not be analysed. Greater sample numbers would be required to determine whether subsite and stage influences the level of suppression induced by Tregs. In addition, this would help establish whether Treg suppressive activity varies between tumour infiltrated lymph nodes or the primary tumour site.

5.4.4.1 Comparison of the suppressive activity of Tregs in the tumour microenvironment and peripheral circulation of HNSCC patients

With the tumour microenvironment shown to elevate the proportion of Tregs in the infiltrating lymphocyte population compared with the peripheral circulation it was of interest as to whether the tumour also affected the suppressive capacity of Tregs.

It was hypothesised that Tregs isolated from the tumour microenvironment would induce a significantly greater level of suppression as the tumour may directly influence these cells in close proximity, promoting actions to thwart the host's anti-tumour response. However, no significant differences were observed between the level of suppression induced by tumour infiltrating Tregs and peripheral Tregs on the proliferation of their respective effector T cell populations. These results were shown for Tregs isolated from the tumour infiltrating lymph nodes and by increasing the sample cohort it would be interesting to determine whether the same statistical trend occurs for Tregs infiltrating the primary tumour microenvironment. Chi *et al.* (2010) also reported no significant differences between the regulatory function of Tregs isolated from the PBMC (n=10) and TIL (n=10) of bladder carcinoma patients when co-cultured with their autologous effector T cells at a 1:1 ratio.

5.4.4.2 The suppressive activity of tumour Tregs on tumour infiltrating and peripheral effector T cells

The level of suppression observed during the co-culture of tumour infiltrating Tregs and infiltrating effector T cells may have been influenced by the susceptibility of the effector population to Treg induced suppression. For example, the effector T cells having infiltrated the tumour may have been 'conditioned' by the tumour's immunosuppressive microenvironment causing them to already have been suppressed *in vivo* and therefore be less susceptible to the suppressive activity of Tregs *in vitro*. Consequently the full functional capacity of tumour infiltrating Tregs might have been masked. To assess this, tumour infiltrating nodal Tregs were also co-cultured with their autologous peripheral effector T cells. However, although not significant, a higher level of suppression was observed during the co-culture of nodal Tregs and nodal effectors compared with the co-

culture of nodal Tregs and peripheral effectors, irrespective of the Treg population ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) analysed or the effector T cell population being suppressed ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$). The same trend was also observed with the primary tumour Tregs. These results imply that the immunosuppressive conditions of the tumour microenvironment may in fact cause the tumour infiltrating effector T cells to become more susceptible to the actions of the tumour Tregs, once again, a larger cohort would be required to confirm this trend.

5.4.4.3 The suppressive activity of tumour and peripheral Tregs on peripheral effector T cells

To directly compare the suppressive capacity of tumour infiltrating and peripheral Tregs, the regulatory populations from the two different environments were both co-cultured with autologous peripheral effector T cells. It was demonstrated that, regardless of the Treg or effector T cell phenotype, the level of suppression induced by peripheral Tregs on peripheral effector T cells was greater than that observed for the co-culture of tumour infiltrated lymph node Tregs and peripheral effectors; this reached significance for the $CD25^{inter}$ Tregs on both effector T cell populations investigated ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) and $CD25^{high}$ Tregs on the $CD4^+CD25^+CD127^+$ effector T cells. The same trend was also shown for the primary tumour Tregs. It was hypothesised that the Tregs isolated from the tumour may have had a greater suppressive ability than the peripheral Tregs due to the immediate tumour influence, however, the results suggest that Tregs isolated from the primary tumour site or tumour infiltrated lymph nodes are not influenced by the tumour to induce a greater level of suppression on the proliferation of effector T cells compared with peripheral Tregs.

Kryczek *et al.* (2009) have also directly compared the suppressive activity of Tregs from the peripheral circulation and tumour microenvironment in a small cohort of patients (n=4) with colorectal cancer by co-culturing the suppressive T cells with effector T cells isolated from a healthy donor. It was demonstrated that peripheral and tumour infiltrating Tregs induced similar levels of suppression on effector T cell proliferation however, it was unclear how many patient's peripheral Tregs were assessed for suppressive activity and whether these patients also had colorectal cancer or a range of various cancer types as the

study had also recruited patients with melanoma, ovarian, renal, hepatic and pancreatic cancer. In ovarian carcinoma, Curiel *et al.* (2004) assessed the suppressive activity of tumour infiltrating (n=5) and peripheral Tregs on the proliferation of effector T cells isolated from tumour ascites. Similar levels of suppression were observed demonstrating that tumour infiltrating Tregs were as efficient as peripheral Tregs at inhibiting T cell proliferation however, it was unclear whether the peripheral and tumour cohorts consisted of the same patients.

Unfortunately due to insufficient cell numbers it was not possible to co-culture tumour infiltrated nodal and peripheral Tregs with effector T cells isolated from the tumour microenvironment. This would have helped establish whether Tregs optimally suppress the proliferation of effector T cells from the same environment from which they were isolated.

The experiments conducted during this study have assessed the suppressive activity of Tregs based on their ability to suppress the proliferation of effector T cells. However, Tregs also have the ability to suppress the functional actions of effector T cells such as the secretion of cytokines and it may be this which the Tregs are influencing in the tumour microenvironment. As shown by other research groups tumour infiltrating Tregs isolated from melanoma (Viguier *et al*., 2004), bladder (Chi *et al.*, 2010), breast (Gobert *et al.*, 2009), hepatocellular (Zhou *et al*., 2009), pancreatic (Liyanage *et al.*, 2002) and renal (Liotta *et al.*, 2010) cancer have the ability to suppress the secretion of IFN-γ by $CD4^{+}CD25^{-}$ infiltrating effector T cells. It has been reported in the metastatic lymph nodes of melanoma patients that at low co-culture ratios, 1:8 and 1:10 (Treg:effector T cell), the inhibition of cytokine secretion was still marked, however the inhibition of proliferation was weaker (Viguier *et al.*, 2004). This suggested to Viguier and colleagues that the tumour infiltrating Tregs may be more suppressive on effector T cell function rather than proliferation. To the author's knowledge, the inhibition of IFN-γ secretion induced by Tregs isolated from the tumour microenvironment of HNSCC patients has not previously been assessed, or compared to that exerted by peripheral Tregs. Examining this association in the HNSCC microenvironment would possibly elucidate a further mechanism tumour infiltrating Tregs could use to promote an immunosuppressive environment.

It is likely in the tumour microenvironment that the suppressive role of Tregs covers many aspects of effector T cell biology including activation, function and proliferation in

attempts to inhibit the actions of the host's anti-tumour response and promote the growth and progression of the tumour.

5.4.5 Conclusion

The current study has observed a significant increase in the proportion of Tregs in the infiltrating lymphocyte population compared with the peripheral circulation of newly-presenting HNSCC patients, regardless of the Treg phenotype examined. For the first time $CD127^{low/-}$ Tregs have been identified and isolated from the TIL of head and neck cancer patients and it has been demonstrated that the level of suppression induced by tumour infiltrating Tregs and peripheral Tregs on the proliferation of their respective effector T cell populations was similar. However, it is likely that there is an overall greater level of suppression induced by Tregs in the tumour microenvironment compared with the peripheral circulation due to the elevation of the proportion of Tregs infiltrating the tumour microenvironment.

With similar levels of suppression induced by tumour infiltrating and peripheral Tregs it could be suggested that a number of different suppressive mechanisms are employed by Tregs to promote an overall immunosuppressive environment favourable to the growth and progression of HNSCC, including the suppression of proliferation, activation or function of immune cells. By investigating different methods of Treg suppression, such as the secretion of cytokines by effector T cells, and assessing them with respect to clinicopathological features, the role of infiltrating Tregs in HNSCC would be further elucidated. This would help establish whether the presence or function of Tregs could be confidently utilised as a reliable prognostic determinant as well as assisting with the development of new therapeutic strategies and targets.

Chapter 6.

The secretion of TGF-β and IL-10 by HNSCC and the influence of HNSCC conditioned medium on Treg suppressive activity

6.1 Introduction

Tumour growth, development and survival is dependent on the creation of a favourable microenvironment, this is driven by a number of factors including the accumulation of genetically altered cells (Section 1.3), infiltration of immune cells which can enhance the survival of the cancer cells (Section 5.1), the supportive role of the stroma and the cytokine network. Chapter 5 studied the infiltration of Tregs into the HNSCC microenvironment and this chapter will continue investigations into the head and neck tumour microenvironment by focusing on the presence of the immunosuppressive cytokines TGF-β and IL-10. These cytokines are known to have a role in the induction of Tregs (Section 1.11.2) and the suppressive activity exerted by the regulatory population (Shevach, 2009), as well as being involved in numerous other mechanisms to promote tumour progression (see below).

The tumour microenvironment has a plethora of soluble mediators, that can have pro-inflammatory (IL-1, IL-2, IL-6, IFN-γ, TNF-α), anti-inflammatory (IL-4, IL-10, TGF-β, VEGF) and immunosuppressive activity [IL-10, TGF-β, prostaglandin E2 (PGE2)], to assist with the promotion of tissue growth, angiogenesis and inhibition of anti-tumour immune responses (Pries and Wollenberg, 2006; Wang *et al.*, 2009). As previously mentioned (Section 5.1), HNSCC patients have been shown to have a circulating cytokine profile which is more reflective of a Th2-like response (IL-4, IL-6 and IL-10) that is thought to be tumour promoting, as opposed to a Th1-like profile (IFN-γ and TNF-α) that assists the host's anti-tumour immune responses; this trend has been supported by a number of HNSCC studies assessing the cytokines secreted by PBMC and the circulating level of cytokines in the plasma and serum of patients (Bose *et al.*, 2008; Jebreel *et al.*, 2007; Lathers and Young, 2004). A wide range of cytokines have been found to be secreted from HNSCC tumour specimens and cell lines including IL-1, IL-4, IL-6, IL-8, granulocyte macrophage-colony-stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF), TGF-β and VEGF (Chen *et al.*, 1999; Korampalli *et al.*, 2011; Lu *et al.*, 2004; Pries and Wollenberg, 2006; Woods *et al.*, 1998), however, the exact role of these cytokines is unknown. The main aim of the work conducted in this thesis was to assess the frequency, function and role of Tregs in HNSCC, therefore to continue to assess the suppression of the host's anti-tumour response the study has also examined the secretion of

immunosuppressive cytokines TGF-β and IL-10 that are known to have a role in both Treg development and function as well as promoting a microenvironment that favours tumour progression.

The role of TGF-β in the growth and development of tumours is complex, including the initial actions to inhibit epithelial growth, however, during tumour progression the cancerous cells become partly or completely resistant to TGF-β growth inhibition due to disruptions in the TGF-β signalling pathways (Bierie and Moses, 2006; Pasche, 2001). In HNSCC this includes the mutation and down regulation of TGF-β receptors (Pasche, 2001; Wang *et al.,* 1997). Disruption in the TGF-β pathway means that the tumour can hijack the cytokine for its own use as a promoter of tumour progression. TGF-β is a pluripotent cytokine with the ability to disrupt the function and proliferation of TIL, including the anti-tumour actions of cytotoxic $CD8^+$ T cells. In addition, the cytokine can stimulate the migration of monocytes, neutrophils and fibroblasts, decrease the expression MHC class I and II genes and reduce NK cell activation (Bierie and Moses, 2006; Zamarron and Chen, 2011). Furthermore, TGF-β has been shown to promote the generation of Tregs from $CD4^+CD25^-$ T cells (Section 1.11.2; Chen *et al.,* 2003; Liu *et al.,* 2007,). Whilst these actions likely favour tumour growth, TGF-β can also functionally inhibit macrophages by decreasing the production of inflammatory cytokines, such as IL-1β, TNF-α and IL-6, which are associated with inflammatory driven tumour progression (Zamarron and Chen, 2011).

The function of IL-10 in tumour growth and development is also not completely understood but it is commonly regarded as an immunosuppressive Th2-like cytokine. With a role in tumour immune evasion and progression, IL-10 can reduce the production of IL-2 and IFN-γ by Th1 lymphocytes, hinder antigen presentation by dendritic cells and promote tumour cell proliferation and survival (Mocellin *et al.,* 2005; Zamarron and Chen, 2011). Furthermore, IL-10 has been shown to be involved in the generation of induced Tregs, Tr1 cells (Section 1.11.2). However, the role of IL-10 is also complex as it displays the ability to inhibit tumour progression by enhancing NK cell cytotoxicity, $CD8^+$ anti-tumour activity and promoting antigen uptake by dendritic cells (Mocellin *et al.,* 2005).

To further understand the role of the HNSCC tumour microenvironment in establishing and maintaining tumour growth, the current study examined the secretion of TGF-β and IL-10 by head and neck cancer cell lines and freshly dissociated tumour tissue

which were collected during resection surgery from patients with HNSCC arising from different tumour subsites. In addition, it was also investigated whether the secretions from HNSCC cell lines and/or tumour specimens influenced the functional activity of Tregs, making them more or less suppressive towards the proliferation of autologous effector T cells. This was investigated by isolating Tregs and effector T cells from healthy controls and culturing the Tregs with or without cultured medium from HNSCC cell lines or tumour specimens prior to assessing the suppressive activity of the regulatory population using a CFSE assay. It was important to use Tregs and effector T cells from healthy controls and not HNSCC patients as these peripheral T cell populations would not previously have been influenced by the presence of cancer, therefore the direct effects of HNSCC secretions on the Treg population could be observed.

6.2 Materials and methods

6.2.1 HNSCC cell lines and collection of conditioned medium

The head and neck cancer cells lines UMSCC-11b, UMSCC-12a, UMSCC-4, UMSCC-81b and UMSCC-47 (Table 6.1; kindly supplied by Dr. T. Carey, University of Michigan) were cultured and maintained as outlined in Section 2.1. In preparation for the collection of conditioned medium at specific time points the HNSCC cell lines were cultured as outlined in Section 2.5. Briefly, 1 x 10^6 cells, in 1ml of complete growth medium, were added to each well of a 6 well plate. At 24, 48, 72 and 96hrs conditioned medium was removed from the cultured cells, centrifuged at 300 x *g* for 3 minutes to pellet cell debris, aliquoted (250µl) and stored at -80^{o}C (Section 2.5) for use in cytokine ELISAs and the assessment of conditioned medium on Treg functional activity.

Table 6.1 HNSCC cell lines

HNSCC cell line name	Gender of patient	Age of patient	TNM	Anatomic site of origin	Specimen site
UMSCC-11b	Male	65	T2N2aM0	Larynx	Larynx
UMSCC-12a	Male	71	T2N1M0	Larynx	Larynx
UMSCC-4[a]	Female	47	T3N2aM0	Tonsil (Oropharynx)	Back of tongue
UMSCC-81b	Male	53	T2N0M0	Tonsil (Oropharynx)	Tonsillar pillar
UMSCC-47[b]	Male	53	T3N1M0	Lateral tongue (Oral cavity)	Lateral tongue

[a] HPV negative [b] HPV positive

6.2.2 HNSCC tumour specimens and collection of conditioned medium

Following ethical (Yorkshire and the Humber research ethics committee; REC reference 10/H1304/7) and NHS Trust R and D (R0988) approval, and obtaining written informed consent, tumour infiltrated nodal specimens from newly-presenting head and neck cancer patients with HNSCC arising from the larynx and oropharynx were collected during resection surgery (Table 6.2). As outlined in detail in Section 2.14, tumour specimens were dissociated, washed in dissociation medium and plated overnight in a 25cm^3 culture flask. Following overnight incubation, the medium was collected, centrifuged at 400 x *g* for 4 minutes and the supernatant was aliquoted (500µl) and stored at -80^{o}C (Section 2.14.1) for use in the assessment of cytokine secretion and the influence of conditioned medium on Treg functional activity.

Table 6.2 Clinicopathological characteristics of HNSCC patients recruited to obtain overnight conditioned medium from dissociated tumour infiltrated nodal specimens

Anatomic site of origin	Gender	Age	Tumour stage	Nodal status
Oropharynx	Male	50	T3	Node (N2c)
Larynx	Male	68	T3	Node (N2c/b)

6.2.3 Assessment of TGF-β and IL-10 in conditioned medium using ELISA

The concentration of TGF-β and IL-10 present in the conditioned medium of head and neck cancer cell lines (Table 6.1) and overnight culture medium from dissociated HNSCC tumour specimens (Table 6.2) were determined using ELISA as described in Section 2.6. Following absorbance measurement at 450nm with wavelength correction at 570nm a standard curve was generated using a four parameter logistic curve-fit and used to determine the level of the cytokine present in the conditioned medium samples. For TGF-β the derived concentrations were multiplied by a dilution factor of 1.4 due to the pre-treatment step.

6.2.4 Isolation of Treg and effector T cell populations by FACS

Tregs and effector T cells were isolated from two healthy donor lymphocyte cones which were obtained from the blood donation service. In a class II biological safety cabinet (Airstream, Esco), the cones were securely clamped to a stand and the contents of the cone were allowed to flow into a 50ml polypropylene tube before being flushed out with PBS. The lymphocytes were subsequently isolated and cryopreserved using the same method of PBMC isolation from whole blood samples, as outlined in Section 2.13.

Following retrieval from cryopreservation, approximately 1 x 10^8 cells were labelled with the appropriate amount of Treg sorting cocktail (BD Biosciences) to enable the isolation of Treg ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) and effector T cell populations ($CD4^+CD25^-CD127^{-/+}$ and $CD4^+CD25^+CD127^+$) by FACS, as outlined in Section 2.16.

6.2.5 Culture of peripheral Tregs with conditioned medium

Immediately following the flow sorting of a lymphocyte cone, the concentration and viability of the isolated peripheral effector T cell ($CD4^+CD25^-CD127^{-/+}$ and

$CD4^+CD25^+CD127^+$) and Treg ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) populations was determined by trypan blue exclusion (Section 2.4).

As outlined in Section 2.17, approximately 5 x 10^5 effector T cells were cultured in a single well of a flat bottom 24 well tissue culture plate in 750µl of culture medium whilst 2 x 10^5 Tregs were cultured in 134µl of the same culture medium in a single well of a round bottom 96 well tissue culture plate. Both Tregs and effector T cells were cultured with a final concentration of 100U/ml recombinant human IL-2. After 24 hours, half of the wells containing Tregs had 66µl of conditioned medium, collected following either 48 hours of incubation with cell lines (UMSCC-4 or UMSCC-12a) or following overnight incubation with a dissociated tumour specimen (laryngeal or oropharyngeal), added to the appropriate wells. The remaining half of the Treg cell cultures had the same volume of culture medium added to provide a control. Following 48 hours incubation with conditioned or culture medium a CFSE assay was performed (Section 6.2.6).

6.2.6 Assessment of the suppressive activity of Tregs cultured with conditioned medium using a CFSE assay

As outlined in Section 2.18, following 48 hour incubation with conditioned or culture medium the concentration and viability of effector T cell and Treg populations were determined by trypan blue exclusion. Due to the low number of Tregs, the majority of CFSE assays were successfully run at a 1:1 ratio using 2 x 10^4 autologous effectors and 2 x 10^4 Tregs cultured in each well of a 96 well plate. The CFSE assay was set up and analysed as outlined in Section 4.2.4 with each effector T cell population co-cultured with Tregs that had been cultured with and without conditioned medium (Table 6.3). Percentages of suppression were calculated based on the proliferation indexes determined by ModFit^{TM} software (Verity Software House, Section 2.19).

Table 6.3 Combination of cultures used in the CFSE assay to determine the influence of conditioned medium on the suppressive activity of Tregs

Number	Culture combinations
1	Non-labelled effector T cells
2	CFSE labelled effector T cells (no stimulation)
3	CFSE labelled effector T cells
4	Tregs ($CD25^{inter}$) without conditioned medium pre-treatment and CFSE labelled effector T cells (1:1)
5	Tregs ($CD25^{inter}$) **with** conditioned medium pre-treatment and CFSE labelled effector T cells (1:1)
6	Tregs ($CD25^{high}$) without conditioned medium pre-treatment and CFSE labelled effector T cells (1:1)
7	Tregs ($CD25^{high}$) **with** conditioned medium pre-treatment and CFSE labelled effector T cells (1:1)

All wells, except well 2, were stimulated with Human T-Activator CD3/CD28 dynabeads and 100U/ml IL-2 (Section 2.18).

6.3 Results

6.3.1 Assessment of TGF-β and IL-10 secretion by HNSCC cell lines and tumour specimens

The concentration of TGF-β and IL-10, secreted over a 96 hour period by five HNSCC cell lines (Table 6.1) and after overnight incubation from two HNSCC tumour specimens (Table 6.2), was determined using ELISA. For the HNSCC cell lines UMSCC-11b and UMSCC-81b, the ELISAs were repeated by another PhD student (Joanne Smith) in the laboratory, therefore the results from these two cell lines are an average of four concentration values. The lowest standard for both TGF-β and IL10 was 31.25pg/ml, therefore for graphical purposes cytokine levels detected below this concentration were plotted as 0, and considered not detectable.

6.3.1.1 TGF-β and IL-10 secretion by HNSCC cell lines

All five HNSCC cells lines secreted detectable levels of TGF-β over the 96 hour culture period (Figure 6.1). The highest concentrations of the cytokine were observed in the laryngeal cell line, UMSCC-12a (Figure 6.1a), where the highest level of TGF-β (96 hours of culture) was over four fold higher than the other cell lines. The concentration of TGF-β for UMSCC-12a was approximately 690pg/ml, even after only 24 hours of culture, which was higher than the maximum concentrations determined for the remaining four cell lines (Figure 6.1b). To confirm the high concentrations of TGF-β in the conditioned medium of UMSCC-12a, the ELISA was repeated and again similar high levels were obtained. However, the variation between duplicates was poor and therefore these results are not represented in Figure 6.1a. For the remaining four cells lines similar concentrations of TGF-β were measured (range 0-416pg/ml). A similar pattern of secretion was observed for the oropharyngeal cell line UMSCC-4 and the laryngeal cell line UMSCC-11b where the concentration of TGF-β in the conditioned medium was low initially, peaking at 48 hours before declining over the remaining culture period (Figure 6.1b). For the oropharyngeal cell line UMSCC-81b, laryngeal cell line UMSCC-11b and oral cavity cell line UMSCC-47 similar concentrations of TGF-β were measured at 48 hours of culture. (Figure 6.1b). For UMSCC-47 and UMSCC-4 the level of TGF-β measured at 96 hours of culture was also similar (Figure 6.1b).

None of the HNSCC cell lines secreted detectable levels of IL-10 (not above 31.25pg/ml) over the 96 hour culture period (data not shown).

6.3.1.2 TGF-β and IL-10 secretion by dissociated tumour infiltrated nodal specimens

The conditioned medium collected from overnight incubation of the dissociated laryngeal tumour sample demonstrated a low concentration of TGF-β (mean concentration ±SEM; 39.29 ± 6.48pg/ml) whereas no detectable level of TGF-β was found to be secreted from the oropharyngeal tumour sample, not above the lowest standard 31.25pg/ml. Both laryngeal and oropharyngeal tumour samples secreted similar concentrations of IL-10 (Mean concentration: 281.45±21.72pg/ml and 307.93±14.58pg/ml respectively).

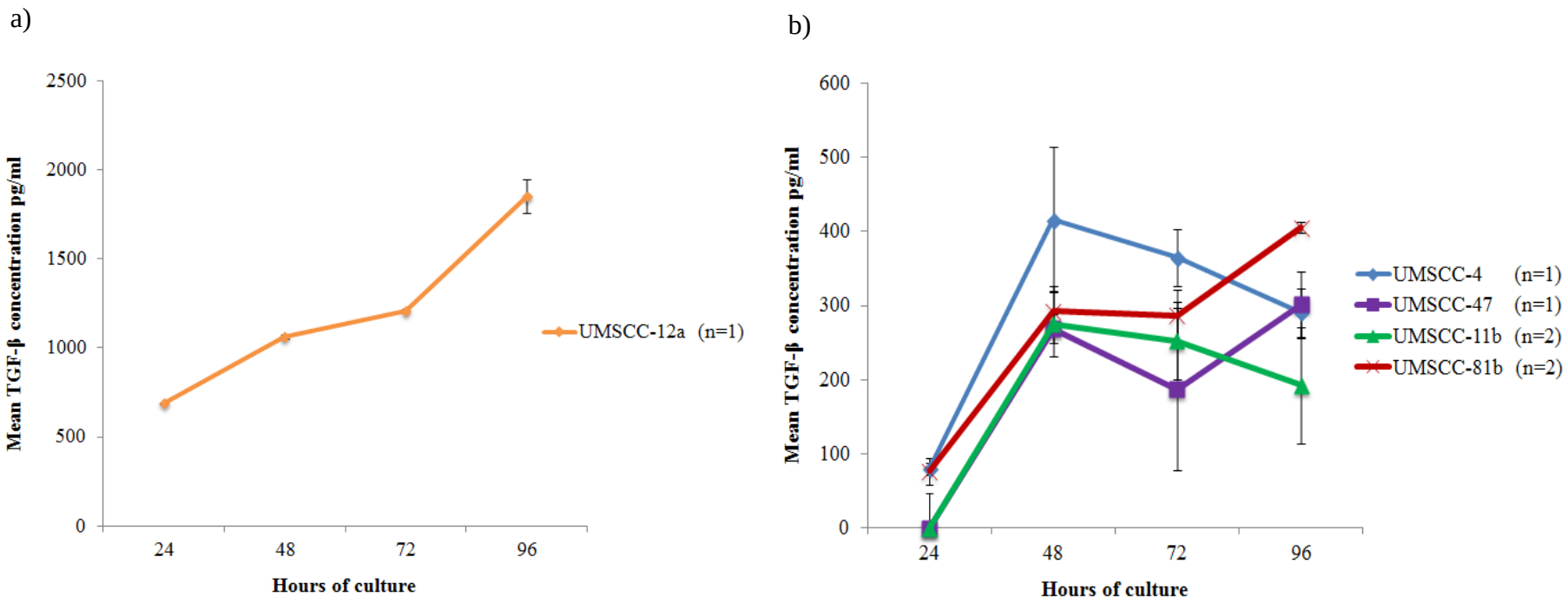


Figure 6.1 Concentration of TGF-β in the conditioned medium of five HNSCC cell lines over a 96 hour culture period
Following the culture of cells at 24, 48, 72 and 96 hours, conditioned medium was removed and assessed for the concentration of TGF-β (pg/ml) by ELISA (a) UMSCC-12a (laryngeal) (b) UMSCC-4 (oropharyngeal), UMSCC-47 (oral cavity), UMSCC-11b (laryngeal), UMSCC-81b (oropharyngeal; mean ± SEM).

6.3.2 Optimising the culture of effector T cells for the subsequent assessment of Treg suppressive activity

Tregs and effector T cells were sorted at the same time from the same lymphocyte cone, therefore in order to use both in the CFSE proliferation assay it was important to maximally maintain effector T cells during the 72 hours of culture of Tregs with or without conditioned medium.

Initially the sorted effector T cells were cultured immediately after isolation with Human T-Activator CD3/CD28 dynabeads and 100U/ml IL-2 for the 72 hour period, before being used in the CFSE assay. However, during the analysis of effector T cells for CFSE content by flow cytometry, it was observed that there was no narrow peak of non-dividing effector T cells (parent population) for the cells which had received stimulation with dynabeads and IL-2 during the 72 hour 'waiting' period before being cultured without stimulation during the CFSE assay (Figure 6.2a). The narrow parent peak was always observed in the numerous CFSE assays conducted previously when the effector cells were used immediately after isolation in the assay (Figure 6.2b).

Therefore, to determine the optimum culture conditions for the effector T cell population during the 'waiting' period and for a successful CFSE assay, $CD4^+CD25^+CD127^+$ effector T cells were cultured for the 72 hour 'waiting' period in three different conditions: CD3/CD28 dynabeads and IL-2, IL-2 only, and no stimulation, before the CFSE assay was performed. In addition, some $CD4^+CD25^+CD127^+$ effector T cells were also frozen down during the 'waiting' period. The same results for the parent population were obtained for the $CD4^+CD25^+CD127^+$ effector T cell population cultured during the 72 hour period with CD3/CD28 dynabeads and IL-2 (Figure 6.2a). However, for the effector population cultured with IL-2 only, no stimulation or those that had been frozen down, a narrow parent peak representing cells that had not undergone proliferation was observed, similar to that shown in Figure 6.2b. The viability of cells was poorer for effector T cells cultured without stimulation or frozen down and therefore for subsequent experiments effector T cells were cultured with IL-2 only during the 72 hour 'waiting' period.

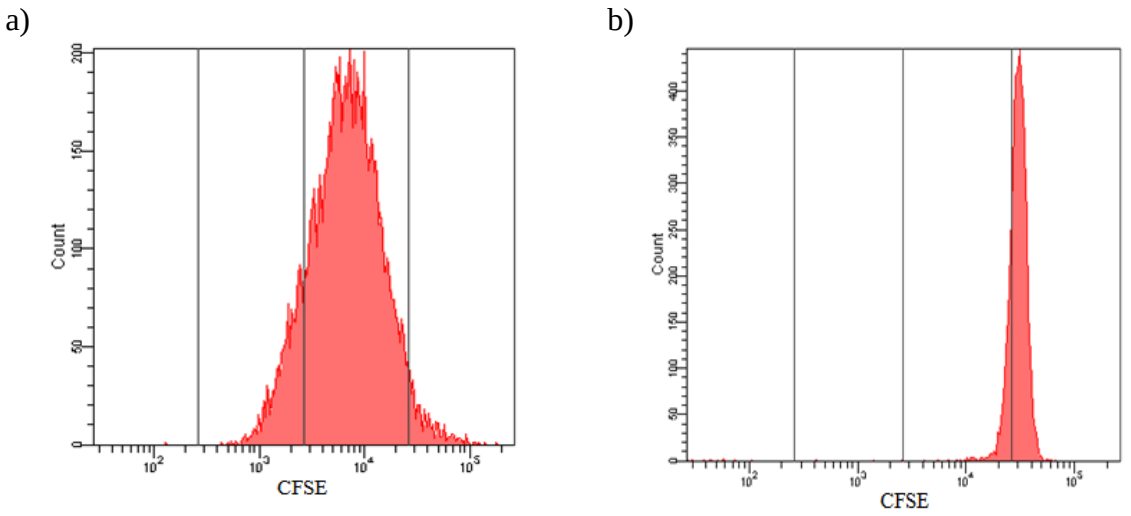


Figure 6.2 CFSE parent populations obtained during the different culture conditions of effector T cells
Effector T cells were isolated by FACS before being immediately cultured with CD3/CD28 dynabeads and IL-2 for 72 hours prior to the set up of the CFSE assay. a) CFSE content for the parent population of effector T cells cultured with stimulation during the 72 hour 'waiting' period but with no stimulation during the assay (representative of two independent experiments) compared with b) the parent population obtained from previous CFSE assays where the effector population was used immediately in the assay.

6.3.3 Assessment of the influence of HNSCC conditioned medium on Treg suppressive activity

Tregs isolated from two lymphocyte cones were pre-treated, during separate experiments, with conditioned medium from HNSCC cell lines (laryngeal, UMSCC-12a and oropharyngeal, UMSCC-4) and dissociated tumour infiltrated nodal specimens (1 x laryngeal and 1 x oropharyngeal). The levels of suppression induced by Tregs pre-treated with conditioned medium from each cell line or tumour specimen for both lymphocyte cones were combined to give an average of two results. The percentages of suppression induced by Tregs incubated with unconditioned culture medium provided the control. Due to the low number of lymphocyte cones assessed statistical analysis was not performed. With large error bars for some of the experiments conducted, further repeats with different lymphocyte cones would be needed to confirm the trends observed.

The percentages of suppression induced by Tregs were calculated from the proliferation indices determined by the ModFit software as outlined in Section 2.19 and

Section 3.2.3. To illustrate this further Table 6.4 demonstrates the calculations conducted to determine the percentages of suppression induced by Tregs cultured without conditioned medium (control; green) and with oropharyngeal cell line conditioned medium (UMSCC-4; red) on the proliferation of effector T cells. The use of colour in the table relates to the colours used in the bar charts on the following figures.

Table 6.4 Calculating the percentages of suppression induced by Tregs cultured without and with oropharyngeal cell line conditioned medium on the proliferation of effector T cells

Culture combination	Proliferation index (ModFit)	Percentage of proliferation	Percentage of suppression
CFSE labelled effector T cells	20.88	100	0
Tregs ($CD25^{inter}$) without conditioned medium pre-treatment and CFSE labelled effector T cells	16.54	79.4	20.6
Tregs ($CD25^{inter}$) **with** conditioned medium pre-treatment and CFSE labelled effector T cells	17.59	84.0	16.0
Tregs ($CD25^{high}$) without conditioned medium pre-treatment and CFSE labelled effector T cells	19.39	92.6	7.4
Tregs ($CD25^{high}$) **with** conditioned medium pre-treatment and CFSE labelled effector T cells	19.51	93.5	6.5

6.3.3.1 Influence of HNSCC cell line conditioned medium on Treg suppressive activity

Tregs ($CD4^+CD25^{inter}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}$) pre-treated with conditioned medium from both the laryngeal (UMSCC-12a) and the oropharyngeal cell line (UMSCC-4) induced similar levels of suppression on the proliferation of $CD4^+CD25^-CD127^{-/+}$ effector T cells compared with Tregs of the same phenotype with no conditioned medium pre-treatment (control; Figure 6.3). In addition, no obvious differences in the levels of suppression were demonstrated between the oropharyngeal and the laryngeal conditioned medium pre-treatment, regardless of the Treg phenotype (Figure 6.3).

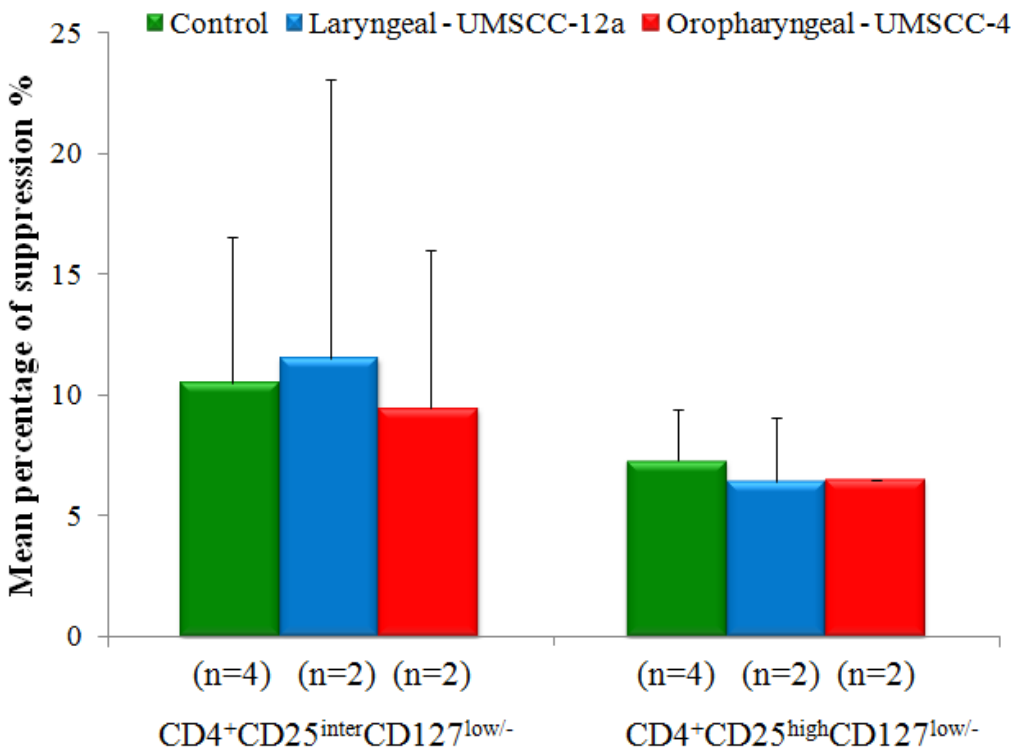


Figure 6.3 Mean percentage of suppression on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells by Tregs pre-treated with or without conditioned medium from HNSCC cell lines

Tregs were isolated by FACS from a lymphocyte cone and pre-treated for 48 hours without (control; green) or with conditioned medium collected from the HNSCC cell lines UMSCC-12a (laryngeal; blue) and UMSCC-4 (oropharyngeal; red). Tregs were assessed for suppressive activity on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells using a CFSE proliferation assay.

Equally, irrespective of the conditioned medium used, similar levels of suppression were induced on the proliferation of $CD4^{+}CD25^{+}CD127^{+}$ effector T cells by $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs which had been pre-treated with and without conditioned medium (Figure 6.4). Pre-treatment of $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs with conditioned medium from the oropharyngeal cell line (UMSCC-4) however, induced a lower level of suppression compared with Tregs of the same phenotype incubated with UMSCC-12a conditioned medium (Figure 6.4).

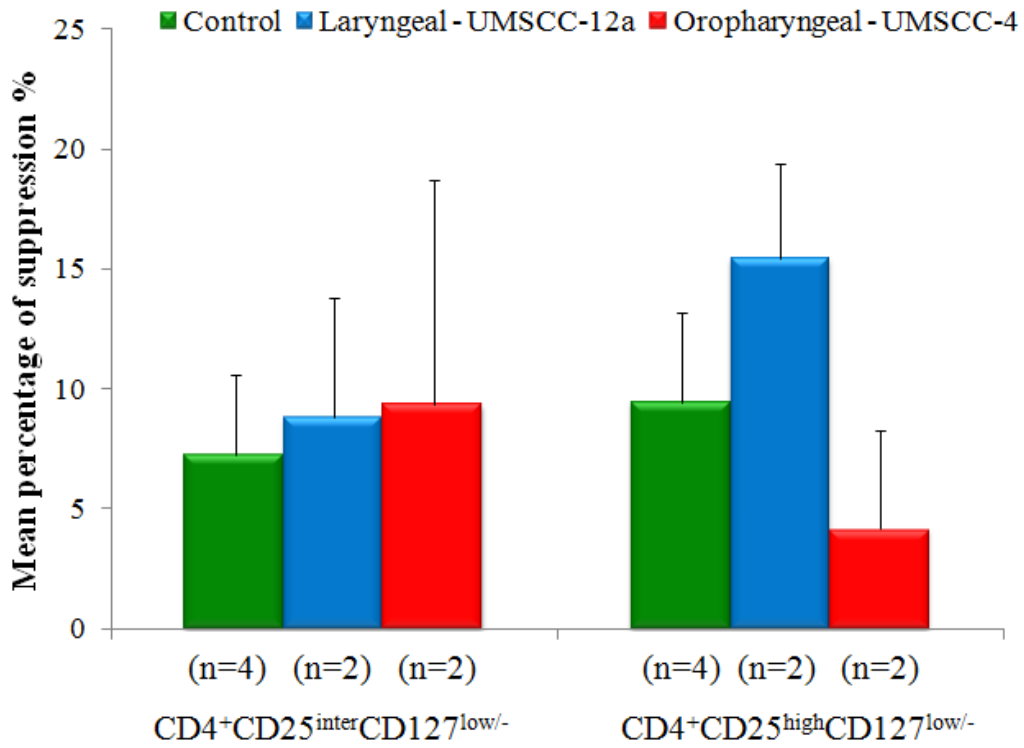


Figure 6.4 Mean percentage of suppression on the proliferation of $CD4^+CD25^+CD127^+$ effector T cells by Tregs pre-treated with or without conditioned medium from HNSCC cell lines

Tregs were isolated by FACS from a lymphocyte cone and incubated for 48 hours without (control; green) or with conditioned medium collected from the HNSCC cell lines UMSCC-12a (laryngeal; blue) and UMSCC-4 (oropharyngeal; red). Tregs were assessed for suppressive activity on the proliferation of $CD4^+CD25^+CD127^+$ effector T cells using a CFSE proliferation assay.

6.3.3.2 Influence of conditioned medium collected from dissociated HNSCC specimens on Treg suppressive activity

$CD4^+CD25^{inter}CD127^{low/-}$ Tregs pre-treated with conditioned medium from dissociated tumour had similar suppressive activity on the proliferation of $CD4^+CD25^-CD127^{-/+}$ effector T cells compared with control Tregs of the same phenotype, regardless of the conditioned medium used (Figure 6.5). Pre-treating $CD4^+CD25^{high}CD127^{low/-}$ Tregs with conditioned medium from a dissociated laryngeal tumour specimen tended to induce a lower level of suppression on the proliferation of $CD4^+CD25^-CD127^{-/+}$ effector T cells compared with Tregs of the same phenotype incubated without conditioned medium (control) or with conditioned medium from the oropharyngeal tumour specimen; whereas

the conditioned medium from the oropharyngeal tumour appeared to have no effect on Treg suppressive activity (Figure 6.5).

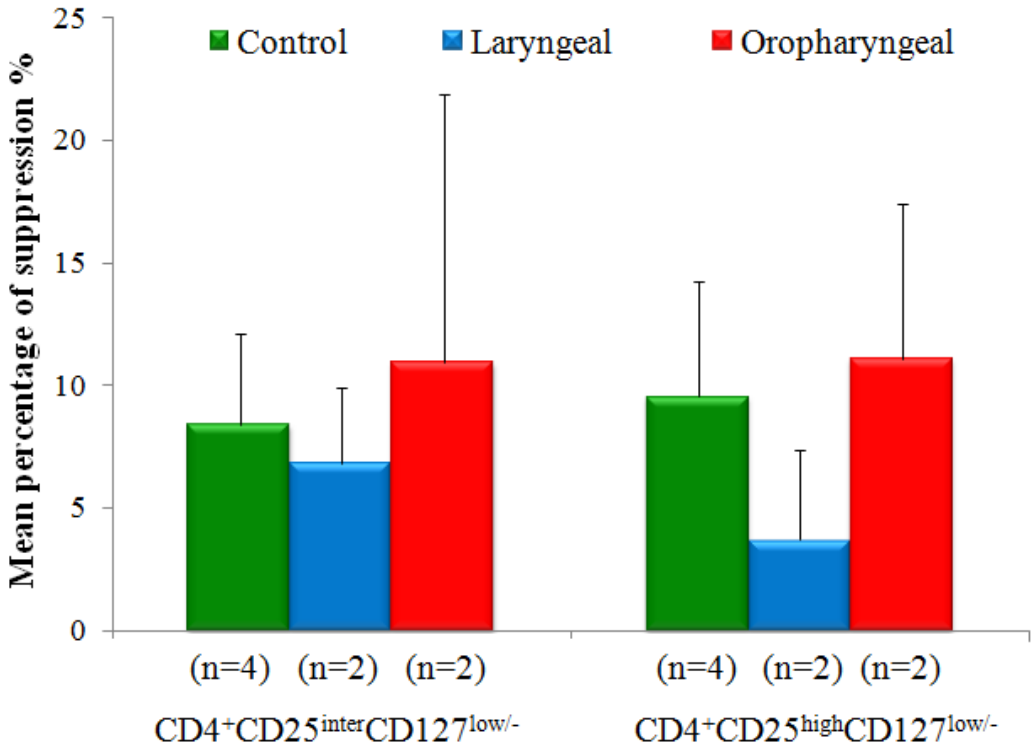


Figure 6.5 Mean percentage of suppression on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells by Tregs pre-treated with or without conditioned medium from dissociated tumour specimens
Tregs were isolated by FACS from a lymphocyte cone and incubated for 48 hours without (control; green) or with conditioned medium collected from dissociated laryngeal (blue) or oropharyngeal (red) tumour specimens incubated overnight. Tregs were assessed for suppressive activity on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells using a CFSE proliferation assay.

$CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs incubated with conditioned medium from a dissociated laryngeal tumour specimen appeared to have slightly greater suppressive activity on the proliferation of $CD4^{+}CD25^{+}CD127^{+}$ effector T cells compared with Tregs of the same phenotype which had not been pre-treated with conditioned medium or incubated with conditioned medium from an oropharyngeal tumour specimen (Figure 6.6). In contrast, the $CD4^{+}CD25^{high}CD127^{low/-}$ Treg population had similar suppressive activity pre-treated with or without conditioned medium, irrespective of the tumour subsite from which the conditioned medium was obtained (Figure 6.6).

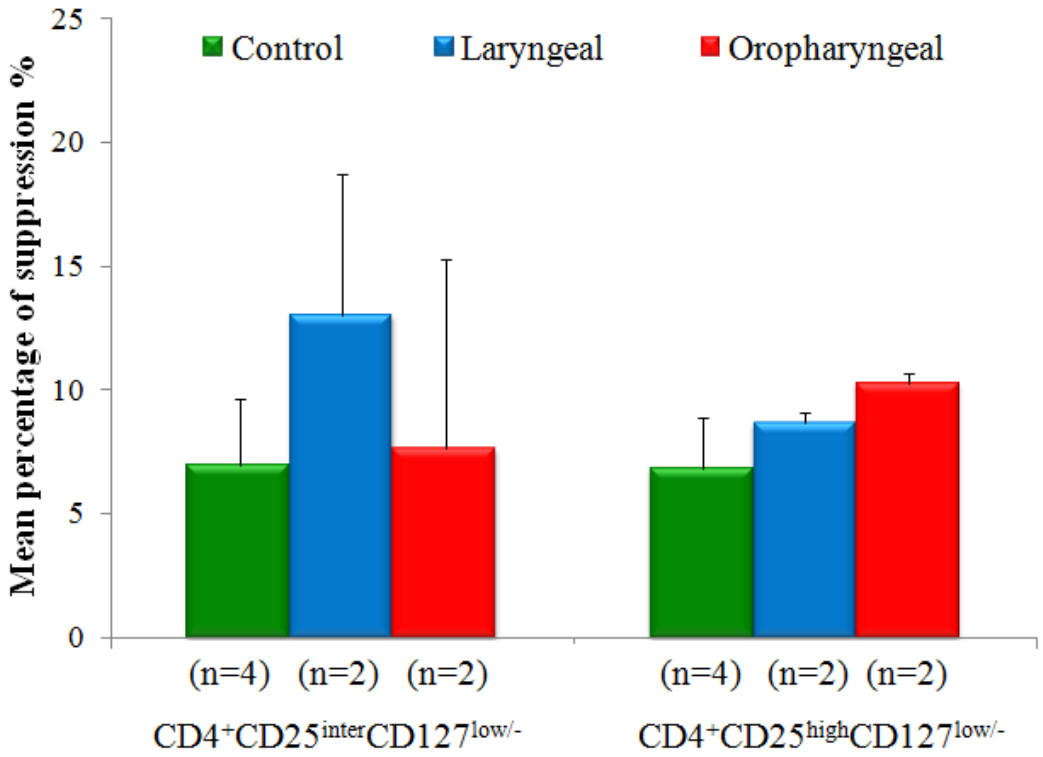


Figure 6.6 Mean percentage of suppression on the proliferation of $CD4^+CD25^+CD127^+$ effector T cells by Tregs incubated with or without conditioned medium from dissociated tumour specimens

Tregs were isolated by FACS from a lymphocyte cone and incubated for 48 hours without (control; green) or with conditioned medium collected from dissociated laryngeal (blue) or oropharyngeal (red) tumour specimens incubated overnight. Tregs were assessed for suppressive activity on the proliferation of $CD4^+CD25^+CD127^+$ effector T cells using a CFSE proliferation assay.

6.4 Discussion

Numerous cytokines have been identified in HNSCC patient samples, both in the periphery and the tumour microenvironment, however their precise role in both progression and inhibition of tumour growth is not fully understood. The aim of the current study was to examine the secretion of two immunosuppressive cytokines, TGF-β and IL-10, by HNSCC cell lines and freshly dissociated tumour tissue from different head and neck subsites. In addition, the influence of secretions from cell lines and tumour specimens on the suppressive activity of Tregs was also investigated. To the author's knowledge this is the first study to assess the effect of cancer cell line/tumour specimen secretions on Treg function.

6.4.1 Secretion of TGF-β and IL-10 by HNSCC cell lines and tumour specimens

Cancer cell lines provide a snap shot of what might have been occurring in the tumour at the time of resection and present an important starting point in the search for therapies and investigation into tumour biology when clinical samples are less readily available. However, cell lines can alter over time of culture and therefore after initial studies clinical specimens should be used to validate findings (Lansford, 1999; Lin *et al.*, 2007).

6.4.1.1 Secretion of TGF-β

The head and neck cancer cell lines were originally derived from different HNSCC patients with variations in the specimen site, staging and HPV status, even so, all five cell lines were observed to secrete detectable levels of TGF-β over the 96 hour culture period. It was noted that the HPV negative cell line (UMSCC-4) secreted slightly higher levels of TGF-β compared with the HPV positive cell line (UMSCC-47) although the concentration of TGF-β measured following 96 hours of culture was similar. The concentration of TGF-β increased steadily over 96 hours for UMSCC-12a and the oropharyngeal cell line UMSCC-81b whilst UMSCC-4 and UMSCC-11b reached a peak concentration at 48 hour of culture before the levels decreased over the remaining 48 hours; this may have been due to the culture wells becoming confluent and overcrowded, with the death of cells causing the secretion of TGF-β to decrease. The oral cavity cell line UMSCC-47 showed varying concentrations of the cytokine over the culture period and a repeat of the ELISA would confirm the trend of TGF-β secretion over 96 hours of culture. The dissociated laryngeal tumour infiltrated nodal specimen, cultured overnight, secreted low but detectable levels of TGF-β however, the oropharyngeal tumour infiltrated nodal specimen did not secrete any detectable levels of TGF-β which is in contrast to the results shown by the oropharyngeal cell lines UMSCC-4 and UMSCC-81b. The differences in the levels of TGF-β measured in the HNSCC cell lines and freshly dissociated tumour specimens could be attributed to the length of culture, 96 hours compared with overnight. Additionally, the culture conditions were different, with the cell lines cultured in a 6 well plate in 1ml of medium (1×10^6

squamous carcinoma cells) whereas the tumour specimens were cultured in a $25cm^3$ flask in 5ml of medium (approximately 2×10^7 cells); it could therefore be suggested that in 5ml of medium any secreted TGF-β may be too dilute to be measured by ELISA. However, having only assessed one laryngeal and one oropharyngeal tumour sample increasing the number of patient specimens assessed would help establish the secretion of TGF-β by dissociated tumour samples and determine whether there may be subsite differences.

The fact that TGF-β was detected in HNSCC cell line conditioned medium is in contrast to Chen and colleagues (1999) who reported that TGF-β was not detected by ELISA in their head and neck cancer cell line supernatant samples, but does agree with the findings of Young *et al.* (1996) who detected TGF-β in five head and neck cancer cell lines and freshly excised cultured head and neck cancer specimens (primary tumour and metastatic nodal specimens). The differences reported could be due to the use of different HNSCC cell lines, however with Chen and colleagues and the current study both analysing UMSCC-11b, it could also be attributed to the different methods of culture and time of supernatant collection. For example, Chen *et al.* (1993) cultured the cell lines in 12ml of medium and collected supernatant after 48 hours, whereas Young and colleagues and the current study cultured in 1ml of medium and collected supernatant after 24 hours and over a 96 hour period, respectively. In clinical samples, TGF-β has been shown to be secreted by HNSCC tumour samples (Gasparoto *et al.,* 2010; Lu *et al.,* 2004) and the mRNA of the cytokine was also demonstrated to be expressed by oral SCC tumour specimens (Lee *et al.,* 2010a).

6.4.1.2 Secretion of IL-10

None of the HNSCC cell lines investigated secreted detectable levels of IL-10 over the 96 hour culture period. However, both the laryngeal and oropharyngeal dissociated tumour specimens were observed to secrete detectable levels of the cytokine at similar concentrations.

These results are in agreement with Chen *et al.* (1999) who also failed to detect the secretion of IL-10 in the supernatant of HNSCC cell lines. The absence of IL-10 secretion may be due to the squamous carcinoma cells not producing the cytokine or the concentration of the cytokine being too low for detection by ELISA. However, in line with

the current study, IL-10 has previously been detected in freshly excised cultured head and neck cancer specimens (n=12, Gaparoto *et al.,* 2010; n=283, Young *et al.,* 1996). Due to the secretion of the cytokine by freshly dissociated tumour specimens and not in all HNSCC cell lines it is suggested that the cytokine is secreted by infiltrating immune cells or fibroblasts from the stroma instead of the squamous carcinoma cells.

To further understand the immunosuppressive microenvironment created within HNSCC the secretion of TGF-β and IL-10 was investigated. It is well known that a plethora of cytokines are present in the tumour microenvironment which may play a role in influencing both the promotion and inhibition of HNSCC growth. However, it was decided to start assessing the cytokine network by focusing on the secretion of two immunosuppressive cytokines known to be involved in Treg induction (Section 1.11.2) and Treg suppressive activity (Shevach, 2009).

6.4.2 The influence of HNSCC conditioned medium on the functional capacity of Tregs

Subsequent to the assessment of cytokine secretion by HNSCC cell lines and tumour specimens it was questioned whether the head and neck cancer secretions, including TGF-β and IL-10, would influence the suppressive activity of Tregs, making them more or less suppressive on the proliferation of autologous effector T cells. This was examined by incubating Tregs with culture medium from HNSCC cell lines or tumour infiltrated nodal specimens before assessing their suppressive activity using a CFSE proliferation assay.

6.4.2.1 Conditioned medium from HNSCC cell lines and tumour specimens

To continue with the study's aim of examining different HNSCC subsites a laryngeal and an oropharyngeal cell line and tumour specimen were chosen for assessment of their effects on Treg suppressive activity. The laryngeal cell line UMSCC-12a and the oropharyngeal cell line UMSCC-4 were chosen due to TGF-β being detected at high levels in their conditioned medium. UMSCC-4 is a HPV negative cell line and was chosen due to

the HPV status of the tumour specimens in the current study being unknown. In future work it would be interesting to assess whether the HPV status of a tumour influences Treg suppression, however due to time limits this was not able to be investigated.

Tregs were cultured with conditioned medium from the HNSCC cell lines following 48 hours of culture due to this culture period having detectable levels of TGF-β for both cell lines (UMSCC-12a and UMSCC-4), reaching a peak concentration for UMSCC-4. Previous studies in the laboratory, incubating PBMC with head and neck cancer cell line conditioned medium, showed that the greatest level of proliferative activity was at 48 hours of incubation compared with measurements taken at 24, 72 and 96 hours. Consequently, isolated Tregs were incubated with conditioned medium, collected following either 48 hours of culture with HNSCC cell lines or following overnight incubation with a dissociated tumour specimen, for 48 hours before the CFSE assay was performed. The conditioned medium was diluted to prevent any adverse effect from toxic waste products secreted by the cells.

6.4.2.2 Optimisation of effector T cell culture for subsequent assessment of Treg activity

To assess the suppressive activity of Tregs on autologous effector T cell proliferation it was important to maximally maintain the effector populations during the 72 hours that the Treg were pre-incubated with or without conditioned medium. To the author's knowledge this method has not previously been performed by other research groups and therefore with no protocols for comparison the method had to be optimised.

Initially the sorted effector T cells were cultured with Human T-Activator CD3/CD28 dynabeads and 100U/ml IL-2 for the 72 hour 'waiting' period. However, it was observed that there was no narrow parent peak during the CFSE assay for the effector T cells which had received stimulation during the 72 hour 'waiting' period before being cultured without stimulation during the CFSE assay. The parent peak represents the population of cells that have not undergone division and provides the reference peak needed to calculate the proliferation index of dividing effector T cells, and is therefore essential in determining the suppressive activity of Tregs (Section 3.2.3). The absence of a narrow parent peak was possibly due to the effector T cells receiving stimulation during the

'waiting' period and that the residual presence of CD3/CD28 dynabeads caused the effector T cells to continue to divide, even though they were under non-stimulatory conditions in the CFSE assay.

To 'recover' the parent population several different culture conditions for the effector T cells during the 72 hour 'waiting' period were set up: CD3/CD28 dynabeads and IL-2, IL-2 only, no stimulation and freezing down of the effector population. A broad parent peak was again observed for the effector cells stimulated with dynabeads and IL-2 during the 72 hour 'waiting' period whilst the remaining culture conditions provided a narrow parent peak during the CFSE assay. However, for the effector T cell population which had been frozen down a number of cells had died due to the freezing process and the cells cultured with no stimulation did not look as 'healthy' when stained with trypan blue. It was therefore decided that the effector T cells would be cultured with IL-2 only for the 72 hour 'waiting' period prior to the set up of the CFSE assay as this culture condition gave the essential parent peak whilst maintaining the population at a good cell number sufficient for use in the assay. To remain constant, Tregs were also cultured with IL-2 only during the 72 hour incubation period with or without conditioned medium.

6.4.2.3 Suppressive activity of Tregs cultured with HNSCC conditioned medium

IL-10 and TGF-β are known to have a role in the induction of Tregs and the suppressive activity exerted by Tregs on effector populations (Section 1.11.2; Shevach, 2009). It was therefore of interest whether IL-10 and TGF-β, measured in the conditioned medium of dissociated tumour specimens and HNSCC cell lines, along with the plethora of secretions within the tumour microenvironment, would have a direct influence on the functional activity of Tregs. If the secretions in the tumour environment were to affect Treg function it would be hypothesised that their suppressive activity would be enhanced, to continually assist in the suppression of the host's anti-tumour attack.

To investigate this conditioned medium from HNSCC cell lines and tumour specimens were incubated with Tregs before assessing their suppressive activity on the proliferation of autologous effector T cells. The regulatory population was isolated from two different lymphocyte cones which had been donated by healthy individuals. It was important to use Tregs and effector T cells from healthy donors, and not HNSCC patients,

as these populations would not have previously been influenced by the presence of a tumour and therefore the effect of HNSCC secretions could be fully examined. Additionally, the large number of lymphocytes obtained from the cones meant that the same sample could be used to assess the influence of two HNSCC cell lines and two head and neck tumour specimens on Treg function.

The results obtained from the two lymphocyte cones showed that $CD4^{+}CD25^{inter}CD127^{low/-}$ and $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs cultured with and without conditioned medium induce similar levels of suppression on the proliferation of autologous effector T cells, irrespective of the effector cell phenotype and the head and neck subsite from which the conditioned medium was collected; thereby suggesting that tumour secretions perhaps do not directly influence Treg suppressive activity. Due to time constraints no further lymphocyte cones could be assessed but continuing the study would assist in determining whether tumour secretions do affect Treg functional activity.

6.4.3 Conclusion

The current study has demonstrated that HNSCC cell lines and tumour specimens secrete TGF-β whereas only the dissociated tumour samples secrete detectable levels of IL-10. The assessment of head and neck cancer cell lines has provided an important starting point investigating the secretion of immunosuppressive cytokines. However, the use of primary tumour specimens is essential and increasing the number of HNSCC specimens would validate current results and provide a greater overview of the secretion of TGF-β and IL-10 in the tumour microenvironment. Furthermore, by separating immune cells, fibroblasts and squamous carcinoma cells from the dissociated tumour specimens, the cells responsible for the secretion of IL-10 in the HNSCC microenvironment could be determined.

In general, the incubation of Tregs with conditioned medium from HNSCC cell line and tumour specimens does not influence the suppressive activity of Tregs on the proliferation of effector T cells. However, due to the effect of HNSCC conditioned medium only being assessed on two normal PBMC samples a larger study would need to be conducted to confirm this trend. As discussed in Chapter 5, Tregs also have the functional capacity to suppress the secretion of cytokines by effector T cells; therefore the influence of

pre-treated Tregs on cytokine secretion is another mechanism of suppression that could be investigated by future experiments. Additionally, this pilot study has established a successful experimental set up that will enable the influence of HNSCC conditioned medium on the proliferation and functionality of effector T cells along with their conversion to the regulatory phenotype to be assessed by future work.

Chapter 7.

Discussion

Although an estimated 7.6 million people die from cancer each year (Ferlay *et al.*, 2010) the tumour microenvironment remains poorly understood. In fact, the tumour microenvironment is increasingly regarded to be as complex if not more so than that of normal tissue (Hanahan and Weinberg, 2011). A recent review by Hanahan and Weinberg (2011) outlined several hallmarks of cancer that provide the tumour with the capabilities to develop, progress and metastasise including: sustained proliferative signalling, evading growth suppressors, replicative immortality, angiogenesis, invasion, resisting cell death and avoiding immune destruction. Furthering our understanding of the different cancer hallmarks will not only unveil the complex and intricate mechanisms that have remained unclear to scientists for years but will also provide the information necessary to identify novel therapeutic targets, new prognostic markers and the development of treatment strategies that will have the potential to improve cancer survival rates.

The current study focused on one of the mechanisms suggested to be employed by HNSCC to evade the host's anti-tumour immune attack and promote tumour growth and development, the presence of the Treg population. There is a constant and complex interplay between the tumour microenvironment and the immune system, and it is the balance between immune evasion, suppression and attack that influences the success or failure of the host's anti-tumour response.

It was demonstrated that the prevalence of circulating $CD127^{low/-}$ Tregs, regardless of the level of CD25 expression, was similar between HNSCC patients and healthy controls. However, $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs isolated from HNSCC patients induced a significantly greater level of suppression on the proliferation of $CD4^{+}CD25^{-}CD127^{-/+}$ effector T cells compared with those from healthy individuals, reinforcing the fact that function should always be investigated alongside frequency. Patients with nodal involvement were shown to be associated with an elevated frequency and suppressive activity of peripheral $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs compared to patients with no nodal involvement. Additionally, patients with advanced stage tumours were also found to have an increased level of $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs compared with early stage tumours. It was consistently observed that $CD4^{+}CD25^{inter}CD127^{low/-}$ Tregs, for a number of different HNSCC patient cohorts, induced a greater level of suppression compared with $CD4^{+}CD25^{high}CD127^{low/-}$ Tregs. However, it remains unclear whether the presence of the

regulatory population promotes the growth and spread of the tumour or whether these aspects cause an elevation in Treg frequency and/or suppressive activity.

The tumour microenvironment was demonstrated to have a significantly higher percentage of Tregs with a corresponding lower frequency of effector T cells compared to that of their matching peripheral blood lymphocytes, suggesting the generation of a localised suppressive environment within the tumour. However, the levels of suppression induced by tumour infiltrating and peripheral Tregs on their autologous effector T cells was similar. Despite this, the elevated frequency of Tregs, in comparison to effector T cells, infiltrating the tumour microenvironment is likely to induce a greater overall level of immune suppression than that observed in the periphery.

The secretion of two immunosuppressive cytokines that are known to assist with tumour immune suppression, TGF-β and IL-10 was also investigated. In the HNSCC microenvironment detectable levels of TGF-β in the laryngeal specimen and IL-10 in both the laryngeal and oropharyngeal samples were measured, however, only TGF-β was secreted by the HNSCC cell lines suggesting that it may be other cells rather than the tumour epithelial cells that are secreting IL-10. It was shown that HNSCC secretions, from both head and neck cancer cell lines and dissociated tumour specimens, do not directly influence the suppressive activity of Tregs on the proliferation of effector T cells, however, due to time constraints, further experiments would need to be conducted to confirm this trend.

The current study has contributed to our understanding of Tregs in HNSCC and it is important that research continues to develop our knowledge of the head and neck tumour microenvironment. This will not only involve confirming the presence of various cell types but also understanding their function and role in the vast number of mechanisms and pathways that are acting to promote as well as suppress tumour growth; thereby providing the opportunity to translate research observations into successful clinical strategies.

It is due to our increased understanding of the different cancer hallmarks that the use of antibody-based therapies, in combination with radio- and chemotherapy regimes, has successfully become established in cancer treatment strategies over the past 15 years; in HNSCC this has included the introduction of cetuximab which targets the EGFR inhibiting the induction of downstream pathways. The receptor has been implicated as one of the mechanisms used by HNSCC to evade controlled cell growth and high expression of the

receptor is associated with poor prognosis (Ang *et al.,* 2002; Chung *et al.,* 2006; Hitt *et al.,* 2005; Laimer *et al.,* 2007; Rubin Grandis *et al.,* 1998). In combination with radio/chemotherapy, cetuximab has been shown to improve the overall survival rate and is relatively well tolerated by HNSCC patients (Bonner *et al.,* 2010; Reeves *et al.,* 2011). However, the precise mechanisms induced by molecule specific drugs remain unclear, including their immunomodulatory effects. For example, the tyrosine kinase inhibitors imatinib and sunitinib, that induce growth arrest or apoptosis, have also been shown to reduce Treg frequency, modulate their suppressive function and limit their infiltration in to the tumour (Larmonier *et al.,* 2008; Xin *et al.,* 2009), consequently impairing the role of Tregs in tumour mediated suppression. Increasing our knowledge of the different pathways and interactions that occur within the tumour microenvironment and the various factors that influence the immune infiltrate will improve our understanding of the molecular mechanisms influenced by current therapies, enabling optimal treatment strategies to be implemented as well as assisting further in the development of new therapeutic agents.

Despite therapeutic advances in the treatment of HNSCC the 5 year survival rate remains poor. Consequently, research into head and neck tumour biology is also focusing on biomarkers which will provide further information on prognosis, treatment response and assist with the selection of treatment regimes. The HPV status of a patient's tumour has over recent years become a widely accepted prognostic marker that identifies a distinct group of head and neck cancers that are associated with improved overall survival. However, the interactions that occur between the virus and other tumour associated pathways remain unclear (Lucs *et al.,* 2013). Emerging biomarkers include proteins such as the excision repair cross-complementation group 1 enzyme (ERCC1) and Ku80, involved in DNA damage repair networks, along with hypoxia-inducible factor 1, that are all showing the potential to predict HNSCC patient outcome following chemotherapy and/or radiotherapy (Aebersold *et al.,* 2001; Jun *et al.,* 2008; Moeller *et al.,* 2011). There is also the possibility of immune components, such as Tregs, to become established as prognostic determinants.

The ultimate aim of HNSCC research is to provide the knowledge necessary to develop and deliver therapies that are tailored towards the patient's individual biological disease state (Calabrese *et al.,* 2012), improving their quality of life, reducing their chance of recurrence and increasing their overall survival.

7.1 Future work

The current study successfully optimised and established a number of techniques that can now be used to expand investigations into other aspects of Treg function and HNSCC biology.

It was of interest to the study whether individual head and neck tumour subsites would have different effects on the Treg population. Using a stringent Treg phenotype, the results observed using flow cytometry in both the peripheral circulation and tumour microenvironment of HNSCC patients found no subsite differences for either the frequency or the functional activity of Tregs. However, this study focused on only two HNSCC subsites, the larynx and oropharynx, and the HPV status of the patient group was unknown. Therefore broadening the patient cohort in future work to include patients with cancer of the nasopharynx, hypopharynx and oral cavity, as well as determining their HPV status, would determine whether various HNSCC subsites have different influences on Treg frequency or function.

It was important to assess both the frequency and function of Tregs, and it was observed that peripheral and tumour infiltrating Tregs induced similar levels of suppression. If this trend is shown to be maintained through the assessment of further clinical samples, future studies could just investigate peripheral Tregs to give an indication of functional activity. This would reduce both time and cost and would be simpler as the number of lymphocytes isolated from the periphery is not as limited as the tumour microenvironment.

To gain a further insight into the cytokine network within HNSCC, separating immune cells, fibroblasts and squamous carcinoma cells from the freshly dissociated HNSCC specimens would determine which cells are responsible for the secretion of IL-10 in the tumour microenvironment. Additionally, by assessing more clinical samples and increasing the profile of cytokines examined, a greater overview of cytokine secretion in the tumour microenvironment could be determined as well as establishing whether secretions are associated with different clinicopathological features.

To further increase our understanding of the interactions that occur between Tregs and immune cells of the host's anti-tumour response, the suppressive activity of Tregs on $CD8^+$ cytotoxic T cell proliferation could be assessed; to the author's knowledge this has

not previously been assessed with $CD127^{low/-}$ Tregs in cancer studies. Additionally, as outlined in previous chapters, Treg functional activity could also be examined by their ability to suppress the secretion of cytokines by effector T cells, which may be another way by which the Tregs exert their pro-tumour effects. Furthermore, by assessing the influence of HNSCC conditioned medium on Treg functional activity, a method that will enable the influence of head and neck tumour secretions on the effector T cell population has been established, this will enable investigations into their functional activity and their induction to the Treg phenotype, an aspect not known to have been assessed by previous HNSCC studies.

It is currently unclear whether Tregs are associated with a positive or negative prognosis in HNSCC, however, the current study has provided data that could be assessed in retrospective investigations to establish whether the frequency, in particular of $CD4^{+}CD25^{inter}CD127^{low/-}$, or the suppressive activity of Tregs is associated with prognosis. In gastric cancer patients, an elevated frequency of peripheral Tregs was associated with poorer survival compared with patients who had low Treg levels (Kono *et al.,* 2006; Sasada *et al.,* 2003), whereas the opposite trend was reported in renal cancer (Liotta *et al.,* 2010). However, to the author's knowledge these are the only two studies to relate the frequency of Tregs, identified by flow cytometry, from patients with solid tumours to prognosis, and no cancer publications have associated the level of Treg induced suppression to prognosis. It would therefore be of great interest to establish whether Treg frequency or suppressive activity, in both the periphery and tumour microenvironment, has the potential to be used as a prognostic determinant in HNSCC.

7.2 Conclusion

This study has further developed our understanding of the Treg population in HNSCC, highlighting differences between the frequency and suppressive activity of Tregs with respect to tumour stage and nodal involvement and also between the peripheral circulation and tumour microenvironment. By continuing HNSCC research, the cellular pathways, interactions and immunosuppressive strategies implemented by the tumour can be further understood in the hope of generating new therapeutic targets, improving the patient's quality of life and providing better prognostic information.

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Appendix

PBS (1L; Oxoid Limited)

Dissolve 10 tablets in 1 litre of double distilled water

Sterilised using standard protocol for the Boxer autoclave (Lab_3)

PBS/BSA/sodium azide

1L PBS (Oxoid Limited)

2.5g BSA (Fisher Scientific, Thermo Scientific)

6.24ml 10% Sodium Azide (Sigma)

Tris Buffer Solution (TBS; 20x)

121g Trizma base (1M; Sigma)

170g Sodium chloride (3M; Sigma)

Make up to 1L with distilled H_2O

Adjust pH to 7.6 with concentrated hydrochloric acid

Dilute 20x in distilled H_2O for use in immunohistochemistry