THE UNIVERSITY OF HULL

The Effect of HNSCC-derived Soluble Factors on the Proliferation and Function of Immune Cells

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by

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Abstract

Malignant epithelium and associated stromal cells secrete soluble factors which may influence tumour evasion of host immunity. The effect of these factors on the proliferation and function of individual immune cell populations has been investigated along with the role of hypoxia.

The conditioned medium (CM) from four HNSCC cell lines, primary-derived fibroblasts, cultured in both normoxic and hypoxic conditions, and overnight-dispersed tumour CM was collected. Cytokine profiles were determined using a Quantibody cytokine array™ and ELISA. The CM was added to Tregulatory cells (CD4⁺CD25⁺CD127lo), T effector cells (CD4⁺CD25⁻) and cytotoxic T cells (CD8⁺) isolated from healthy donors and HNSCC patients. MTS assays and flow cytometry were used to assess changes in proliferation and percentage of immune cells. CFSE suppression assays, ELISA and flow cytometry were undertaken to measure changes in function of Tregulatory cells, Teffectors cells and CD8⁺ T cells obtained from healthy PBMC.

A significant increase in the proliferation of whole PBMC from patient and healthy donors was observed upon the addition all HNSCC-derived CM, with healthy PBMC proliferating to an even greater extent compared with the patient samples. Tregulatory and Teffector cell percentages within healthy PBMC increased while CD8⁺ T cell percentage decreased in many cases. Also, HNSCC-derived CM was able to increase the suppressive activity of Tregs in 40% of samples. The CM caused an increase in Th1 type cytokines IFN-γ and IL-2 in at least 50% of samples with little change to Th2 cytokine levels and was also able to significantly reduce the function of CD8⁺ T cells in at least 50% of samples.

In conclusion, the secretome of HNSCC epithelial cells, primary-derived fibroblasts and overnight dispersed tumour has the ability to alter the proliferation and function of individual sets of immune cells, potentially to a greater extent in the presence of other cell types.
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<th>Description</th>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CFDASE</td>
<td>Carboxyfluorescein Diacetate Succinimidyl Ester</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein Succinimidyl Ester</td>
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<tr>
<td>CM</td>
<td>Culture Medium</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr Virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra Acetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ENT</td>
<td>Ear, Nose and Throat</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FBs</td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>Foxp3</td>
<td>Forkheadbox Transcription Factor p3</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl) Piperazine-1-Ethanesulfonic Acid</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and Neck Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papillomavirus</td>
</tr>
<tr>
<td>H$_2$SO$_4$</td>
<td>Sulphuric Acid</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>iTreg</td>
<td>Induced T Regulatory Cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>Macrophage Inflammatory Protein-3 alpha</td>
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<tr>
<td>nTreg</td>
<td>Naturally Occurring T Regulatory Cells</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural Killer Cells</td>
</tr>
<tr>
<td>NPC</td>
<td>Nasopharyngeal Carcinoma</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin-Chlorophyll-Protein Complex</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI</td>
<td>Proliferation Index</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma Protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
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<td>SSC</td>
<td>Side Scatter</td>
</tr>
<tr>
<td>TAFs</td>
<td>Tumour Associated Fibroblasts</td>
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<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-beta</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper-1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper-2</td>
</tr>
<tr>
<td>Th3</td>
<td>T helper-3</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper-17</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour Infiltrating Lymphocytes</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFa/β</td>
<td>Tumour Necrosis Factor alpha/beta</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour, Node, Metastases</td>
</tr>
<tr>
<td>Tr1</td>
<td>T Regulatory Type 1</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
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</table>
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Authors 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 classified as cancer that arises in the head and neck region, for example the nasal cavity, sinuses, lips, mouth, salivary glands, larynx and pharynx (O’Rorke et al., 2012; Figure 1.1). Squamous cell carcinomas (SCC) account for 85-95% of all head and neck cancers (HNSCC; O’Rorke et al., 2012; Leemans et al., 2011; Curado and Hasibe, 2009), and originates from the epithelial linings present throughout these regions. Other rarer forms include adenocarcinomas which develop from the lining of glands, sarcomas which arise from muscle or cartilage and melanomas which arise from melanocytes (www.macmillan.org.uk).

Figure 1-1 - Head and neck anatomy; Diagram showing the possible anatomical locations for HNSCC within the head and neck region (Gleeson, 2008)
1.2 – Anatomy of the head and neck

This report is focused on the laryngeal and oropharyngeal HNSCC sub-sites due to availability of patient samples and cell lines. In addition, many HNSCC patients show nodal involvement along with the primary cancer. This section focuses on those anatomical areas, highlighting their normal functions within the body.

1.2.1 – The larynx

The larynx, also known as the voice box, lies between the trachea and the hypopharynx (figure 1.1) and is an essential structure involved in protection of the airway, coughing, swallowing and is also the source of sound (voice). The larynx houses the vocal cords, and by controlling the airflow and pressure within can produce vibration and therefore sound with varying pitch and volume. During swallowing the larynx’s sphincteric action prevents any food debris from entering the trachea; this is an inbuilt reflex to prevent against choking (Burdett and Mitchel, 2011).

The majority of laryngeal cancers are squamous cell carcinoma, in part due to the fact that the majority of the epithelium consists of squamous cells. For the purpose of cancer categorising, the larynx is split into 3 subsites; glottis, supraglottis and subglottis (Gleeson. 2008). Laryngeal cancer is also associated with human papillomavirus infection with up to 24% being HPV positive (HPV; Li et al., 2013; Rautava et al., 2012) though not as frequently as oropharyngeal cancer.

1.2.2 – The pharynx

The pharynx consists of three portions, including the oropharynx the nasopharynx and the hypopharynx (Gleeson, 2008; figure 1.1).

The oropharynx is located between the hard palate of the oral cavity and the hyoid bone of the larynx. For the purpose of categorising cancers it is split into 4 subsites; the anterior wall (base of tongue), the lateral wall (including tonsils), the posterior laryngeal wall and the roof of the oropharynx (Gleeson, 2008).

The pharynx connects the airway of the oral and nasal cavity to the digestive tracts and its main job is to ensure that air and food enter the correct pathway. Food and air will pass through the oropharynx before being directed to the lungs or the digestive system.
The tonsils that reside within the oropharynx also play a role in immunity as they provide a first line of defence against pathogens (Gleeson, 2008).

The location of the oropharynx leaves it highly exposed to air born carcinogens as well as those that are swallowed. The main risk factors for oropharyngeal SCCs include smoking, drinking alcohol and HPV infection (section 1.4; Leemans et al., 2011).

1.2.3 - Lymph nodes

Once a primary tumour has developed in the head and neck region, in some patients cancerous cells can detach from the mass and travel via the lymphatic systems to the surrounding lymph nodes. The head and neck area has an extensive lymphatic system, allowing for a greater level of drainage and therefore spread of metastasising cells (Wenzel et al., 2004). Up to 43% of HNSCC patients have nodal involvement at the moment of diagnosis (Ridge et al., 2013).

Not all HNSCCs result in nodal involvement as it is determined by primary site and possibly by the genetic profile of the tumour (Leemans et al., 2011). The involvement of nodes also has an impact on a patient’s prognosis, where the size and number of nodes involved has an impact (Spriano et al., 2006). Patients with nodal involvement have a reduced treatment success and a reduced survival rate (Leemans et al., 2011; Wenzel et al., 2004)

If nodal involvement occurs in a patient, treatment strategies may be altered. A combination of chemotherapy and the removal of involved nodes along with any primary tumour tissue may be used. The current project includes the use of both primary tissue and nodal tissue.

1.3 - Incidence of HNSCC

In the developed world, cancer is one of the leading causes of death, in part due to ageing populations and life-style choices (Teymoortash et al., 2014; Jemal et al., 2011). There was a slight decrease in cancer incidence in men between 2005-2009 with cases in women remaining stable (Siegel et al., 2013). Despite minimal changes in incidence there are 20% fewer cancer related deaths, in part due to recent cancer control strategies and improved treatments (Siegel et al., 2013).
HNSCC is the seventh most common cancer in the world (Rettig and D’Souza, 2015) with around 50-60% of diagnosed patients of all age groups surviving for 5 years, which gives it one of the lowest survival rates among other major cancer types (Teymoortash et al., 2014; Leemans et al., 2011; Crowe et al., 2002).

In 2009, in the UK there were around 8500 new cases of head and neck cancer diagnosed, 2300 of which were laryngeal cancer and 6200 cases of oral cancer and oropharyngeal cancer (Cancer Research UK).

HNSCC is more common in the ageing population (over 65 years old) with the elderly having a worse prognosis in comparison to younger generations (Teymoortash et al., 2014; Majchrzak et al., 2014; Pulte and Brenner, 2010). Up to 75% of new laryngeal cancer cases are diagnosed in the over 60’s with 40% of oral cancer cases being diagnosed in the over 65 age group (Cancer Research UK). However, in the last 2-4 decades, there has been a steady increase in the incidence of oral cavity and oropharyngeal SCCs in young males between the ages of 18 and 45 years (Majchrzak et al., 2014; Gayar et al., 2014). These cases are non-classical in that they cannot be attributed to alcohol and tobacco use, but to the transmission of viral infection through changes in sexual activity of males in the 18-45 age group (Majchrzak et al., 2014; Gayar et al., 2014; section 1.4.1).

There is also a huge variation of HNSCC incidence rates between different countries. For example between 1983-1987 and 1998-2002 there was an increase in the incidence of laryngeal cancer in the UK, however there was a decrease in the majority of other countries (Simrad et al., 2014). Oropharyngeal cancer rates also increased during the same time span in the UK (Simrad et al., 2014). Despite regional increases, the lowest rates of HNSCC are seen in Europe and North America where only 3-4% of cancers are associated with the head and neck, and the highest rates in southeast Asia and Africa where 8-10% of cancers are from the head and neck region (Choong and Vokes, 2007).

These regional differences are generally attributed to different habits and cultures associated with the distant locations. Countries where there is a culture of tobacco use see an increased prevalence of oral cavity, such as Southeast Asian communities where chewing tobacco is common practice. Oral cavity cancer is on the decline in the UK and
USA in the last 10 years due to the changes in attitudes towards smoking (Jemal et al., 2011). Countries with a higher economic status, such as the UK and the USA tend to see increased prevalence of HPV related oropharyngeal, tongue base and tonsil cancer thought to be due changes in sexual behaviour (Simrad et al., 2014; O’Rorke et al., 2012; Jemal et al., 2011; section 1.4.1). Globally around 25% of HNSCC cancers are thought to be caused by HPV, whereas in the USA it is thought to account for around 60% (Simrad et al., 2014).

Long term survival prospects for HNSCC patients are poor due to the high level of late stage diagnosis and the likelihood of reoccurrence (Ragin and Taioli, 2007), with not much improvement over the last 40 years with mortality rates sitting at around 50% for HNSCC (Stransky et al., 2011) though it varies greatly depending on the region’s healthcare systems. For example in the USA survival rates increased from 52.7% to 65.9% between 1982-1986 and 2002-2006 (Pulte and Brenner, 2010) respectively, suggesting that countries with a higher economic status are more equipped to improve a patient’s chance of survival. Improvements to screening processes and staging are also thought to contribute to improving survival (Pulte and Brenner, 2010). It also depends on cancer sub-site, how early the cancer is detected, treatment and HPV involvement.

Evidence exists to show that the chance of survival increases with the involvement of HPV (O’Rorke et al., 2012; Marur et al., 2010). For example, the five year survival rate of non-HPV associated oropharyngeal cancer is between 25-40%, whereas it is in the region of 70-80% if the patient is HPV positive (O’Rorke et al., 2012). This may be due to HPV related cancers being more susceptible to treatments (Pulte and Brenner, 2010). HPV related HNSCC patients have a better chance of survival as stated above. This may be down to several things; firstly, HPV related SCCs tend to be detected at an earlier stage in younger, healthier patients, meaning treatment can be more successful and metastasis is less likely to have occurred and also due to improved tumour control (Boscolo-Rizzo et al., 2013; Ang and Stugis, 2012).

Around 5-20% of all HNSCC is attributed to HPV infection (Boscolo-Rizzo et al., 2013) with between 40-80% of oropharyngeal SCCs being HPV+ in the USA, though rates can be as high as 90% in some European countries such as Sweden where tobacco use is reduced (Marur et al., 2010). Other non-oropharyngeal sub types of HNSCC are
also found to be HPV+ with around 25% of all laryngeal SCCs testing positive for the virus (Torrente et al., 2011). One group found that out of 30 Laryngeal SCC patients, only 3 were found to be HPV positive, so the percentage varies depending on where the samples were sourced, however in all cases it was substantially lower than with oropharyngeal SCC.

Once a patient is cleared of HNSCC, their mortality rates drops to 20%, and these deaths are normally attributed to the continuation of bad habits like smoking. However, the chance of recurrence and another primary tumour remain (Teymoortash et al., 2014). The longer the patient goes without a recurrence, the better their long term survival rate (Teymoortash et al., 2014).

1.4 - Risk factors/aetiology

There are many known risk factors for HNSCC, which are generally linked to a specific region in the head and neck depending on area of exposure. The main risk factors discussed include: Viral infection, tobacco, alcohol, inherited disorders, occupational exposures, and tooth loss.

1.4.1- The human papillomavirus as a risk factor for head and neck cancer

Viral infections such as those caused by HPV are known to lead to an increased risk of cancer. HPV is most well known for its involvement in cervical cancer; however, it is also implicated in HNSCC in particular oropharyngeal carcinomas which have shown an increase in prevalence over the last few decades (Powell and Evans, 2015). HPV is more commonly associated with cancer of the oropharynx, most likely due to an increased chance of infection due to a general change in the sexual behaviour of individuals. It is suggested that an increase in ‘premarital sex, oral sex and increased number of sexual partners’ causes an increase in the risk of HPV associated oropharyngeal cancer, particularly in middle aged white males (de Souza et al., 2011). An increase in sexual contact is thought to increase the chance of spreading HPV.

HPV is a double stranded DNA virus that targets and infects squamous epithelial cells (Stanley, 2014). There are over 200 different sub-types of HPV which are categorised as either mucosal or cutaneous, depending on which cell type they target (Rautava and Syrjanen, 2012). However, only a small proportion of subtypes have been linked to an
increased risk of head and neck cancer (Furniss et al., 2007). These different subtypes can be categorized as having a high (subtypes 16 & 18), medium (subtypes 31 & 33) or low risk (subtypes 6 & 11) of forming cancer (de Villiers, 1994). The HPV 16 subtype is the most strongly implicated in HNSCC (Gleeson, 2008), and in particular oropharyngeal cancers. Approximately 20-25% of HNSCCs are caused by the HPV, with subtype 16 being responsible for between 90-95% of these (Furniss et al., 2007; Snijders et al., 1996). Up to 64% of oropharyngeal cancer patients were found to be positive for HPV 16 DNA (Pawlita et al., 2007) and these patients tend to be younger adults who do not smoke or drink (Evans and Powell, 2010), with the mean age being around 5-10 years younger than HPV negative cases.

The exact mechanisms behind the entry of HPV into epithelial cells in not fully known, however, a few different receptors have been identified which could involved, such as sulphate proteoglycans (HSPGs; Rautava and Syrjanen, 2012). Once the HPV has targeted and infiltrated the squamous epithelial cell, it will incorporate itself into the cells genome and will hi-jack its replication machinery. HPV does not have its own replication capabilities, so it must utilise the host’s own systems in order to replicate and continue existing (Powell and Evans, 2015). The virus produces proteins such as E6 and E7 which work to disrupt signalling pathways, keeping the cell in a perpetual state of proliferation (Powell and Evans, 2015). E7 interferes with the tumour suppressor retinoblastoma (Rb) and E6 with p53. Tumour suppressor genes produce protein products which prevent cells with damaged or mutated DNA from progressing onto mitosis by using checkpoints within the cell cycle. These interactions greatly reduce the ability of Rb and p53 to act as negative growth regulators causing the cell to become immortalised (Powell and Evans, 2015; Scully, 1993; figure 1.2). However, this is not enough for the cells to transform into malignant cells. It is the constant high levels of proliferation which cause an increased chance of mutations leading to accumulation which cause the cells to transform (Powell and Evans, 2015).

The addition of other risk factors such as smoking and drinking on top of HPV+ infection increases the risk of developing head and neck cancer when compared to non-smoking, non-drinking HPV+ patients (Pawlita et al., 2007). Some authors also argue
that HPV will not act independently and requires one of these other factors to become a risk (Gleeson, 2008).

HNSCC patients that are found to be HPV+ have a better prognosis than HPV- patients. This may be down to the younger demographic of HPV+ patients, but it may be in part due to the lack of mutations within the p53 tumour suppressor gene (Powell and Evans, 2015). In HPV- HNSCC the p53 gene is mutated and non-functional, however, in HPV+ the p53 is merely tampered with and still has some function. This may give therapies a better chance of success.

A vaccine against a specific HPV subtype has been developed recently and issued to individuals, to prevent the onset of cervical cancer (Furniss et al., 2007). However, the effects of this mass immunisation will not be seen for some time to come. If results show a decrease in the number of new cervical cancer cases, it is promising news for the potential development of a vaccine against HPV products which increase the incidence of head and neck cancer, and would provide evidence that not only women should be immunised but males as well (Furniss et al., 2007).
Figure 1-2 - Increased risk of HNSCC once infected by HPV – HPV related proteins E6 and E7 will affect p53 and pRB causing the cells to proliferate unchecked by the cell cycle check points. This will cause an accumulation of mutated cells, leading to tumour formation.
1.4.2 – Tobacco smoking/chewing and HNSCC

The smoking of tobacco has been strongly linked to many forms of cancer since the early 1900’s. In 1985 it was confirmed as a causal agent for various HNSCCs including cancer of the oral cavity, pharynx and larynx (Argiris et al., 2008). More recently it has been implicated in cancer of the nasal cavities and nasal sinuses (Gandini et al., 2008). A seven fold increase in risk of laryngeal cancer was observed in smokers who were non-drinkers compared to non-smokers/drinkers (Hashibe et al., 2007). In terms of relative risk (RR) for current smokers, laryngeal cancer is the most common HNSCC caused by smoking tobacco (RR = 6.98, 95% CI), closely followed by cancer of the pharynx (RR = 6.76, 95% CI), with cancer of the oral cavity yielding a lower relative risk (RR = 3.43, 95% CI; Gandini et al. 2008; table 1.1). RR will also decrease upon cessation from smoking (Hashibe et al., 2007).

Table 1-1 -Relative risk (RR) of developing cancer in smokers and former smokers in difference subsites of HNSCC

<table>
<thead>
<tr>
<th>Anatomical location</th>
<th>RR (95% CI) in current smokers</th>
<th>RR (95% CI) in former smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>3.43</td>
<td>1.4</td>
</tr>
<tr>
<td>Pharynx</td>
<td>6.76</td>
<td>2.28</td>
</tr>
<tr>
<td>Larynx</td>
<td>6.98</td>
<td>4.65</td>
</tr>
</tbody>
</table>

The absolute risk of smoking is hard to distinguish due to its close relationship with alcohol consumption; people who drink heavily will often smoke and vice versa. It is thought that 75% of cases of HNSCC are due to a combination of smoking and alcohol consumption (Hashibe et al., 2007). Some studies have focussed on tobacco smokers that do not consume alcohol to determine how tobacco alone influences the development of HNSCC. For example Hashibe et al (2007) pooled data from 15 different case-controlled studies and found that in 25% of head and neck cancers, tobacco was the sole causal agent. Several studies have also found a positive correlation between increase in frequency, duration and amount of cigarette packs smoked per year
and the risk of HNSSC (Hashibe et al., 2007; Franceschi et al., 1990). Twenty cigarettes or more a day is thought to present a major risk factor for cancer of the oral cavity, pharynx and larynx (Curado and Hashibe, 2009) and there is further evidence that cessation of smoking for 10 years leads to a substantial decline in risk (Franceschi et al., 1990). A study revealed that quitting smoking at an earlier age resulted in a greater drop in risk; for example cessation at 30 years of age reduced risk by 90% but a smaller decrease of 50% was seen in those who quit at 50 years old (Curado and Hashibe, 2009).

Second hand smoke will also increase the risk of some forms of cancer, especially if a person has been in the presence of a smoke filled environment for over 15 years as increased exposure time will increase the chances of cancer formation (Curado and Hashibe, 2009).

Other forms of tobacco consumption increase risk of cancer including smoking cigars or pipes which have been found to be a greater risk for cancer of the oral cavity than smoking cigarettes (Franceschi et al., 1990). Chewing tobacco is highly prevalent in countries such as India, where it is easily available (Sapkota et al., 2007). Zhou et al (2013) found a significant increase in the risk of HNSCC among tobacco chewers who do not smoke tobacco compared to non chewers/smokers.

An altogether new trend in smoking comes in the form of electronic cigarettes. This type of cigarette produces vapour from a solution of nicotine which is inhaled by the user. It is claimed to have lower levels of toxins, including carcinogenic material. Lukasz et al (2014) conducted a series of experiments to test the vapour to identify which toxins, if any, were present. They found a 9-450 x reduction in the levels of toxins in the nicotine vapour compared to cigarette smoke. This may be good news for the incidence rate of HNSCC for the future. It will be interesting to see whether increased uptake of E cigarettes leads to a reduction in the smoking of normal cigarettes, and whether this will lead to a reduction in HNSCC diagnoses.

The carcinogens in tobacco smoke can lead to the development of genetic abnormalities and therefore promote carcinogenesis. The toxins inhaled include nitroamines, acetaldehydes, acetone and formaldehyde (Lukasz et al., 2014). The extent of the build-
up of genetic abnormalities depends on the individual; how susceptible they are and also how much a person smokes. A person can inherit their susceptibility to tobacco related carcinogenesis, as an individual with a first relation who has developed head and neck cancer has a 2-14 fold increased risk (Ho et al., 2007).

The presence of certain enzymes, such as those coded for by the cytochrome p450 (CYP) family can increase the effect of certain carcinogens (Han et al., 2014). A polymorphism in the enzyme CYP1A1 can cause an increase in the activation of nitrosamines for example (Morita et al., 2010; Han et al., 2014). Individuals with these polymorphisms therefore become more susceptible to developing tobacco related cancer.

As mentioned earlier in this section, many people who smoke also drink alcohol. The intake of alcohol can increase the susceptibility of smokers to HNSCC. This will be discussed in the subsequent sections.

1.4.3 – Alcohol consumption and HNSCC

Alcohol presents another risk factor in the development of cancer (Purdue et al., 2009; Hashibe et al., 2007; La et al., 1998) and has been found to be a causal agent in cancer of the head and neck (Curado and Hashibe, 2009). It is thought that alcoholic beverages contain various carcinogens each with the potential to cause malignancy; these include ethanol, tannins and urethane.

The frequency of alcohol consumption increases the risk of HNSCC and consuming larger volumes per day when compared to moderate consumption, does increase risk in comparison to non-drinkers (Hashibe et al., 2009; Hashibe et al., 2007; La et al., 1998). A two-fold increase in the risk of HNSCC is seen in those who drink three or more drinks per day when compared with non-drinkers, though this figure varies between different anatomical locations in the head and neck. For example, consuming 1-2 alcoholic drinks per day increased the risk of pharyngeal cancer in contrast to laryngeal cancer, which requires the consumption of five or more drinks before an increase in risk is seen (Hashibe et al., 2007). The type of alcoholic beverage such as beer, wine or spirits appears to have no bearing on the risk of HNSCC (Purdue et al., 2009), however,
increasing volumes did, requiring health authorities to target education to prevent binge drinking.

Specific genetic abnormalities increase the risk of developing alcohol-related HNSCC such as cancer of the larynx. A defect in an alcohol metabolising enzyme such as aldehyde dehydrogenase confers an increased risk of developing cancer. Presumably, because the carcinogen (ethanol) is present for a longer period of time within the body (La et al., 1998)

Exposure to alcohol has been shown to increase the activity of cytochrome p450 proteins, which as previously stated, activates tobacco carcinogens, which lends to the multiplied risk when both alcohol and tobacco are consumed. Alcohol also increases the ability of carcinogens to pass into cells, making it more likely that they will cause genetic abnormalities. When alcohol is consumed it is converted into a product called acetaldehyde which is the carcinogenic factor of alcohol. The accumulation of acetaldehyde can be increased by the increased metabolism of alcohol, the increased intake of alcohol and the hampered degradation of the product. If someone were to have these problems they would become more susceptible to developing alcohol related carcinogenesis. Acetaldehyde will bind to cell’s DNA and will cause hypomethylation which affects the transcription of genes leading to possible genetic aberrations (Figure 1.3).
The increased consumption of alcohol will increase levels of acetaldehyde and therefore increase its metabolism. This build up can lead to genetic abnormalities and eventually to the development of cancer. Alcohol can also increase the risk of smoking by sensitising the body to the tobacco carcinogens. (ADH-antidiuretic hormone, ALDH-aldehyde dehydrogenase, GSTM1 - Glutathione S-transferase Mu 1).
1.4.4 – Genetic susceptibility and Inherited disorders

Certain inherited disorders such as Fanconi’s anaemia can increase the risk of developing cancer, with HNSCC the most frequent type (Leemans et al., 2011). Fanconi’s anaemia is a rare, autosomal recessive disorder described as a ‘progressive lethal anaemia’ (Schethenbach et al., 2012; Lustig et al., 1995) with one of its main characteristics being the development of cancer at an early age (Kutler et al., 2003). The disorder causes cells to become unable to maintain their genome’s stability causing instability of chromosomes and increasing cancer susceptibility (Kutler et al., 2003).

Kutler et al (2003) used the ‘international Fanconi’s Anemia registry’ to select 754 patients and found that 3% of these patients had been diagnosed with HNSCC, presenting an increased risk of developing HNSCC when compared to the general population. Only 16% of these patients had other contributing factors such as tobacco use, exposing a link between Fanconi’s anaemia and HNSCC. It was also found that up to half of these patients died as a direct result of the HNSCC (Schethenbach et al., 2012).

1.4.5 – Occupational exposures

Some occupations lead to greater levels of exposure to carcinogens and are therefore at a higher risk of developing cancer such as HNSCC. One example is the exposure to asbestos and cement dust which has been found to increase the risk of laryngeal cancers and pharyngeal carcinoma (Langevin et al., 2013; Purdue et al., 2006).

Though there is plenty of literature on the effect of occupational exposures on the prevalence of HNSCC, studies often do not take into account smoking and alcohol drinking habits. So it is difficult to estimate the overall effect of certain exposures.

1.4.6 – Tooth loss

Oral health and tooth loss has been shown to have an association with HNSCC (Curado and Hashibe, 2009; Guha et al., 2007). Tooth loss is more often found in the elderly and is normally due to the spread of bacterial infection and inflammation, causing the onset of periodontitis. Periodontitis has been linked to various chronic diseases including HNSCC (Hiraski et al., 2008). Oral cancer in particular is associated with poor oral
hygiene as the presence of bacterial infection in the oral cavity increases levels of potential carcinogens such as nitrosamine, which when present at high levels can cause a significant increase in the risk of developing cancer (Hiraski et al., 2008). It has also been suggested that tooth loss may also cause the patient to eat a less healthy diet, adding to the associated risks (Hiraski et al., 2008).

1.5 – Pathogenesis and Staging

1.5.1 - Pathogenesis

It is generally accepted that cancer arises from a single cell which has undergone a genetic aberration, causing a growth advantage over neighbouring cells. It is thought that several of these aberrations must occur for a tumour to develop (Hardisson, 2003; Klein and Grandis, 2010) and these genetically modified cells will accumulate replacing existing cells within the tumour area. There are several paths that can lead to genetic alteration; some of which are detailed below.

Multiple genes are involved in the pathogenesis of head and neck cancer. How each carcinogen leads to genetic abnormalities which can then develop into head and neck cancer is becoming more clear (Singh, 2008).

One of the first mutations to be identified was p53, which is associated with the development of many human cancers (Hardisson, 2003). P53 is a tumour suppressor gene located on chromosome 17q13 which is responsible for identifying DNA damage and preventing those cells from continuing to proliferate (Klein and Grandis, 2010). It does this by arresting the cell cycle until the DNA has been repaired or if it cannot be repaired it is involved in the initiation of apoptosis (Riaz et al., 2014; Klein and Grandis, 2010). The most recent figures suggest that between 46 and 73% of HNSCC patients have mutations in the p53 gene (Riaz et al., 2014) suggesting that it plays a major role in the progression of HNSCC. Up to 75% of these are missense mutations in the DNA binding domain which will alter or inactivate the function of p53, allowing for unchecked cell proliferation and subsequent development of a tumour mass (Rothenberg and Ellisen, 2012; Hardisson, 2003). The oncogenic proteins E6 and E7 from HPV infection also down regulate p53 (Rothenberg and Ellisen, 2012; Klein and Grandis, 2010; Section 1.4.1). There are several other less common mutations that can
lead to the development of HNSCC, including the CDKN2A gene, EGFR and Rb pathways (Suh et al., 2014; Rothenberg and Ellisen, 2012; Klein and Grandis, 2010).

1.5.1.1 – Recurrence

Recurrence rate for HNSCC tumours in the same region as the primary tumour, is between 25 and 50% depending on the sub-site (Ho et al., 2014). There are two main theories as to why head and neck cancers have a high recurrence rate of a second tumour in the same region; field cancerization and micrometastatic lesions.

The field cancerization theory states that “multiple areas of genetically unrelated aberrations appear in the same field of tissue which have been exposed to carcinogens”. It is thought that extended exposure to carcinogens preconditions the area making it more susceptible to further genetic abnormalities and development of cancerous lesions (Tabor et al., 2001). It has been observed that abnormal cells can be seen at the surgical margin and that secondary tumours are very common and will often appear in the same anatomical location (Tabor et al., 2001). Also the cells within the tumour lesions have been found to be heterogeneous; likely originating from different genetic aberrations. This theory would also explain the high rate of patients developing a second tumour, as it is possible that not all lesions were removed during surgery, however, although there is evidence to support this theory, more work needs to be done (Lydiatt et al., 1998).

The other theory states that micrometastatic foci which have migrated from the primary site to a new site in the same area are the cause of high rates of secondary tumours. It is thought that the tumour could spread through the mucosa creating a genetically related tumour within the same vicinity as the primary tumour. In some patients it has been proven that a primary and local secondary tumour have developed from a single clonal population, by examining the cells genetic profile (Bedi et al., 1996).

Recurrence is one of the main factors in the poor long term survival of HNSCC patients and requires a multidisciplinary team in order to maximise the patient’s chance of long term survival (Ho et al., 2014; Arnold et al., 2004). Treatment of recurrence can be difficult, leading to poor success rate, which is discussed in more detail in section 1.7.
1.5.2 – Staging

HNSCC is classified using the TNM (tumour, node, metastases) staging system developed by the UICC (International union against cancer) and the AJCC (American joint committee on cancer). TNM classification uses the size of the primary tumour (T), the involvement of nodes (N) and discovery of metastases (M) as a way of giving patients final stage. Definitions of each stage can vary slightly depending on the sub-site. Table 1.2 highlights the stages for oropharyngeal and laryngeal HNSCCs (Gleeson, 2008).

The classification of head and neck cancer allows clinicians to make decisions, regarding treatment plans and estimate what a patient’s prognosis might be. It also allows information to be passed between centres making it much easier to transfer patients and for research to be compared. There are limitations, including the inevitable human error involved as the tumour must be assessed then classified based on human judgment; therefore there will always be a degree of variability.
**Table 1-2 - TNM staging and there descriptions**

<table>
<thead>
<tr>
<th>TNM designation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T = Tumour</strong></td>
<td></td>
</tr>
<tr>
<td>Tx</td>
<td>The primary tumour cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>There is no evidence of a primary tumour</td>
</tr>
<tr>
<td>Tis</td>
<td>Primary tumour is in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Primary tumour is &lt;2cm</td>
</tr>
<tr>
<td>T2</td>
<td>Primary tumour is &gt;2cm but no more than 4cm</td>
</tr>
<tr>
<td>T3</td>
<td>Primary tumour is &gt;4cm</td>
</tr>
<tr>
<td>T4a</td>
<td>Primary tumour has become invasive and is described as moderately advanced local disease</td>
</tr>
<tr>
<td>T4b</td>
<td>Primary tumour has invaded further and is described as very advanced</td>
</tr>
<tr>
<td><strong>N = Regional lymph node involvement</strong></td>
<td></td>
</tr>
<tr>
<td>Nx</td>
<td>Lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No lymph node involvement</td>
</tr>
<tr>
<td>N1</td>
<td>Metastases in a single lymph node &lt;3cm</td>
</tr>
<tr>
<td>N2a</td>
<td>Metastases in a single lymph node &gt;3cm but no more than 6cm</td>
</tr>
<tr>
<td>N2b</td>
<td>Multiple lymph node involvement, none &gt;6cm</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in lymph node &gt;6cm</td>
</tr>
<tr>
<td><strong>M = Distant metastasis</strong></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

* Modified from National Comprehensive cancer network*
1.6 – Symptoms, diagnosis and prognosis of HNSCC

The symptoms of head and neck cancer depend on the primary site, the involvement of metastases and the stage to which the cancer has developed. HNSCC typically presents with either red/white sores in the mouth that do not heal well, lumps in the neck area, persistent sore throats and hoarseness of voice. It can lead to difficulty talking, swallowing, eating and pain within the ear and jaw area (Gleeson, 2008). The symptoms are not specific to head and neck cancer, so it is important to use additional evidence to diagnose a patient.

In order to diagnose head and neck cancer there are several tests which can be carried out. First, a detailed history is taken, recording habits such as smoking, alcohol intake and age, in order to assess the potential risk for developing head and neck cancer. A physical examination then looks for lumps within the neck area and the oral cavity. If the problem cannot be viewed directly, an endoscope can be passed into the nasal cavities and throat in order to gain a better view. To determine whether HNSCC is present, a biopsy must be taken and sent to the laboratory for testing; however, in some cases biopsy is not possible. Certain imaging techniques can be used to look at those tumours which cannot be biopsied and also to look at the rest of the body to locate metastases. These tests can determine whether the patient has cancer, the stage and therefore which treatment options will work best for each individual patient (Gleeson, 2008).
1.7 – Treatment/ follow up

Once a diagnosis has been made a treatment plan can be developed to give the patient the best chance of survival. The three main options are surgery, chemotherapy and radiotherapy or a combination of these, each having their own disadvantages and advantages. Each NHS trust will have a slightly different protocol for treatment, with different drugs available for chemotherapy, and different attitudes towards when surgery should be performed. Determining whether there is nodal involvement is also of importance, as it can change the way in which a patient is treated (Roepman et al., 2005).

In the UK T1 and T2 tumours are treated with radiotherapy or chemotherapy. Surgery will be performed if there is involvement of neck node prior to any other treatment. Any larger, later stage tumours are removed using surgery followed by radiotherapy and chemotherapy. The aim as always is to maintain patient quality of life.

The best treatment option for most HNSCC is a combination of radiotherapy and immunotherapy in the form of a drug called Cetuximab. Cetuximab is an antibody directed against the epidermal growth factor receptor (EGFR) and works to sensitize cancer cells to the radiation treatment (Tejani et al., 2010). New therapies are constantly being researched to improve the survival rate of HNSCC patients.

After intervention for the primary tumour, patients are monitored for recurrence regularly, due to the high likelihood of them developing. Treatment for recurrence can be very difficult. Factors including previous treatment, length of time since last treatment and the subsite can make choosing the most appropriate course of action difficult. Also, during the first round of therapy, cells undergo a selection process, whereby the most resistant cells endure, leading to more resistant recurrences (Ho et al., 2014). Previous treatment with chemotherapy and radiotherapy leads to toxicity, so if the recurrence appears soon after treatment for primary, the patients may not be able to deal with further doses. The favoured path is to surgically remove the new lesions, if the mass is resectable, though often radio and chemotherapy is first tried in order to maintain the patient’s quality of life (Ho et al., 2014; Arnold et al., 2004).
Surgical removal can often result in reduced quality of life for patient, for example, in laryngeal SCC recurrence, it may be necessary to perform a complete laryngectomy, which will leave the patient unable to speak (Ho et al., 2014). Success for the treatment of recurrence is dependent on sub-site. Recurrence of laryngeal SCC has better survival rates compared to other subsites with up to 60% of patients surviving for 5 years or longer (Arnold et al., 2004). Whereas oropharyngeal SCC recurrence has a poor survival rate after intervention, with a 5 years survival rate of around 16% (Arnold et al., 2004).

1.8 - The immune system

The immune system is a set of biological structures, cells and processes that work together to protect the body against foreign organisms such as bacteria and viruses, and can also work to prevent the development of cancer cells. In order to work efficiently the immune system must be able to distinguish between foreign antigens and self antigens thus preventing damage to tissue and it does this using cell surface markers. If the immune system fails to recognise and avoid self antigens, it can cause autoimmune disease and inflammatory diseases. Immune deficiencies increase the likelihood of infection which can increase the chance of developing cancer (Furniss et al., 2007). The immune system contains multiple cell types, all with highly specialised functions which are able to work together by communicating via cell to cell contact and soluble factors. Immune cells originate from the bone marrow where they differentiate from stem cells by haematopoiesis. The cells then either mature within the bone marrow (B cells) or pass into the blood stream where they can travel to the thymus to become one of a selection of T cells. Immunity can be either innate or adapted and the processes involved with each are summarised in (Koch and Radtke, 2011).

1.8.3 - The immune system and cancer

Cancer develops from host cells that have undergone certain changes allowing them to proliferate unchecked. Every step of a tumour’s development requires avoiding the intervention of the immune system, leading to a myriad of different avoidance techniques (Visser et al., 2006). Cancer cell express tumour-associated antigens (TAA) which can be recognised by circulating T cells and cause their activation (Restifo et al., 2012). Development and metastasis of a tumour is therefore a process which requires
the ability to escape detection of the immune system. There are many theories as to how this immune evasion is achieved, though it is likely that a plethora of mechanisms are involved (Table 1.3).

**Table 1-3** – Showing the various techniques employed by tumours to evade immune detection

<table>
<thead>
<tr>
<th>Evasion technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down regulation of adhesion molecules on tumour cells</td>
<td>Igney and Krammer, 2002</td>
</tr>
<tr>
<td>Impaired antigen expression leads to the tumour being undetectable by the immune system. Also expressing heterogeneous antigen reduces the chance of a strong specific response from immune cells.</td>
<td>Igney and Krammer, 2002</td>
</tr>
<tr>
<td>Reduced MHC-1 expression on malignant cells, which leads to a reduced immune response.</td>
<td>Igney and Krammer, 2002</td>
</tr>
<tr>
<td>Expression of immunosuppressive factors (e.g. TGF-β and IL-10) by tumours cells and other cells in the tumour microenvironment such as immune cells and stromal cells.</td>
<td>Igney and Krammer, 2002</td>
</tr>
<tr>
<td></td>
<td>Croci and Salatino, 2011</td>
</tr>
<tr>
<td></td>
<td>Chew et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Young, 2006</td>
</tr>
<tr>
<td>Reduced expression of co-stimulatory molecules on the surface of tumour cells, which lead to T cell anergy.</td>
<td>Igney and Krammer, 2002</td>
</tr>
</tbody>
</table>
The tumour microenvironment push’s the immune response from Th1 type to Th2 type response, which is thought to be pro-tumour. Igney and Krammer, 2002

The tumour stimulates T regulatory cells which dampen down the immune response, and will therefore prevent the immune response against the tumour cells. Igney and Krammer, 2002

Chew et al., 2012

Strauss et al., 2007

The tumour alters molecules in its apoptosis pathways to prevent its destruction upon detection by immune cells. Cancer cells can also produce anti-apoptotic molecules. Igney and Krammer, 2002

Chew et al., 2012

As time goes by, malignant cells become more resistant to immune attack due to immune selection. Whereby the immune system destroys susceptible cells and leaves behind resistant cells which will then multiply. Igney and Krammer, 2002

Chew et al., 2012

Cancer associated fibroblasts in the local area produce growth factors which will promote the development and growth of the cancerous cells. Chew et al., 2012

Evidence implicates the immune system directly in the development and progression of cancer (Whiteside, 2010; Visser et al., 2006). Tumour associated macrophages (TAM) are known to aid the development of cancer by their involvement in angiogenesis and favouring T regulatory cells particularly in late stage cancer (Visser et al., 2006; Zamarron and Chen, 2011). An increase in T regulatory cells then goes on to reduce the
effectiveness of other CD4$^+$ T effector cells, giving the tumour free reign to carry on proliferating (Zamarron and Chen, 2011). TAMs are members of the innate immune system, as are other tumour promoting cells, such as myeloid-derived suppressor cells.

It is widely believed that transformed cells will arise on a regular basis within the body and that the immune system will destroy these cells before they can develop into cancer (Kim et al., 2007). There has been evidence found of a weak immune response against TAAs in some cancer patients, and the presence of tumour infiltrating lymphocytes (TILs) within the tumour mass is believed to be a good prognostic sign (Whiteside, 2010), although the evidence for this is sometimes conflicting and it might be that the relationship between TILs and prognosis depends on the type of immune cells present. So if the immune system is able to target tumour cells how is it that a tumour can develop fully without being destroyed?

There are thought to be three steps during tumour development relating to its interactions with the immune system; elimination, equilibrium and escape (Kim et al., 2007).

The immune response will involve both the innate and adaptive immune system, and will result in the destruction of the offending cells; this process is called elimination. The process of elimination is thought to cause immune selection, where only the most immune-reactive cells are targeted and destroyed, leaving behind the more resistant cells which subsequently emerge. This balance between tumour cells being eliminated and replaced by less immune-reactive cells is the equilibrium phase, which is thought to be the longest stage. Finally the tumour develops further and creates a tumour microenvironment (TME) which will suppress the local immune cells via cell to cell interactions and soluble factors such as cytokines. One of the TMEs purposes is to limit any further immune reaction, and it is thought to do this by releasing immunosuppressive factors rendering the TILs phenotypically and functionally altered, so reducing their capability of providing an immune response (Whiteside, 2010). The TME is a complex arena of cells and soluble factor interactions, which are still being investigated and the communication between the tumour cells and the immune system via soluble factors forms the focus of this thesis. Subsequent sections will highlight what is known so far and what this project aims to elucidate.
1.8.4 - The immune system in HNSCC

Just like many other cancer types, HNSCC patients have been found to harbour a compromised immune response, which lead to a reduced response to TAAs. However, like other tumours, HNSCC-associated antigen have been identified and it has been shown that CD4+ and CD8+ T cells can recognise them, they are just not able to build up enough of an immune response to dispatch the tumour (Strauss et al., 2007). Despite various immune cells being found within the HNSCC tumour microenvironment, it has been shown that TILs (such as T effector cells) lose their ability to react to TAA.

The tumour cells are able to evade the immune system and actively reduce any anti-tumour response present, using different techniques (Alhamarneh et al., 2008; Whiteside, 2005; table 1.3). HLA class I molecules are involved in the presentation of endogenous proteins to cytotoxic T lymphocytes leading to immune activation; one way in which HNSCC cells (and other cancer cells) avoid immune detection is by the down regulation of the HLA class I molecules on their surface (Young, 2006; Whiteside, 2005). The secretion of immunosuppressive factors such as TGF-β and IL-10 by cells in the HNSCC tumour microenvironment reduces the activity of reactive T cells leading to a further reduction in the immune response (Alhamarneh et al., 2008; Young, 2006).

There is also evidence that the HNSCC environment enhances the body’s natural ability to suppress immune responses, by the stimulation of regulatory T cells (Treg; Young, 2006). Treg cells have been found in elevated levels in both the tumour microenvironment and the circulation of HNSCC patients (Strauss et al., 2007). This increase in Treg cells will have a negative impact on the ability of T effector cells and CD8+ cytotoxic T cells to eradicate the tumour cells. However, there is still allot of work to be done regarding the mechanisms.

The HNSCC microenvironment is able to promote the generation of immunosuppressive Tregs which produce the immunosuppressive factors TGF-β and IL-10 (Strauss et al., 2007). A culmination of these events has the potential to lead to the induction of immune suppression by the tumour allowing immune evasion and tumour growth and spread. This thesis concentrates on how the tumour interacts with
immune cells via soluble factors, focusing on Tregs, T effector cells and CD8+ cytotoxic T cells (section 1.9.2 onwards).

1.9 - Tumour microenvironment and its contributors

The tumour microenvironment is a term used to describe the non-cancerous components within the TME and consists of a plethora of different cells including stromal cells, immune cells and soluble factors which have the potential to create an environment which aids the tumour to evade the immune system.

1.9.1 - Stroma

A large proportion of solid tumour is made up of stroma which co-evolves alongside malignant epithelial cells to produce a pro-tumour microenvironment (Li et al., 2007). The stroma is made up of fibroblasts, epithelial cells, immune cells, fat and muscle cells along with extra-cellular matrix which can take up a large proportion of a solid tumours mass (ECM; Li et al., 2007). This thesis focuses on cancer associated fibroblasts as they are the most predominant cell type within the stroma.

1.9.1.1 - Cancer associated fibroblasts

The role of fibroblasts in healthy tissue is to maintain the ECM and therefore structure within the local area (Li et al., 2007). They constantly renew the ECM in a very controlled and regulated manner and are also involved in wound healing, where they become activated by molecules such as TGF-β and migrate to the site of injury (Li et al., 2007). Activated fibroblasts can be characterised by their morphology and their expression of α-smooth muscle actin (α-SMA; Östman and Augsten, 2009; Li et al., 2007).

During the development of a tumour, cells in the local area are affected by cell to cell interactions and the milieu of different soluble factors released in the microenvironment by the malignant epithelial cells. Cancer-associated fibroblasts are cells that have been altered phenotypically and functionally under the influence of the tumour microenvironment and have acquired the characteristics of activated fibroblasts (Li et al., 2007). The exact origin of CAFs is still under investigation, however, there are a few theories including local fibroblasts, epithelial cells that have undergone an epithelial
to mesenchymal transition, local malignant cells and endothelial cells (Östman and Augsten, 2009; Li et al., 2007; figure 1.4). Mueller et al (2007) have provided evidence that local fibroblasts convert into CAFs in colorectal liver metastases by showing that these are phenotypically and functionally similar to a population of normal fibroblasts in close proximity. It is likely that there are multiple sub-types of CAFs which vary depending on the origin of the cancer (Östman and Augsten, 2009; Li et al., 2007).

Figure 1-4 - Schematic of the proposed origins of cancer-associated fibroblasts. It is thought they can originate from normal/ malignant epithelial cells, endothelial cells, mesenchmal precursor cells and local fibroblasts. Adapted from Li et al., 2007 and Östman and Augsten, 2009.

CAFs are known to be pro-tumour, releasing elevated levels of various growth factors including TGF-β and epidermal growth factor (EGF) which promote the growth of malignant cells in the tumour microenvironment (Östman and Augsten, 2009; Rosenthal et al., 2004). In addition CAFs can have metabolic pathways which compliment malignant cells; they act to remove toxic metabolites, buffering acidity to maximise tumour growth and can promote angiogenesis (Östman and Augsten, 2009). CAFs are
thought to exert an immunosuppressive effect within the tumour microenvironment by the release of TGF-β, for example, Rosenthal et al (2004); using ELISA, found that CAFs isolated from HNSCC samples produced a significantly higher amount of TGF-β compared to normal fibroblasts, even when cultured in isolation from malignant cells.

CAFs lie in close proximity to cancerous epithelial cells yet it is still not fully clear which causes the other to appear, whether they develop in tandem and how one influences the other (figure 1.5).

![Figure 1-5; Photomicrograph showing tumour epithelial cells and stromal cells within the HNSCC tumour microenvironment (x400; courtesy of Dr Samantha Drennan)](image)

**1.9.2 - T regulatory cells**

T regulatory cells and their immunosuppressive function were first identified by Sakaguchi et al (1995), and have since been the focus of much research, in particular, their role in tumour progression. Treg cells account for 7-8% of all CD4+ T cells in the periphery and have also been found in the tumour microenvironment suggesting a major role to play (Oleinika et al., 2013; Liu et al., 2006). There are two main ways in which Treg cells can exert their immunosuppressive powers;
CD4\(^+\) Treg cells are found naturally (nTreg) within the body and are able to suppress components from both the innate and adaptive immune system to prevent an autoimmune reaction and maintain immune homeostasis (Vignali \textit{et al}., 2008; Wing \textit{et al}., 2006; Lan \textit{et al}., 2005; Fontenot and Rudensky, 2004). For example, Treg cells can disrupt the function of dendritic cells and NK cells from the innate immune system and T effector cells, CD8\(^+\) cytotoxic T cells from the adaptive immune response (Vignali \textit{et al}., 2008). They are also involved in the control of immune reactions against pathogens and the tolerance of indigenous flora (Fontenot and Rudensky, 2004). The importance of Tregs has been demonstrated in Scurfy mice which lack the fork head box P3 transcription factor (FOXP3), essential for Treg development, where the onset of autoimmune disease was observed with uncontrolled lymphocyte proliferation (Lan \textit{et al}., 2005; Fontenot and Rudensky, 2004; Dieckmann \textit{et al}., 2001). In contrast the transfer of CD4\(^+\)CD25\(^+\) Treg cells into Scurfy mice was found to inhibit the onset of such autoimmune diseases and in some cases reverse it (Wing \textit{et al}., 2006; Fontenot and Rudensky, 2004). These diseases closely resemble those seen in humans with regulatory cell dysfunctions and the extent of Treg depletion is closely linked with the severity of such diseases (Wing \textit{et al}., 2006). The investigation into the role of Tregs has been hampered somewhat by the lack of expression of specific Treg marker and by the realisation that there is more than one subset of Tregs.

### 1.9.3 - T regulatory cell subsets

Tregs can be sub-divided into naturally occurring Tregs (nTregs; CD4\(^+\)CD25\(^+\)FOXP3\(^+\)) and inducible Tregs (iTregs; CD4\(^+\)CD25\(^+\)) both of which have very different developmental pathways and methods of suppression (Chatenoud, 2011; Adalid-Peralta \textit{et al}., 2011; Wing \textit{et al}., 2006).

#### 1.9.3.1 - nTregs

nTregs are developed to maturity within the thymus before being released into the circulation (Adalid-Peralta \textit{et al}., 2011; Lan \textit{et al}., 2005; Figure 1.6). Within the thymus nTregs develop their ability to regulate self-tolerance and it has been found that to
develop and survive they require molecules such as CD28, CD40 and IL2 (Lan et al., 2005). It has been suggested that in the thymus a high affinity for TCR interaction may control the expression of the intracellular molecule FOXP3, an important factor in the development of nTreg (Vignali et al., 2008; Fontenot and Rudensky, 2004). The lack of FOXP3+ Tregs has been shown to lead to autoimmune diseases highlighting its importance during development and for Treg function (Vignali et al., 2008). nTregs comprise 7-8% of the CD4+ T cell population in healthy humans and are able to regulate an array of different immune cells such as T effector cells and CD8+ cytotoxic T cells (Wing et al., 2006; Liu et al., 2006; Lan et al., 2005). nTregs can be distinguished from other CD4+ T cells by their high levels of CD25 expression, which is the α subunit of the IL-2 receptor, located on their surface and also the expression of intracellular FOXP3 (Lan et al., 2005). It is thought that nTregs principally use cell-cell contact to suppress other immune cells (Wing et al., 2006; Lan et al., 2005).

CD4, CD25 and FOXP3 expression were used for a substantial amount of time to identify T regulatory cells. It was found that this was not selective enough to distinguish between normal and regulatory cells of the same expression pattern as up to 20% of all CD4+ T cells in the periphery are CD25+ (Seddiki et al., 2006). Due to the need for a more precise biomarker, several groups have identified another marker which can distinguish T regulatory cells; CD127 (IL-7 receptor; Seddiki et al., 2006; Liu et al., 2006). T regulatory cells express low levels of CD127, whereas other T cells have a much higher expression. CD127 is inversely correlated with FOXP3 and it is thought that the presence of FOXP3 may prevent the expression of CD127 (Liu et al., 2006). In order to test whether these CD4+CD25+FOXP3+CD127lo cells were in fact T regulatory cells Seddiki et al (2006) used thymidine and CFSE assays to track the proliferation of CD45RA+CD25+ cells in the presence of CD127hi and CD127lo cells. They found that only the CD127lo cells were able to suppress the cells activity. This new biomarker gives the ability to sort T regulatory cells more specifically, allowing us to test their function more precisely. For the purpose of this thesis, the biomarkers CD4, CD25 and CD127 were selected. It was unfeasible to stain for FOXP3 every time due to the fact it is an internal marker, and the T regulatory cells were to be cultured.
**1.9.3.2 - iTregs**

Inducible Tregs develop from naïve T cells in the periphery when the right conditions are present such as the presence of VEGF, IL-10, TGF-β and dendritic cells (Whiteside, 2014; Lan et al., 2005; figure 1.7). They can be divided into IL10 producing Tr1 cells and TGF-β producing Th3 cells (Adalid-peralta et al., 2011; Wing et al., 2006). Tr1 can develop from naïve T cells in an environment containing IL10 and have relatively poor proliferative activity (Strauss et al., 2007; Wing et al., 2006) and differ from nTregs in that they do not suppress via cell-cell contact, but use the release of cytokines; they also do not express high levels of CD25 or FoxP3 (Lan et al., 2005; Dieckmann et al., 2001). Th3 cells produce TGF-β which in turn drives the differentiation of further naïve T cells into Th3 iTregs (Wing et al., 2006). Th3 cells, like nTregs have CTLA-4 (cytotoxic T lymphocyte-associated molecule 4) on their surface which is a molecule involved in the secretion of TGF-β which is the main suppressive mechanism of these cells. CTLA-4 appears to be an important molecule in the function of T regulatory cells, as it is involved in T cell homeostasis (McCoy and Le Gros, 1999). It is thought to have a negative effect on the activation of T cells, however, this research is still controversial with other suggesting the opposite (McCoy and Le Gros, 1999).
Figure 1-6: Diagrammatic representation of the origin of nTregs and their method of suppression.
Figure 1-7: Diagrammatic representation of the origin of iTregs and their method of suppressing their target.
1.9.3.3 - T regulatory cells in cancer

The increased numbers of Tregs within the circulation of cancer patients and in the tumour microenvironment is partly responsible for preventing the immunosurveillance and elimination of newly emerging cancer cells and also prevents an immune response against already established cancer tissue (Shen et al., 2010; Chaput et al., 2009; Yang et al., 2006).

Several studies have identified the increased prevalence of Tregs (between 25-30%) within the periphery and in the tumour in many types of cancer including early-stage non-small cell lung cancer, ovarian cancer (Woo et al., 2001), colorectal cancer (Chaput et al., 2009), hepatocellular carcinoma (Shen et al., 2010; Yang et al., 2006) and head and neck cancer (Schaefer et al., 2005). Shen et al (2010) harvested culture medium from hepatocellular carcinoma tissue and found that it had a greater chemo-attractant effect on the migration of Treg cells when compared to culture medium collected from benign tissue suggesting that the tumour is actively recruiting Tregs into the microenvironment to help with immune evasion. A study by Chaput et al (2009) looked at the presence and function of CD4^+CD25^+FOXP3^+ Tregs in the periphery and in the tumour of 40 colorectal patients and compared them to samples from healthy donors. They found that in addition to the ability of the Tregs to suppress the proliferation of T effector cells (CD4^+CD25^-), T regulatory cell number increased in both the periphery and the tumour tissue compared to the controls and that the overall numbers showed a positive correlation with the stage of the tumour, suggesting that Tregs accumulate as the tumour progresses. Many other groups have also shown that Tregs from cancer patients will suppress the activity of CD4^+CD25^- cells (Shen et al., 2010; Woo et al., 2001), whereas some have shown that they will also inhibit the proliferation of CD8^+ cytotoxic cells in hepatocellular carcinoma, which will prevent their action against the tumour tissue (Yang et al., 2006).

The presence of Treg cells in the TME can effect a patients overall survival rate. In some cancers such as ovarian, the presence of Tregs leads to a poor prognosis and survival rate (Gao et al., 2007), whereas in HNSCC Badoual et al (2006) found infiltrating Tregs had no significant effect on the overall survival of patients. Gao et al
(2007) have suggested that it is the balance of Treg cells and CD8+ T cells in the TME that is more suggestive of a patient’s chance of survival.

To determine the role of Tregs in the development of tumours, anti-CD25 IL-2Rα monoclonal antibodies were administered into mice with a range of tumours including leukaemia, myeloma and sarcoma causing a reduction in the number of CD4+D25+ Tregs and regression of the tumours in 6/8 cases (Onizuka et al., 1999).

1.9.3.4 - T regulatory cells in head and neck cancer

Immune dysfunction in HNSCC patients has been well documented and it has been found that HNSCC patients have an overall lower level of CD4+ cells in their peripheral circulation (Schaefer et al., 2005; Hoffmann et al., 2002; Reichert et al., 2002). However, many groups have found a significant increase in the frequency of Tregs within the PBMC in patients with active HNSCC compared with controls and they suggested that as well as aiding in the development of the tumour, their presence may also contribute to the progression of recurrences (Strauss et al., 2007; Schaefer et al., 2005). The same group has demonstrated that there is no difference in the level of Tregs in the circulation of HNSCC patients with active disease and those who have had treatment with no further sign of disease, suggesting that the level of Tregs do not normalise rapidly following tumour removal, which may be the reason for such high recurrence rates (Strauss et al., 2007; Schaefer et al., 2005).

Strauss et al (2007) found that the CD4+CD25hiFoxp3+ cells localise within the HNSCC tissue. These cells secrete IL-10 and TGF-β into the tumour microenvironment and therefore have a strong suppressive ability. Treg present in the tumour, differ from those found in the periphery, as they share common characteristics with activated memory T cells but also have similarities with Tr1 cells (Strauss et al., 2007), so it is unclear whether these cells are recruited into the HNSCC tumour microenvironment, or whether the environment converts CD4+CD25- cell into Treg.
1.9.3 - CD4$^+$ T effector cells

Both CD4$^+$ and CD8$^+$ T cells start life as lymphoid progenitors in the bone marrow with no expression of either surface marker. The progenitor cells then migrate to the thymus where they begin to express both CD4$^+$ and CD8$^+$ markers (double positive) as well as the T cell receptor (TCR; Germain, 2002). They are then exposed to MHC class I and MHC class II molecules on the surface of cells within the thymus. If the TCR binds preferentially to MHC class II the cells become exclusively CD4$^+$ T cells and if they bind MHC class I they become CD8$^+$ T cells. They drop the opposing marker to become single positive before migrating out of the thymus and into the periphery (Germain, 2002; figure 1.8).
Figure 1-8 - Showing the origin and selection of CD4$^+$ and CD8$^+$ T cells. Progenitor cells in the bone marrow are double negative (−−) for CD4$^+$ and CD8$^+$ before travelling to the thymus where they gain the TCR and CD4$^+$ and CD8$^+$ becoming double positive (++)$. They become exposed to epithelial cells expressing MHC class I and MHC class II molecules. If the TCR preferentially binds MHC class II they drop the CD8$^+$ marker and become CD4$^+$ T cells. If the TCR preferentially bind MHC class I they drop the CD4$^+$ marker and become CD8$^+$ T cells. They then migrate from the thymus into the periphery. Adapted from Germain (2002).

Once the CD4$^+$ T cells are in the peripheral blood they are able to undergo antigen-dependant differentiation. An APC presents an antigen (e.g. a tumour associated antigen) to the CD4$^+$ T cells causing activation into cells with full effector function (Pepper and Jenkins, 2011). Then depending on the local cocktail of cytokines they can differentiate into an array of different subtypes (Zhu et al., 2010; figure 1.9). The three sub-types of consequence in this thesis are Th1, Th2 and Th17 T cells, though others do...
exist (Luckheeram et al., 2012; figure 1.9). Once activated the T effector cells express homing receptors and begin to migrate to the site of origin of the antigen where they can begin the process of eradicating the foreign particles (Pepper and Jenkins, 2011). T effector cells are involved in stimulating further immune responses by the release of cytokines into the local environment (Luckheeram et al., 2012). After the foreign particles have been eradicated up to 90% of the CD4+ T effector cells die, leaving behind a small number of memory cells, which live longer in order to quickly respond to repeat episodes (Pepper and Jenkins, 2011).

**Figure 1-9 -** Showing the antigen driven differentiation of naïve CD4+ T cells into Th1, Th2 and Th17 cells, cytokine secretion and function. The cytokine environment helps determine the subtype. Cytokine profiles of Th1, Th2 and Th17 are a distinguishing feature of each subtype. Adapted from Luckheeram et al (2011), Pepper and Jenkins (2011) and Nakayamada et al (2012).
1.9.3.1 - CD4+ T cell sub-sets

Although distinct T helper cell sub-types exist, it is thought that they exhibit high plasticity, whereby they can convert into other sub-types depending on the local environment (Nakayamada et al., 2012). For example, there is evidence that Th17 cells can convert into Th1 cells and start to produce IFN-γ when presented with the correct environment (Nakayamada et al., 2012). This paints the picture of a very adaptable system, which can change quickly when new threats arrive leading to an effective immune response in a healthy individual. Although each subtype can be identified by their cytokine profile, caution is needed when using certain cytokines due to them being widely released by many different cells (Nakayamada et al., 2012; Zhu et al., 2010). This is why it is often best to look at more than one cytokine to identify each sub-type. They can also be identified by a transcription factors; Th1 express T-bet and Th2 express cMAF (Schreck et al., 2009).

Another point to interest is the fact that Th1 and Th2 cells secrete the cytokines needed to cause their differentiation from naïve T cells forming a positive feedback loop, whereby the cells are amplifying themselves (Zhu et al., 2010).

1.9.3.1.1 - Th1 cells

If the local environment is rich in IL-12 and IFN-γ, naïve T cells are likely to differentiate into Th1 cells. Th1 cells are involved in the response against intracellular pathogens following activation by APCs and induction of antibody production (Luckheeram et al., 2012, Pepper and Jenkins, 2011). They are thought to increase the uptake of infected cells when compared to Th2 cells (Knutson and Disis, 2005). As with all the T cell sub-types they release a wide range of different cytokines, but they can be identified by the secretion of IFN-γ, and IL-2 (Luckheeram et al., 2012, Nakayamada et al., 2012, Zhu et al., 2010). IL-2 is involved in the survival of nTreg and the proliferation of CD8+ T cells and so is thought to aid the cytotoxic immune response (Luckheeram et al., 2012). IFN-γ can reduce the proliferation of cancer cells and promote their apoptosis (Ruffell et al., 2010).
1.9.3.1.2 - Th2 cells

Th2 cells develop in the presence of IL-4 and IL-2 (Zhu et al., 2010). They are involved in the response against extracellular pathogens and are known to down-regulate Th1 cells (Luckheeram et al., 2012; Pepper and Jenkins, 2011). They down-regulate cell-mediated immunity and promote humoral immunity by inducing the production of IgE (Johansson et al., 2008; Sparano et al., 2015). Th2 cells can be identified by their secretion of IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 (Luckheeram et al., 2012; Nakayamada et al., 2012; Zhu et al., 2010). IL-4 along with IL-13 will work to decrease pro-inflammatory cytokines and at the same time, increase immunosuppressive cytokines, so giving Th2 cells a indirect immunosuppressive capability (Ruffell et al., 2010). IL-4 is also involved in promoting the differentiation of naïve T cells into Th2 cells, which creates a positive feedback loop at the same time as reducing the numbers of Th1 cells and the function of CD8+ cytotoxic T cells (Luckheeram et al., 2012; Ruffell et al., 2010).

1.9.3.1.3 - Th17 cells

Naïve T cells differentiate into Th17 cells in the presence of IL-6, IL-21, IL-23 and TGF-β all of which are involved in different steps during the process. Th17 cells are in fact very similar to Treg cells in that they are induced by TGF-β (Luckheeram et al., 2012). Their function is to aid in the immune response against extracellular bacteria and fungi and are identified by their release of IL-17α, IL-17F, IL-21 and IL-22 (Luckheeram et al., 2012; Nakayamada et al., 2012; Zhu et al., 2010). IL-17 is involved in promoting the production of pro-inflammatory cytokines such as IL-6 and TNF-α (Luckheeram et al., 2012).

1.9.3.2 - T effector cells in cancer

Though there is evidence of an anti-tumour response and CD4+ cells are found within the TME, the function of these T effector cells is reduced, allowing the tumour to grow and become invasive. Increased numbers of Treg cells within the TME are able to suppress the function of T effector cells. This is thought to be one explanation for the immune evasion by the tumour (Shen et al., 2010; Chaput et al., 2009; Yang et al., 2006).
The Th1/Th2 balance is also thought to play a role in CD4+ effector cells reduced ability to eradicate cancer cells (Ruffell et al., 2010) with the ratio of Th1 and Th2 cells having more of an effect than absolute numbers (Monte et al., 2011). For example Th1 cells exhibit anti-tumour effects by their ability to activate CD8+ cytotoxic T cells against cancer cells whereas Th2 cells are thought to promote tumour progression (Sparano et al., 2015; Ruffell et al., 2010; Schreck et al., 2008; Johansson et al., 2008). It is thought that the tumour controls the local environment thus influencing the types of immune cells which might be present. More detail is given in chapter 6.

1.9.4 - CD8+ cytotoxic T cells

CD8+ cytotoxic T cells have a wide range of important roles within the body. Their principle function is the detection and destruction of foreign matter, such as bacteria, viruses and cancerous cells without damaging healthy tissue (Coico et al., 2003; Janeway et al., 2001). CD8+ cells are also referred to as cytotoxic T lymphocytes (CTL) due to their method of destroying target cells. Just like CD4+ T cells CTL also have the TCR targeted at a specific antigen, however unlike CD4+ cells which recognise antigens presented by MHC class II, CTL recognises specific antigens presented by MHC class 1 molecules, on the surface of antigen presenting cells such as dendritic cells (APCs; Milstein et al., 2011; Bennett et al., 1998). Binding of the CD8+ cell to the target causes the cytotoxic cell to release effector molecules such as perforin and granzymes, which are stored in granules within its cytoplasm, inducing cell death by apoptosis (Coico et al., 2003; Janeway et al., 2002; Bennett et al., 1998; figure 1.10). CD8+ cells can also kill by interaction of CD178 (Fas ligand) present on their surface, with CD95 (Fas) found on the APC which induces apoptosis, by activating proteolytic enzymes within the target cell (Coico et al., 2003).

Along with the effector molecules, CD8+ cytotoxic T cells also release cytokines such as IFN-γ, TNF-α and TNF-β. These cytokines aid in the general immune response and have multiple roles. For example; IFN-γ will elevate MHC class I expression, so increasing the ability to mark infected cells and activates macrophages in the area (Janeway et al., 2002; See cytokine table in appendix 1 for details of the function of cytokines).
CD8⁺ cells also originate from the thymus emerging inactive and become activated following antigen stimulation and exposure to co-stimulatory molecules such as IL-2 produced by CD4⁺ helper T cells (Hannigan et al., 2000). Dendritic cells can also activate CD8⁺ cells by a method called cross-priming where they present viral antigens and by using B7 on their surface as a co-stimulatory molecule (Coico et al., 2003).

Overall, CTLs are an effective component of the immune system and are essential for maintaining health. This ability is somewhat hampered in certain conditions such as cancer.

Figure 1-10 - Diagram showing the method by which cytotoxic T lymphocytes (CTLs) cause apoptosis in tumour cells. Dendritic cells (DC) present tumour associated antigen (TAA) to CD8⁺ T cells located in lymph nodes where the CTL recognises the antigen and becomes activated. It then migrates to the site of the tumour where CD8⁺ T cells release granzymes and perforin in the surrounding area. These chemicals induce the apoptosis of the tumour cells.
1.9.4.1 – CD8+ cytotoxic T cells in cancer

The role of these CD8+ T cells in the tumour microenvironment has not been completely elucidated, though it is clear that in cases where tumours grow they fail in their job to eradicate cancer.

CTLs have been found to infiltrate the tumour of various cancers upon activation by tumour-antigen presenting cells including HNSCC (e.g. dendritic cells; Pittet, 2009; Ferris et al., 2006; Zippelius et al., 2004). For example, Chaput et al (2009) found the CD8+CD25+Foxp3+ T cell sub-type in colorectal tumour tissue in 96% (31/32) of patients investigated, whereas they were only present in 34% (12/32) of normal colorectal tissue.

However, once within the tumour microenvironment, CTLs lose their full ability to induce apoptosis of the tumour cells (Zippelius et al., 2004). Though a response has been seen, the killing mechanism is slow and in most cases not enough to halt the progression of cancer (Pittet, 2009). In contrast, the CTLs from the periphery of cancer patients are fully functional as demonstrated by Zippelius et al (2004) who conducted a study to investigate CTLs cytotoxic activity obtained from melanoma patients. They cultured CTLs from the periphery and the TME before assessing their ability to release IFN-γ, granzyme B and perforin; all important molecules involved in cytotoxic activity. They found that CTLs obtained from the tumour released relatively lower levels of all three molecules when compared to that of the peripheral CTLs suggesting that the tumour microenvironment is responsible for the loss of function, possibly a combination of both cell to cell interaction and action of soluble factors.

Cells within the tumour microenvironment have been shown to have an effect on CTL. For example, T regulatory cells which accumulate in the TME have been shown to reduce the ability of CTLs to release perforin and granzyme B, molecules which are essential for inducing the apoptosis of cancer cells (Pittet, 2009).

Cytokines such as TGF-β also affect the function of CTLs. TGF-β, which is found in greater levels in the TME and known for its immune-suppressive action, suppresses the expression of perforin, granzyme B and IFN-γ, therefore hindering the CTLs ability to induce apoptosis (Rabinovich et al., 2007).
The effect of the TME on the CTLs however appears to be reversible, therefore it would be beneficial to try and utilise their abilities using immune-therapies. This study aims to further uncover the effects of the tumour microenvironment on the function of CTLs.

1.10 - Soluble Factors within the Head and Neck Tumour Microenvironment

Alongside the various cells within the head and neck tumour microenvironment there are also a myriad of soluble factors being released by these cells. Their presence is thought to contribute to the immunosuppressive microenvironment. A table of some of the cytokines found in the TME can be found in appendix 1 which shows their origin and their function. This thesis is mainly concerned with the immunosuppressive factors IL-10 and TGF-β and will therefore go into more detail about these cytokines.

1.10.1 - Transforming growth factor β (TGFβ)

1.10.1.1 - Normal Function of TGFβ

TGF-β is a cytokine, which has a plethora of different functions within the body. It exists in three isoforms (TGFβ1, TGFβ2 and TGFβ3) and is produced by nearly every cell in the human body having the potential to alter the function of any cell which has the TGF-β receptor (Pasche and Smith, 2001; Kreutzer, 1996). TGF-β is secreted by cells in a latent form, bound to a latency associated protein (LAP), which prevents it from binding to its receptors (Pasche and Smith, 2001), and covalently bonded to several different carrier proteins which are involved in its activation (Beck et al., 2001). The latent form of TGF-β has no known function, however once the LAP has been cleaved by proteases (e.g. plasmin), TGF-β has the ability to affect nearly every cell type (Beck et al., 2001). Once bound to its receptor, TGF-β is involved in the cell growth, differentiation and function of many cells types, including the proliferation of epithelial cells, and is also known to suppress lymphocyte activation thus playing a role in the protection against autoimmune disease (Pickup et al., 2013; Beck et al., 2001). Kulkarni et al (1993) demonstrated that TGF-β is essential for immune suppression by showing that TGF-β null mice died of massive lympho-proliferation. Another known function of TGF-β is its involvement in the proliferation of endothelial cells and so
plays a role in angiogenesis (Beck et al., 2001; Pasche and Smith, 2001) and the formation of extracellular matrix (Matrix et al., 1986).

1.10.1.2 - The role of TGF-β in cancer

The exact role of TGF-β in the tumour microenvironment is hard to define, as there is a vast amount of cross talk between the different components in the locality and therefore there are likely to be numerous ways in which TGF-β exerts its affects (Bierie and Moses, 2006). It is released by many cells within the tumour microenvironment including immune cell, epithelial cells and fibroblasts (Ostman and Augsten, 2009; Rosenthal et al., 2004).

TGF-β has a dual role in the progression of cancer; in early stage cancer it is anti-tumour, then as the cancer progresses it can become pro-tumour (Pickup et al., 2013). In early stage cancer, TGF-β can target receptors on the surface of the cancer cells causing the induction of cell cycle arrest. It has been shown that TGF-β is a strong inhibitor of the growth of normal stroma, immune cells and epithelial cells (Pickup et al., 2013; Pasche and Smith, 2001). However; during the development of cancerous cells they become unresponsive to the mechanisms used by TGF-β, possibly as a result of alterations in their surface receptors and signalling pathways (Pickup et al., 2013; Beck et al., 2001). It has been shown that around 80% of colon cancer have a mutation in at least one component of the TGF-β signalling pathway (Grady et al., 1999) and that this is the case for 100% of pancreatic cancer in humans (Paules et al., 1998), suggesting that this lack of responsiveness to TGF-β is essential for tumour development.

It has been demonstrated that tumours including, breast, colon, lung (Bierie & Moses 2006) and head and neck cancer (Lu et al., 2004) release a greater amount of TGF-β compared to normal tissue, particularly in the later stages of the disease. This increased release contributes to the immunosuppressive microenvironment inhibiting immune cell functions and angiogenesis, therefore allowing the tumour to avoid destruction (Bierie and Moses, 2006; Pasche and Smith, 2001). TGF-β exerts its immunosuppressive actions in a few different ways: it can prevent the production and release of perforin and granzyme A from cytotoxic T cells, preventing these cells from acting against cancerous cells (Bierie and Moses, 2006; Thomas and Massagué, 2005). It also has the ability to
decrease expression of MHC molecules on the surface of tumour cells which will decrease the activation rate of tumour targeting immune cells (Bierie and Moses, 2006).

The over-expression of TGF-β by the tumour also contributes to the overall invasiveness of the tumour and also the metastasis to secondary sites (Pasche and Smith, 2001). It has been found that tumours which have a greater amount of TGF-β in their tumour microenvironment possessed a growth advantage over those that had lower expression (Beck et al., 2001). All this evidence suggests that TGF-β plays an essential role in the process of tumour progression and could potentially be used as a therapeutic target for cancer therapies.

Many studies have investigated the production of TGF-β by head and neck cancer both using cell lines and fresh tumour samples. One group looked at the ability of head and neck cell lines to express TGF-β by using reverse transcription PCR to detect TGF-β-mRNA. They found that 7 or 8 cell lines (MDA Tu cell lines) were producing TGF-β (Wood et al., 1998). In addition, mutations in the TGF-β type II receptor has also been detected in HNSCC cell lines (Garrigue-antar et al., 1995). There are also examples of work on fresh tumour samples; Kreutzer (1996) looked at 47 different HNSCC samples and found that 40 had no TGF-β type I receptor and 43 had no TGF-β type II receptor. Wang et al (1997) also looked at fresh HNSCC samples, and found that 6/28 (21%) samples had a mutation in the TGF-β Type II receptor, but found in a subset of samples that 20/23 (87%) of samples had a decrease in the expression of the TGF-β type II receptor.

The overall picture is that head and neck squamous cells have mutations which alter the TGF-β receptors meaning that TGF-β can no longer work to prevent the over proliferation of these cells. As TGF-β can no longer act, the cells can potentially over proliferate to form a tumour. As previously stated, tumour cells will secrete greater level of TGF-β, and though it can no longer effect the epithelial cells, it will still have an effect on various immune cells within the tumour microenvironment to cause immune-suppression (Kreutzer, 1996).
1.10.2 - Interleukin 10 (IL10)

1.10.2.1 - Normal function of IL10

IL-10 is an anti-inflammatory cytokine involved in the Th2 immune response (Alhamarneh et al., 2008; Couper et al., 2008), which is an antibody mediated immune response against infection and is involved in maintaining the proliferation of epithelial cells. It is released by a plethora of different cells including dendritic cells, B cells and many subsets of CD4+ and CD8+ T cells and can impact the function of many cells types (Couper et al., 2008). Its main function in the normal human body is to regulate immune-suppression and to limit and even halt inflammatory responses caused by pathogens in order to prevent damage to self tissue (Moore et al., 2001; Nemunaitis et al., 2001). This suppression of the pro-inflammatory response may be due to the ability of IL-10 to inhibit the activation of certain cells such as T effector cells, monocytes and macrophages (Moore et al., 2001; Kühn et al., 1993). IL-10 can prevent CD4+ T cells from releasing important pro-inflammatory cytokines such as IL-2, IFN-γ and TNF-α (Couper et al., 2008). In addition IL-10 can also regulate the growth of cytotoxic T cells, mast cells, dendritic cells and endothelial cells (Moore et al., 2001). More recently IL-10 has been found to be released by Tr1 Tregs, contributing to their suppressive ability (Levings et al., 2002). Due to the number of cell types that release IL-10 and its ability to effect different cells, it is thought that IL-10 producing cells will regulate themselves in a normal functioning system (Couper et al., 2008). The function of both IL-10 and TGF-β is thought to be interlinked with IL-10 enhancing the production of TGF-β and vice versa, possibly using a positive feedback loop in order to become more effective (Mocellin et al., 2005).

The importance of IL-10 is clear when the effect of IL-10 deficiency in murine models is investigated: these mice appear to have normal lymphocyte development, but have stunted growth and are likely to develop conditions such as chronic enterocolitis, due to the unsuppressed inflammatory reaction causing damage to surrounding tissues (Kühn et al., 1993).
1.10.2.2. - The role of IL10 in cancer

As mentioned previously, IL-10 is part of the Th2 cytokine profile, a group of cytokines secreted by cells involved in the humoral immune response, which has been shown to be favoured in many types of cancer (section 1.9.3.1.2). IL-10 is able to inhibit the production of the Th1 cytokines (e.g. IL-2 and IFN-γ; Alhamarneh et al., 2008), reducing the effectiveness of the cytotoxic Th1 response on the removal of cancerous cells, favouring tumour evasion from the immune system. IL-10 has also been found to promote the production of TGF-β from Tregs, which will in turn increase the level of the immunosuppressive cytokine TGF-β. In addition, when exposed to IL-10, dendritic cells become less able to present TAA, meaning that fewer tumour cells will be destroyed (Mocellin et al., 2005).

As well as being released by certain immune cells, IL-10 can also be released by malignant cells into the tumour microenvironment where it can also stimulate the proliferation of malignant cells, preventing their apoptosis, so contributing to the growth of the tumour in a more direct way (Mocellin et al., 2005).

As with other kinds of cancer, IL-10 is elevated in the tumour microenvironment of head and neck tumours where there is again a bias towards the Th2 cytokine profile (Alhamarneh et al., 2008; Pries and Wollenberg, 2006). It has been demonstrated that high levels of IL-10 are correlated with a reduced level of CD8+ cells and lower levels of IL-2 and IFN-γ, normally released by CD4+ helper T cells (Young, 2006).

Pries et al (2006) looked at the production of IL-10 in four different head and neck cell lines and found no release suggesting that the immune cells might be the main source. However, in fresh head and neck tumour specimens a significant levels of IL-10 was observed (Young, 2006), which correlated with reduced levels of CD8+ cells and also lower levels of IL-2 and IFN-γ, which are normally released by CD4+ helper T cells.

The presence of high levels of IL-10 in the serum of late stage head and neck cancer patients was correlated to poor prognosis/survival (Alhamarneh et al., 2008). This poor prognosis was found to be true for laryngeal and oropharyngeal patients but not for patients with cancer of the oral cavity (Alhamarneh et al., 2008). A higher systemic
level of IL-10 (measured in the patient’s serum) is also correlated with poor survival, not just IL-10 within the tumour microenvironment (Nemunaitis et al., 2001).

IL-10 is an immunosuppressive cytokine which is present within the tumour microenvironment, so elucidating its exact role it essential in understanding the mechanisms behind tumour immune evasion.

1.11 – Hypoxia and the tumour microenvironment

Reduced oxygen levels are characteristic of most tumour microenvironments, and play a vital role in maintaining the immune-suppressive microenvironment (Kumar and Gabrilovich, 2014; Janssen et al., 2005). Levels of oxygen can vary in different tissue but is anywhere between 5-10%, however, this can drop below 5% in the tumour microenvironment (Palazon et al., 2012).

Tumours develop regions of hypoxia due to the poor formation of vasculature and a high rate of proliferating cells with those cells further away from vessels becoming increasingly hypoxic (Kumar and Gabrilovich, 2014; Janssen et al., 2005). During hypoxia, hypoxia-inducible factors (HIF) are released into the TME which regulate genes relating to metabolism and angiogenesis (Kumar and Gabrilovich, 2014; Palazon et al., 2012; Koukourakis et al., 2002). Cells begin to produce ATP anaerobically, with cells that adapt well surviving over those that perish leading to a more aggressive tumour type (Janssen et al., 2005; Koukourakis et al., 2002). What’s more, this anaerobic environment is detrimental to a patient’s prognosis, due its negative effect of radio and chemotherapies and the tumours increased chance of metastasising (Kumar and Gabrilovich, 2014; Janssen et al., 2005). Any oxygen levels below 5mmHg causes progressively worse patient responses to radio and chemotherapies (Janssen et al., 2005).

HIFs can target numerous immune cells, contributing to the immune-suppressed TME. HIF-1α is known to target T effector cells, cancer-associated macrophages and T regulatory cells (Kumar and Gabrilovich, 2014). It is able to inhibit T effector cell function by reducing their ability to become activated through TCR and CD28 (Kumar and Gabrilovich, 2014; Palazon et al., 2012). HIF-1α is also able to up regulate CD73 and CD39 which increases levels of adenosine which is known to reduce the function of
T cells (Palazon et al., 2012). HIFs are able to initiate the degradation of proteins inducing FOXP3, which leads to a decrease in T regulatory cell development (Kumar and Gabrilovich, 2014; Palazon et al., 2012).

It remains important to assess the effect hypoxia has on the cytokine profile of cancer cell lines and primary fibroblasts and whether their CM causes any differential effect on the function of immune cells compared to CM collected under normoxic conditions. Many experiments are carried out under atmospheric conditions which are not realistic, due to lower oxygen levels within living tissue. So to better understand how hypoxia effects the results, this thesis will do all experiments using CM collected from both normoxic and hypoxic conditions, so differences may be observed.

1.12 – Aims

The aim of the current project is to determine the cytokine profiles of different cells within the HNSCC tumour microenvironment with a view to understanding which cytokines might be involved in creating a suppressive tumour microenvironment. The role of the soluble factors released from each of the cell types within the TME will then be investigated by looking at the effect on proliferation of whole PBMC populations and individual sorted CD4^+CD25^- T effector cells, Tregs and CD8^+ T cells. The effect on the function of each of these immune cell populations will also be assessed using cell type specific assays to identify changes in their functional activity. Healthy human PBMC will be used throughout so that these cells are encountering the pseudo in vivo TME for the first time and should therefore give the greatest effect. However, the results will also be compared to those cells isolated from patient PBMC to determine whether these cells have been pre-primed by the TME. The role that hypoxia plays in creating an enhanced suppressive TME will also be investigated.
Chapter 2 -
Methods
2.1 – Cell culture

Cell culture work was carried out in a class II biological safety cabinet under sterile conditions and all surfaces were swabbed with 70% ethanol before and after use to maintain sterility.

Plastic-ware was purchased sterile from Sarstedt (Leicester; unless otherwise stated) and opened in a sterile environment.

Contaminated plastics and spent medium were soaked in a solution of 2% virkon overnight before being disposed of in the clinical waste to be incinerated.

2.1.1 – Maintenance of cell lines

HNSCC cell lines and primary fibroblasts were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM- PAA, Yeovil, UK) containing final concentrations of; penicillin/streptomycin (0.1U/ml and 0.1mg/ml respectively; PAA), L-Glutamine (0.2mM; PAA) and foetal bovine serum (FBS; 10%; Biosera-Boussens) to create complete DMEM. Fungizone® Amphotericin B (final concentration 2.5μg/ml; Gibco® Life technologies Ltd – Paisley, UK) was added to the complete DMEM for the culture of primary fibroblasts.

2.1.1.1 – Passaging cell lines

In order to maintain sufficient quantity of viable cells, it was necessary to passage them regularly.

On reaching 80-90% confluence the medium was removed from the cells and the monolayer was washed with phosphate buffered saline (PBS). Adherent cells were then detached from the flask surface by adding an appropriate volume of trypsin/ ethylenediamine tetra acetic acid (EDTA; 0.5mg and 0.22mg/ml respectively; PAA), to cover the surface, at 37º C for approximately 5 minutes. Following incubation, the flasks were agitated to ensure all cells were detached. To inhibit the trypsin-EDTA, complete DMEM was added before cells were centrifuged at 400 x g for 5 minutes to pellet the cells. The supernatant was discarded and the cells re-suspended in complete DMEM, the cells were then divided and transferred into sterile 75cm² cell culture flasks for further culture.
2.1.1.2 – Cell storage

Cell lines and primary-derived fibroblasts were frozen and stored in liquid nitrogen when not in use. A confluent flask of cells was trypsinised and centrifuged as described in section 2.1.1.1. Pelleted cells were re-suspended in freeze medium (complete DMEM plus 10% dimethyl sulphoxide – DMSO for cryopreservation; Sigma-Aldrich-Dorset, UK) before being transferred into sterile cryovials. Cells were cooled at a rate of 1°C/min in cryopreservation vessels at -80°C, before being transferred into liquid nitrogen for long term storage.

2.1.1.3 – Cell counting using trypan blue exclusion

Cells were counted using trypan blue exclusion to detect dead cells. Trypan blue enters dead cells through their disrupted membranes but is excluded from live cells allowing viability to be calculated. Equal volumes of cell suspension and trypan blue solution (0.4%; Sigma-Aldrich) were mixed and applied to two chambers of a haemocytometer (figure 2.1). All cells within the 5x5 grid (with a volume of 1 x 10⁻⁴ cm³) were counted and an average of two counts was taken. The concentration of cells/ml was determined using the following equation:

\[
\text{Average cell count} \times 2(\text{dilution factor}) \times 10^4
\]

Figure 2-1 - An example of one of the grids found on a haemocytometer. All cells within the highlighted area were included in the count.
2.1.1.4 – Obtaining cell line and primary fibroblast culture medium

A confluent 75cm$^2$ flask of cells was trypsinised (section 2.1.1.1) and counted using trypan blue exclusion (section 2.1.1.3) before being re-suspended at a concentration of 1 x 10$^6$ cells/ml in complete DMEM. The cell suspension (1ml) was transferred into sterile 6 well plates and a further 1ml of medium added to each well and incubated at 37$^\circ$ C. The medium was harvested at four time points (24, 48, 72 and 96 hours), centrifuged at 400 x g for 5 minutes to remove cell debris and conditioned medium (CM) was aliquoted and stored at -80$^\circ$ C for future use.

2.1.1.4.1 – Culture under hypoxic condition

CM was also obtained from cells cultured as described in section 2.1.1.4, however they were also incubated in hypoxic conditions. Cells were incubated for 24, 48, 72 and 96 hours in a hypoxic incubator at 4% oxygen levels before the CM was collected, aliquoted and stored at -80$^\circ$ C for future use.

2.1.1.6 – Mycoplasma testing

Mycoplasma infection of cell cultures is an infestation of parasites (mollicutes), whose presence is known to change cellular characteristics; therefore it was essential to ensure that the cells were free of infection prior to use.

Mycoplasma tests were carried out every 3 months on all epithelial cell lines using the MycoProbe™ Mycoplasma Detection assay (R&D systems, Abingdon, UK); a colorimetric microplate assay. This assay targets 16S ribosomal RNA from 8 of the most common mollicutes, which account for approximately 95% of infections. A negative result confirmed that cell lines could be used for further assays, however if the result was positive they underwent a course of treatment, however all cell lines used in this thesis tested negative for mycoplasma infection.

2.2 – Processing clinical samples

Processing of blood, tumour and lymphocyte cone samples was carried out in a class II biological safety cabinet.
Blood and HNSCC tissue samples were collected from patients with newly presenting HNSCC following resection surgery. Patients were consented prior to the operation. Information on patients can be found in the specific methods sections for each results chapter.

Lymphocyte cones were purchased and collected from the blood bank and were blood samples from healthy individuals. No further information as available about the donors.

2.2.1 – Obtaining peripheral blood mononuclear cells (PBMC) from blood samples

Venous blood was drawn into heparin (2ml; 5000IU/ml) coated syringes from consenting pre-operative patients with confirmed head and neck cancer.

Blood was diluted at a 1:1 ratio with PBS before being carefully layered onto an equal volume (20ml) of lymphocyte separation medium (PAA) ensuring that the two layers did not mix. The lymphocyte separation medium creates a density gradient separating out the different components of the blood when centrifuged at 400 x g for 30 minutes (Figure 2.2). The cloudy ‘buffy coat’ layer was harvested using a serological pipette and added to an equal volume of PBS before being centrifuged at 400 x g for 10 minutes to wash the cells. Supernatant was discarded and the cell pellets re-suspended in a further 10ml of PBS before being counted with trypan blue exclusion (section 2.1.1.3). Cells were centrifuged again, the resultant pellet re-suspended in freeze medium (FBS containing 10% DMSO) and cryopreserved (section 2.1.1.3).

Figure 2-2 - Lymphocyte Separation Tube – Layers of cells and other blood components seen after centrifugation of diluted blood with lymphocyte separation medium.

The white cloudy ‘buffy coat’ layer contains the PBMC to be harvested.
2.2.1.1 – Isolating PBMC from lymphocyte cones

Lymphocyte cones were used as non-cancer controls, and were obtained anonymously from the NHS Blood and Transport service under ethical approval. A lymphocyte cone is a filter which is used to trap and deplete leukocytes from a single donated whole blood sample prior to transfusion and as such is an ideal source of PBMC.

On receipt, the cone was cleaned using 70% alcohol before being secured in place using a clamp stand. The sealed tubing entering and exiting the cone were cut and the contents of the cone allowed to drain into a sterile tube. The cone was then washed through with PBS to ensure maximum yield.

Lymphocytes from the cone sample were isolated and collected as described in section 2.2.1. Harvested lymphocytes were re-suspended in PBS and re-suspended in freeze medium (FBS containing 10% DMSO) at a concentration of $5 \times 10^7$ cells/ml before being cryopreserved (section 2.1.1.3).

2.2.2 – Processing tumour samples

All centrifugation steps in this protocol were carried out at 400 x g unless otherwise stated.

Tumour samples were obtained from consented patients during resection surgery and transported in complete DMEM (plus fungizone®). Fat, blood and necrotic tissue were removed from the sample before it was weighed. Tissue was then minced using scalpels into approximately 1mm$^3$ pieces and washed in PBS (plus 1% fungizone® and penicillin/streptomycin). Following washing the tissue was centrifuged for 3.5 minutes and the supernatant discarded. The pellet was re-suspended in complete DMEM (plus fungizone®) containing collagenase IV (0.05%; Sigma-Aldrich) and DNase (0.02%; Roche, West Sussex, UK) to disaggregate the tissue into a single cell suspension. The disaggregation mix was placed on a MACs Mix$^\text{TM}$ Tube Rotator (Miltenya Biotec, Surrey, UK) in the 37$^\circ$C incubator for 2 hours to provide constant agitation (figure 2.3).

Disaggregated tissue was washed 4 times using DMEM (plus fungizone®) and centrifuged for 3.5 minutes, the final pellet of cells was re-suspended in complete...
DMEM (plus fungizone®) before being counted using trypan blue exclusion (Section 2.1.1.3). The cell suspension was transferred into a 25cm² culture flask and incubated at 37°C overnight to allow adherence of epithelial cells, fibroblast cells and monocytes.

Post incubation, the non-adherent fraction, containing the tumour infiltrating lymphocytes (TILs) was harvested, placed into a fresh tube and centrifuged for 3.5 minutes to pellet the cells. The supernatant was equally divided into microfuge tubes before being stored at -80 °C for future use and labelled ‘overnight dispersed tissue conditioned medium’ (ON CM). The cell pellet was re-suspended in complete medium (plus fungizone®) and viability was assessed using trypan blue exclusion (Section 2.1.1.3). Cells were washed three times in PBS followed by centrifuging for 3.5 minutes, before being cryopreserved (section 2.1.1.2).

Complete DMEM (plus fungizone®) was added to the 25cm² flask to encourage the growth of fibroblasts.

2.2.2.1 – Depleting lymphocytes in tumour samples

Lymphocytes were depleted in head and neck tissue samples to determine whether they were responsible for the release of certain cytokines.

In preparation a total of 1 x10⁷ Dynabeads® (pan mouse IgG – Invitrogen; Life Technologies Ltd, Paisley, UK) were washed in 1ml of isolation buffer (PBS with 0.1% BSA and 1% EDTA) and recovered by attraction to a magnet before the supernatant was discarded (all washes were executed in this way). Dynabeads® were re-suspended in isolation buffer and incubated with 1µg of anti-human CD45 mouse IgG antibody for 30 minutes at 2-8°C to label the beads. Beads were washed four times to remove excess antibody before final re-suspension in 1ml of isolation buffer.

Tissue was divided into two equal halves by weight before being dissociated and the final pellets were either transferred to a flask for immediate culture (section 2.2.2) or re-suspended in 1ml of isolation buffer ready for lymphocyte depletion. Dynabeads® were added to the suspension and incubated for 30 minutes at 2-8 °C to allow the beads to bind CD45⁺ lymphocytes. Beads and bound lymphocytes were removed by attraction to a magnet and the supernatant transferred to a clean tube. Cells within the supernatant
were washed three times as previously described and re-suspended in complete medium (plus fungizone®). Following overnight incubation the CM was harvested, labelled as lymphocyte depleted overnight night dispersed tumour CM and stored at -80°C.
Figure 2-3 - Flow chart demonstrating the process of disaggregating tumour tissue and depletion of lymphocytes. Tissue was disaggregated into a single cell suspension using enzyme and half is placed in a cell culture flask immediately, the other half was depleted of lymphocytes using mouse anti-human CD45 coated Dynabeads®.
2.3 - Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to determine the concentration of IL-2, IL-4, IL10, TGF-β1 and IFN-γ in CM. DuoSET ELISA development kits (R&D systems, Abingdon, UK) were employed using a sandwich based technique (figure 2.4).

Figure 2-4 - Schematic diagram of the DuoSET ELISA development kit ELISA (R&D systems website- ELISA development kit guide). The diagram highlights the steps involved in this sandwich based method.
The ELISAs were performed according to the manufacturer’s instructions. All wash steps were done using a plate washer (Thermo Fisher Scientific- Well wash 4 – Loughborough, UK) using 400µl of wash buffer (0.05% Tween 20 (Sigma) in PBS) per well followed by aspiration of wells, for a total of four washes.

Briefly, a Maxisorp immuno-plate (Nunc, Thermo Fisher scientific) was coated with capture antibody (table 2.1) and incubated overnight at room temperature to allow adsorption to the plate. The wells were washed before blocking buffer (table 2.1) was added and incubated for 1 hour at room temperature.

Cytokine standards were prepared using serial dilutions at the recommended concentrations (table 2.1; figure 2.5).

**Table 2-1** - Specific concentrations of capture and detection antibodies, block buffer and the high standard used for each ELISA kit.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Capture antibody conc/well (µg/ml)</th>
<th>Detection antibody conc/well (ng/ml)</th>
<th>Block buffer</th>
<th>High standard (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-2</strong></td>
<td>4</td>
<td>200</td>
<td>1% BSA in PBS</td>
<td>1000</td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td>4</td>
<td>12.5</td>
<td>1% BSA in PBS</td>
<td>2000</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td>2</td>
<td>150</td>
<td>1% BSA in PBS</td>
<td>2000</td>
</tr>
<tr>
<td><strong>TGF-β1</strong></td>
<td>2</td>
<td>300</td>
<td>5% Tween 20 in PBS</td>
<td>2000</td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td>4</td>
<td>200</td>
<td>1% BSA in PBS with 0.05% NaN₃</td>
<td>1000</td>
</tr>
</tbody>
</table>
Block buffer was removed and the plate washed before the standards and samples were added to duplicate wells, and incubated at room temperature for 2 hours to allow the analyte to bind to capture antibody. Complete medium was also included as a control to negate any signal caused by its constituents.

Prior to loading the plate with samples, the TGF-β1 ELISA kit required an additional activation step. Samples were incubated for 10 minutes with 1N hydrochloric acid (HCl; 20µl/100µl sample), converting TGF-β1 into its active form and 1.2N sodium hydroxide (NaOH) and 0.5M 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, Sigma; 20µl/100µl sample) was added to neutralise the reaction.

Following blocking the wells were washed and detection antibody added (table 2.1) to all wells and incubated at room temperature for two hours.

One to two hours prior to use, 2% heat inactivated goat serum was added to the detection antibody for the IFNγ ELISA kit to prevent non-specific staining.

Streptavidin-horseradish-peroxidase (HRP; 1:200) was added to all wells following further washes, and incubated for 20 minutes at room temperature in the dark. A final
wash was performed before 3,3',5,5'-tetramethylbenzidine solution (TMB; Vector Laboratories, Peterborough, UK) was added to each well and incubated for 20 minutes at room temperature in the dark. Stop solution (2N sulphuric acid; H₂SO₄) was added to prevent further colour change.

The absorbance of each well was read at 450nm with wavelength correction at 570nm using a plate reader (Thermo Scientific Multiskan FC) and blank subtraction performed. A standard curve was plotted using the 4 parameter logistic curve-fit and concentrations calculated using the plate reader software.

Due to the additional activation steps for the TGF-β1 ELISA, all concentrations were multiplied by 1.4.
2.4 - Quantibody® Human Th17 array

The Quantibody® human Th17 array (RayBiotech- Wembley, UK) was used to quantify 20 different cytokines (figure 2.6) within CM utilising multiplexed sandwich ELISA-based technology.

![Diagrammatic representation of the Quantibody® human Th17 array](image)

**Figure 2-6** - Diagrammatic representation of the Quantibody® human Th17 array - Capture antibody is spotted in quadruplicate onto glass slides, which binds the cytokine of interest in the test sample. A biotinylated detection antibody is detected with a streptavidin labelled Cy3 equivalent dye. The intensity of fluorescence is directly proportional to cytokine concentration.

The Quantibody® human Th17 array was performed according to the manufacturer’s instructions and all reagents were supplied and reconstituted as stated. Incubation steps were carried out under gentle orbital rotation unless otherwise stated.

Briefly, glass slides were equilibrated to room temperature before being removed from their protective wrapping and air dried at room temperature for 1-2 hours. The lyophilised cytokine standard mix was reconstituted with sample diluent to make a stock solution of each cytokine (Table 2.2). Seven standards were prepared using serial dilution of the stock solution in sample diluents and sample diluent alone was included as a negative control.
The Quantibody® arrays were blocked with sample diluent at room temperature for 30 minutes. Wells were decanted before seven standards, sample diluent and test samples (CM) were added to appropriate wells and incubated overnight at 4 °C.

Wells were washed a total of seven times, with the first five washes involving the addition of 1x wash buffer I with gentle rotation for 5 minutes. This was then repeated twice more using 1x wash buffer II. All subsequent washes were performed in this way unless otherwise stated.

Detection antibody was reconstituted with sample diluent and added to each well before incubation for 2 hours at room temperature. Following washes, reconstituted Cy3
equivalent dye-conjugated streptavidin was added to each well and incubated in the dark for 1 hour. Wells were decanted and washed five times using 1x wash buffer I.

The chamber block was dismantled from the slides before they were washed in 50ml centrifuge tubes in 1 x wash buffer I protected from the light. The tubes were gently rotated at room temperature for 15 minutes. A further wash for 5 minutes in wash buffer II preceded centrifugation at 1000 rpm for 3 minutes without buffer to dry the slides. Any further residue was removed by gently drawing droplets away with absorbent material, being careful not to disturb the test area.

2.4.1 – Quantibody® data analysis

The Axon Instruments GenePix personal 4100A array scanner was switched on 15 minutes prior to scanning to allow lasers to warm up.

Gene Pix Pro 4.1 software was used to analyse the results. All work was carried out in the dark to preserve fluorescence on the slides.

Array slides were placed in the scanner and preview scans were performed at 532nm (Cy3) with different photomultiplier settings to identify the optimum level to minimise background staining and maximise brightness of sample spots. A full resolution scan was performed and hardware setting and images were saved.

For each set of well data, blocks were generated and aligned over the features as shown in figure 2.7. Each spot was analysed generating a table of results with background mean, background standard deviation, fluorescence mean and fluorescence standard deviation. Background data was subtracted from all test wells.

Data was exported into specifically designed Q analyser software (Excel based) and standard curves were drawn in linear and logarithmic form for each cytokine and the most appropriate on was selected. The concentration of each cytokine in the unknown samples was determined from the standard curve.
2.5 - Phenotypic marker analysis by flow cytometry

The FACS Aria™ II (BD Biosciences, Oxford, UK) was used for detecting the expression of markers on cells for both phenotypic analysis and cell sorting and was set up following the procedure supplied by the manufacturer. Briefly, the FACS flow and ethanol tanks were filled and waste tank emptied. The analyser was switched on before being put through fluidics start-up and an additional aseptic clean protocol and swabbing all surfaces with 70% ethanol when cell sorting to ensure cells were uninfected for culture afterwards.

The stream was started and observed to ensure it was stable and the drops were breaking off the stream at the correct point (figure 2.8). Cytometer Setup & Tracking Beads

Figure 2-7 - Block generation on scanned slide using Gene Pix Pro 4.1 software

– A full resolution scan of the slide (580µM PMT) was taken and blocks generated. Blocks were aligned with the features (fluorescent spots) and any fluorescence contained within them was analysed.
(CS&T) were run through the analyser in order for the lasers to be correctly aligned. The beads are automatically tracked and characterised in order to obtain reproducible results from one experiment to another. The FACS Aria™ II is then ready for flow cytometry or for further set up when continuing on to cell sorting.

![Figure 2-8](image)

Figure 2-8 - A screen shot of the stream on the FACS Aria™ II. Frequency and amplitude of the drops can be adjusted in this window. The drops must form individual droplet below the grey cut of line.

### 2.5.1 – General flow cytometry

Flow cytometry can be used to determine the presence of surface and internal markers on cells, which is useful in the identification of cell types and to look at changes in expression after treatments.

To stain for an external marker, the cells of interest were harvested (section 2.1.1.1) and counted using trypan blue exclusion (section 2.1.1.3) before the concentration was adjusted to $1 \times 10^6$ cells/ml and separated out into the desired number of tubes. The cell suspension was incubated with the appropriate amount of fluorochrome conjugated antibody against the desired marker (e.g. FITC-CD8) for 20 minutes in the dark. The appropriate concentration of Ab was determined by titration, whereby a selection of
concentrations was used and the results assessed to determine optimum amounts. All Abs used were optimised in this way. The suspension was centrifuged at 400 x g for 5 minutes to pellet the cells. Supernatant was discarded and the cells were washed three times using PBS with centrifugation steps in-between each wash at 400 x g for 5 minutes. The final cell pellet was prepared for analysis by re-suspending in PBS/BSA/azide and placing in the dark until needed.

An isotype control was also added to a separate aliquot of cell suspension. This was to exclude any positive results obtained from non-specific binding of the antibody to different proteins. The appropriate amount of isotype control was added to the cell suspension and treated in exactly the same manner as above.

There was also a tube that contained cells only, which had also been washed as above but contained no antibody or isotype control. This was to set the parameters on the FACS machine.

The FACS Aria was set up as described in section 2.5 and fluorochromes parameter selected depending on which were present in the samples (e.g. FITC). The cells only sample was initially run to allow the forward and side scatter (FSC and SSC) to be set to allow visualisation of the cell population of interest (figure 2.9). When possible, it was preferred to run at least 10000 cells (events) in order to gain a better idea of the spread of readings. The population of interest was then gated as shown in figure 2.9 (P1), which allowed all further graphs to be plotted from this population, thereby excluding any unwanted cells.

The second tube to be run was the iso-type control. Once 10000 events had been recorded the resultant peak was shifted on the graph to below the $10^2$ point on the FITC axis by adjusting the FITC parameter until the peak fell into the desired location. A marker was then drawn on that would incorporate any positive results that fell to the right of that peak (P2; figure 2.9).

The cell samples containing the antibody were then run. Any events that fell to the right on the isotype control peak were deemed positive and included in the result. Figure 2.9 shows an example where PBMC had been stained with FITC-CD8. The final tube
shows that 22.2% of the selected cells in the P1 gate were positive for CD8 and that there was a mean FITC-fluorescence of 1203 which is useful to determine density of the marker on the cell surface.

To label internal markers there are a few more steps to include. Buffer A (100μl) was added to the cell suspension and incubated for 15 minutes before being washed with PBS/BSA/Azide and centrifuged at 400g for 5 minutes to pellet the cells. Supernatant was discarded before 100μl of buffer B was added and incubated with the cells for 30 minutes at room temperature. The cells were washed and centrifuged as before and supernatant discarded. Cells were then stained and incubated as described above with the appropriate amount of antibody. All subsequent steps were the same as for external markers.

2.5.2 – For fluorescent cell sorting

Once the cells have been labelled, and interrogated by the flow cytometer laser, a charge is applied to them and deflection plates used to direct the charged cells into appropriate tubes, with unlabelled cells being deflected either into the waste or into another tube (figure 2.10).
Figure 2-9 - Screenshots of data obtained from the FACS Aria. A) Cells only results showing the gating (p1) of the cell population of interest, with SSC and FSC adjusted to allow correct visualisation of the cells. b) Results from the isotype control which shows the resultant peak (drawn from P1) positioned below 10^2 and the marker (p2) set to the right of the peak. The data shows the % of events to the right of the P2 marker and mean fluorescence. c) Results from the test sample (drawn from P1) containing the FITC-CD8 antibody showing a strong positivity for the CD8 marker. The data shows that 22.1% of P1 show positivity for CD8 with a mean fluorescence of 1105.
Figure 2-10 - Schematic of cell sorting process by the FACS Aria. The sample enters a chamber where a laser is used to excite the fluorochromes present on some of the cells. The data is transmitted to the deflection plates through the photomultiplier causing selected cells to be charged. This allows the analyser to direct charged cells into different collection tubes. If the cells have no charge they will enter the waste unless otherwise instructed.
In addition to the start-up procedure described in section 2.5 an aseptic clean of the flow cytometer was performed for cell sorting as well as a thorough swab of all surfaces with 70% ethanol. The sort collection device was also fitted to the analyser.

To ensure all the streams were entering the correct collection tubes, the voltage was turned on and a test sort performed, so the streams could be visualised and adjusted if necessary (figure 2.11).

![Figure 2-11](image)

**Figure 2-11** - Visual of the four streams and the central waste stream upon initialising a test sort. The position of the stream was adjusted using the sliders if the stream wasn’t entering the tube (FACS Aria II handbook: BD Biosciences).

Whilst visualising the streams, the amplitude was increased and decreased until the streams became distorted and values recorded, this was the amplitude range and a mid-value was calculated and entered into the analyser. The amplitude determines the drop drive of the stream, which is where the drop breaks off and becomes charged. The Amplitude range will only be calculated once at the start and will be maintained using the sweet spot function.

Accudrop beads (BD Biosciences) are fluorescently labelled particles which allow illumination of the streams and allow the machine to calculate the drop delay which is the point at which the cells are charged. Calculation of the optimal drop delay ensures maximum deflection of positive cells into collection tubes with minimal waste and was calculated prior to every sort.
2.6 – Sorting T regulatory cells, T effector cells and CD8+ T cells from PBMC

All reagent used were from BD biosciences unless otherwise stated. The FACS Aria™ II was set up for cell sorting as described in section 2.5.

2.6.1 – Preparing PBMC for sorting

PBMC from healthy controls (lymphocyte cones; section 2.2.1.1) and HNSCC patients were washed and re-suspended in filtered wash buffer (fWB), 5% human Ab serum (Invitrogen; Life technologies Ltd) in PBS, before being counted using trypan blue exclusion (Section 2.1.1.3). Cells were re-suspended at a concentration of $2 \times 10^7$ cells/ml before being fluorescently labelled using a human regulatory T cell sorting cocktail (anti-CD127-APC, anti-CD4-PerCpCy5.5 and anti-CD25-PE) and an anti-CD8-FITC Ab. 40µl of Ab was added per $2 \times 10^7$ cells. Cells were incubated for 30 minutes at room temperature in the dark, washed with fWB and re-suspended at a concentration of $7.5 \times 10^6$ cells/ml before being passed through a 35µm nylon mesh filter (BD Biosciences) into a sterile polystyrene tube to remove any clumps of cells. Cells were then ready to be sorted using the FACS Aria™ II.

Treg medium was prepared as follows; 50ml of X-VIVO 20 (Lonza, Slough, UK), 5% heat inactivated AB serum (invitrogen) along with penicillin/streptomycin (concentration 0.1U/ml and 0.1mg/ml respectively; PAA) and recombinant human IL-2 (100U/ml, AbD Serotec). This was then passed through a 0.2µm filter to remove and contaminants.

Four polypropylene tubes (Sarstedt) to capture sorted cells were coated in Treg medium to prevent adherence of the cells on to the side of the tube. These tubes were placed into the sort collection device and fitted to the FACS Aria™ prior to sorting.

2.6.2 – Setting up compensation on the FACS AriaTM II

When using a cocktail of Abs conjugated to a range of different fluorochromes it is essential to compensate for any overlap in their emission spectrum.
2.6.2.1 – Setting compensation

BD compensation beads were coated with the antibodies used for staining of cells (anti-CD127-APC, anti-CD4-PerCP-Cy5.5, anti-CD25-PE and anti-CD8-FITC) and acquired through the FACS Aria™ II. The automatic compensation program was used to adjust parameters to compensate for any overlap in emission spectrum.

Tubes were set up containing 100µl of PBS/BSA/Azide, one for the negative control and one for each of the different antibodies used in the cocktail. One drop of negative control beads was added to each tube and 1 drop of anti-mouse Igk was added to every tube except for the negative control. The relevant antibodies were then added to each tube (20µl of anti-CD127-APC, anti-CD4-PerCP-Cy5.5, anti-CD25-PE and 1.25µl of anti-CD8-FITC) before being incubated at room temperature in the dark for 20 minutes. Beads were washed with PBS/BSA/Azide before being centrifuged at 300g for 10 minutes. Supernatant was discarded and beads re-suspended with PBS/BSA/Azide before being run through the analyser as instructed by the automatic compensation calculation software. Once all the compensation controls had been assayed, the analyser calculated the compensation values and automatically applied them to any further work.

2.6.3 – Gating the populations to be sorted

PBMC labelled with the Treg sorting cocktail (section 2.6.1) were initially run through the FACS Aria™ in order to gate the required populations for sorting. The first plot of forward scatter vs. side scatter was used to isolate the lymphocyte population from other cells present in the PBMC, such as monocytes (figure 2.12). All subsequent plots were drawn from the P1 gated lymphocyte population. Figure 2.13 a, b, c, d, e & f show the gates used to isolate CD8+ T cells, T effector cells and T regulatory cells. Figure 2.14 shows population hierarchy.

Figure 2-12 - Dot plot showing the events generated from whole PBMC and the P1 gate isolating the lymphocyte population.
Figure 2-13 - Dot plots showing gates used to isolate different cell populations within a whole PBMC sample ready for sorting. Dot plots used to isolate 4 populations of cells (P4 – CD8⁺ T cells, P8 – CD4⁺CD25⁺CD127⁺ Tregs, P9 - CD4⁺CD25⁺CD127⁻ Tregs and P10 – CD4⁺CD25⁻ effector T cells). Doublet cells were excluded in the first instance using gates as shown in a) and b). Following doublet exclusion both FITC labelled CD8⁺ T cells and PerCpCy5.5 labelled CD4⁺ cells were gated using plots generated from cells within gate 3 c) and d) respectively. e) is plotted using from the P3 gate and used a quadrant to divide cells that are dead and those that are positive for PE-CD25 and PerCpCy5.5-CD4. Gates P6 are used to define PE-CD25 positive events and mid and high expression of APC. e) is plotted from P5 and is used to define the final populations for sorting. Using E) as a markers for the mid and high APC population, the gates are added.
Once cell populations were gated appropriately the FACS Aria™ identified the cells in the PBMC samples using the fluorochromes on the cells surface and directed them into a collection tubes prepared as in section 2.6.1 (figure 2.10). After the whole PBMC sample was sorted each population was re-analysed to ensure the correct population was isolated and to find out the percent purity (figure 2.15). The CD8⁺ population should appear in the P4 gate, CD4⁺CD25⁺CD127lo Tregs should appear in gate 8, CD4⁺CD25intCD127lo Tregs should appear in gate 9 and CD4⁺CD25⁻ effector T cells should appear in gate 10 (figure 2.15).

Sorted cell populations were used immediately to set up experiments as highlighted in subsequent sections.
2.6.4 – Determining the percentage of cells within a population

It is possible to determine the percentage of cells within a population. Once the population of interest was gated (section 2.6.3) and all histograms drawn, it is possible to use the data supplied to determine the percentage of certain cells within the parent population or a percent of the total sample. For example, looking at figure 2.13 and the population hierarchy in figure 2.14 you can see that the population labelled P8 consists of 6.7% of the parent population P5, and 1.6% of the total sample.

Figure 2.15 - Dot plots generated from sorted PBMC populations to confirm presence of isolated populations - Around 500 events (cells) were recorded when the sorted populations were run back through the FACS Aria™ II to confirm the correct cells had been isolated. A) CD8⁺ population, B) CD4⁺CD25⁺CD127⁻ Tregs, C) CD4⁺CD25⁺CD127⁻ Tregs and D) CD4⁺CD25⁻ effector T cells. The majority of the population was within the gate boundaries.
2.7 –Cell Titer 96® AQueous One Solution cell proliferation assay (MTS assay)

A colorimetric assay was used to determine change in metabolic activity of cells following addition of CM as collected in section 2.1.1.4. Metabolically active cells reduce the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Promega, Southampton, UK) reagent to a soluble formazan product whose absorbance can be measured at 492nm and is directly proportional to the number of active/living cells present (figure 2.16).

![Chemical structure of Cell Titer 96® AQueous and its reduced blue coloured metabolised form; formazan](https://www.promega.co.uk - MTS protocol).

2.7.1 – MTS on whole PBMC and sorted immune cell populations

Normal and patient PBMC samples (section 2.1.1.1) or sorted immune cell populations (section 2.6) were washed and re-suspended in filtered wash buffer (fWB), before cells were counted using trypan blue exclusion (Section 2.1.1.3). Cell concentration was adjusted to 5x10^4 cells per 66μl of Treg medium and each 66μl was added to separate wells of 96 well round bottom culture plate (Sarstedt). Enough wells were plated in order for each test and control to be performed in duplicate. For whole PBMC samples, two wells contained medium alone as a control and two wells contained cells stimulated with Dynabeads® Human T-activator CD3/CD28 (Gibco, Life Technologies Ltd) using a ratio of 3 beads/ cell and IL-2 (100U/ml; Serotec). All subsequent wells had no stimulation. Cells plus medium wells were added followed by wells with the test CM. Figure 2.17 shows a typical 96 well plate layout. For sorted populations, all wells containing cells had the addition of stimulation.
<table>
<thead>
<tr>
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<th>Medium only (blank)</th>
<th>Cells +CM</th>
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</tr>
</tbody>
</table>

**Figure 2-17** - Example of a typical 96 well plate layout. All conditions were assayed in duplicate. The first two wells were medium only, followed by two wells containing cells with Human T-activator CD3/CD28 and IL-2 stimulation. PBMC was with medium only was added to two wells followed by the test conditions which involved the addition of CM.
Cells were incubated at 37˚C and 5% CO₂ in a humidified incubator for 24 hours before the addition of 33µl lymphocyte medium (1:3 ratio with cell suspension) to control wells and 33µl of cell lines, primary-derived fibroblast (normoxic & hypoxic) or overnight dispersed tumour CM to test wells. Cells were incubated for a further 24, 48 or 72 hours at 37 ºC. At the end of each time point, 20µl of CellTiter96 Aqueous solution was added to each well before being incubated for a further 2 hours at 37 ºC. The contents of each well was transferred into a flat bottomed 96 well plate and the absorbance measured at 492nm on a Thermo Scientific Multiskan FC plate reader (Thermo Fisher).

The results obtained from PBMC culture with medium alone were subtracted from test data to ensure it was the soluble factors present in the CM rather than the constituents of the medium having an effect.

2.8 – Carboxyfluorescein succinimidyl ester (CFSE) assay

The function of Treg includes the ability to suppress the proliferation of T effector cells, therefore in order to determine the change in function of Treg upon the addition of HNSCC-derived CM, they were co-cultured with T effector cells labelled with carboxyfluorescein succinimidyl ester (CFSE) and the proliferation of these cells was monitored using flow cytometry.

2.8.1 – Setting up the CFSE assay

CFSE is a fluorescent dye which enters cells through their membrane before binding to intracellular molecules such as lysine residues and is retained within the cell during proliferation. Its signal can be tracked using flow cytometry to determine whether cells have undergone proliferation. Each generation of cells will have approximately half the fluorescence as the previous generation (figure 2.18).
T effector cells (CD4+CD25−) and Treg (CD4+CD25intCD127lo and CD4+CD25hiCD127lo) were isolated from healthy PBMC (lymphocyte cone; section 2.6). Cells from both populations were counted using trypan blue exclusion (section 2.1.1.3) and cell concentration determined.

An aliquot of 5 x 10⁴ T effector cells was placed into a separate tube to act as the non-CFSE labelled control and the remaining cells were labelled with CFSE. All cells were washed three times using pre-warmed PBS/0.1% BSA before being centrifuged at 400 x g for 4 minutes. The pellet was re-suspended in PBS/0.1% BSA at a concentration of 1 x 10⁶ cells/ml and CFSE (Sigma; final concentration 5μM) was added to the labelled-CFSE tube only. Effector cells were incubated in the dark at 37 °C for 10 minutes before the reaction was quenched with 2ml of ice cold Treg medium. Tubes were incubated on ice for a further 5 minutes before being centrifuged at 400 x g for 4 minutes. The labelled cells were washed three times with warm Treg medium before being centrifuged at 400 x g for 4 minutes and re-suspended at a concentration of 5 x 10⁴ cells/66μl of Treg medium.
Tregs isolated above were centrifuged at 400 x g for 4 minutes and the pellet re-suspended at a concentration of 5 x 10^4 cells/66μl of Treg medium.

T effector cells and T regulatory cells were co-cultured at a ratio of 1:1 in 132μl of Treg medium in a round bottom 96 well plate and incubated at 37 ºC and 5% CO₂ in a humidified incubator. Dynabeads® Human T-activator CD3/CD28 (Gibco, Life Technologies Ltd) using a ratio of 3 beads/cell and IL-2 (Serotec; 100U/ml) were added to wells which required stimulation (See bullet points below). After 24 hours, 66μl cell line, primary fibroblast or overnight dispersed tumour CM (normoxic and hypoxic; to create a 3:1 dilution) was added to appropriate wells and the cells incubated for a further 48 hours.

Wells required per healthy PBMC sample for CFSE analysis are summarised below;

1. **Well/tube 1** – Non-CFSE labelled T effector cells WITH stimulation (This is to set the non-fluorescing peak)
2. **Well/tube 2** – CFSE labelled T effectors with NO stimulation (To set the parent peak that will show a non-dividing population).
3. **Well/tube 3** – CSFE labelled T effectors WITH stimulation (a dividing population without the influence of the Tregs).
4. **Well/tube 4** – CFSE labelled T effectors WITH stimulation + Tregs (a dividing population showing the effect of Tregs alone on the proliferation of the effectors).
5. **Wells/tube 5-30** – CFSE labelled T effectorss WITH stimulation + Tregs + various CMs (a dividing population showing the effect of both Tregs and CM on the proliferation of T effector cells).
6. **Wells/tube 31-60** – CFSE labelled T effectors WITH stimulation + various CMs (a dividing population showing the effect of just CM on proliferation).

**2.8.2 –FACS analysis**

Following incubation, the contents of each well was transferred into a FACS tube and centrifuged at 400 x g for 5 minutes. The cell pellet was re-suspended in PBS/BSA/AZIDE ready for analysis on the FACS Aria™ II.
The FACS Aria II was set up for flow cytometry (section 2.6). A dot plot of forward vs side scatter and a histogram with FITC along the X-axis vs cell count (previous figure 2.13) were used to identify the CFSE labelled T effector population. The T effector cells alone without CFSE label but with stimulation (tube 1) was acquired and a gate (p1) was set around the proliferating T effector population (figure 2.19), before 10,000 events were recorded. The second tube containing T effector cells alone without stimulation but with CFSE label (tube 2) was then acquired and a second gate (2) was used to isolate the non dividing T cell population which represented the parent peak, and 10,000 events were recorded. A third tube containing CFSE labelled T effector cells alone with stimulation was acquired to demonstrate 100% proliferation (tube 3). All subsequent tubes contained a 1:1 ratio of Treg and T effectors treated with and without CM, 10,000 events was recorded from each P1 gate and results were exported from the Aria ready for analysis using Modfit software (section 2.8.3).

![Figure 2-19](image.png)

**Figure 2-19** - Dot plot showing the two gates required for recording the CFSE staining of T effector cells. The red gate is P1 and represents proliferating T effector cells. The blue gate represents P2 which isolates non-proliferating T effector cells.
2.8.3 – MODFIT analysis of Treg suppression data

MODFIT is modelling software used for analysing flow cytometry data (Verity House, Topsham, USA). In the current thesis it was used to track the CFSE label on T effector cells as they underwent proliferation. Using labelled T effector cells without stimulation as a 0% proliferation control and T effector cells with stimulation as a 100% proliferation control it is possible to work out the suppressive capacity of Treg when co-incubated with T effector cells. The effect of CM from HNSCC cell lines, primary-derived fibroblasts and overnight dispersed tumour CM on the suppression exerted by the Tregs has also been investigated.

Using the results from the non-labelled T effector cells the data for FITC was converted into a 4 decade log scale and FITC was selected. A FCS (x axis) vs SSC (y axis) dot plot was generated and the lymphocyte population selected (figure 2.20). A second dot plot was drawn using FITC (x axis) vs SSC (Y axis) and a gate positioned to the right of the negative population to exclude unlabelled T regulatory cells from the analysis (figure 2.20).

Figure 2-20 - MODFIT generated dot plots from (Non-CFSE labelled T effector cells WITH stimulation, to set the non-fluorescing peak. A) Forward scatter vs side scatter showing the lymphocyte gate (R1) and B) FITC vs side scatter showing the gate position (R2) to encompass CFSE labelled cells. In this instance all cells were negative for CFSE.
Using the labelled T effector cells alone which had not been stimulated and therefore do not undergo proliferation (the parent population) another plot was generated. FITC was converted to a log scale as previously described and the two gates were defined as before. In this case the lymphocyte population was smaller and lower on the dot plot, but the same gate was used, ensuring the cell population was within the gate boundaries (R1). The software could then generate the parent peak automatically (figure 2.21).

![MODFIT plot showing the non-proliferating T effector cell population](image)

**Figure 2-21** - MODFIT plot showing the non-proliferating T effector cell population (tube 2; parent population). The representative data reveals that 98.78% of the T effector cells were in generation 1 and had not proliferated.

Using the proliferation wizard, the program automatically positioned a marker over the parent peak. As this represents non proliferating T effector cells, any cells that have proliferated should appear to the left of this peak. Tube 3 contained stimulated T effector cells alone which represent the cells which should be undergoing 100% proliferation and 0% suppression. Each generation of proliferating T effectors in a perfect scenario should be represented by a peak with approximately half the amount of fluorescence (figure 2.22). The software automatically calculates how far apart each generation should be using the following calculation;
Spacing = (histogram resolution – 1) * log10 (2.0) /log decades

It assumes that the fluorescent dye on the surface of each cell in successive generations is half that of the previous one. The remaining data for the tubes containing a 1:1 ratio of Treg:Teffectors following various treatments with CM were subsequently run through the Modfit proliferation wizard software.
Figure 2.22 - (a) Histogram plot showing generations of proliferating T effector cells. The far right peak represents the parent peak (figure 2.21) which consists of 18.75% of the total population. The peak to the left are successive generations. The proliferation index is the value of interest highlighted in (a). (b) Dot plot generated alongside the histograms where the colour represents the density of cells within the population. It shows the gated T cell population. (c) Dot plot showing the gated CFSE positive population and the negative T regulatory cell population to the left.
As stated previously, labelled T effectors with stimulation was classed at 100% proliferation and 0% suppression so the PI value for these cells represented the maximum level of proliferation. The level of suppression could then be calculated upon the addition of Treg alone or Treg treated with head and neck cancer derived CM using the PI value. For example;

PI value of labelled, stimulated T effectors was 3.05 = 100% proliferation and 0% suppression

PI value for labelled, stimulated T effector cells + Treg (without CM treatment) was 1.97

Calculation = PI for T effectors & Treg x 100 / PI for T effectors only.

Due to the addition of multiple components to the original experiment, it could have been the CM that directly caused the suppression of T effectors, bypassing the Tregs. So experiments were carried out with CFSE-labelled T effectors and CM alone without the presence of Tregs. The data obtained could then be used to adjust the previous data to ensure the results reflect the function of the Tregs alone.

2.9 – Ethical approval

All patient samples were obtained following informed written consent from patients recruited from the ear, nose and throat department at Castle Hill hospital in Hull.

Ethical approval was granted by the Yorkshire and the Humber research ethics committee REC reference 10/H1304/7. The use of samples was also approved by the NHS trust R & D, see reference R0988.
Chapter 3 -
The release of cytokines within the tumour microenvironment; in relation to HNSCC tumour sub-site and hypoxia
3.1 – Introduction

Patients with HNSCC are known to have a reduced cell-mediated immune response at the primary tumour site as well as within the metastatic nodes and are known to actively avoid the immune system (Wood et al., 1998). In order for HNSCC to develop from a genetically altered cell into a tumour mass, it must first evade detection by the immune system. There are various methods employed by cancer cells, to evade detection or to alter the immune response (Igney and Krammer, 2002; Chen et al., 1999), including cell to cell interaction, whereby tumour cells play a direct role in reducing their delectability by changing surface antigens (Igney and Krammer, 2002). In addition, tumours are thought to secrete a cocktail of cytokines which have the ability to alter the function of infiltrating immune cells (section 1.10). Cytokines are multi-functional peptides which act as a means of communication between cells (Wood et al., 1998), which can be immunosuppressive, leading to dysfunction of the cell mediated response, allowing the tumour to develop further.

Proinflammatory, immunoregulatory and proangiogenic cytokines have been found to be released by primary HNSCC tissue and UM-SCC cell lines in vitro (Chen et al., 1999), suggesting the same may occur in vivo. Chen et al (1999) used ELISA to determine the concentration of cytokines in CM obtained from UM-SCC HNSCC cell lines and freshly isolated primary HNSCC cultures and confirmed their finding using immunohistochemistry. The cytokines detected from HNSCC cell lines from multiple studies include IL-1α, IL-4, IL-6, IL-8, IL-10, GM-CSF, VEGF, PGE₂, bFGF and TGF-β1 (Pries and Wollenberg, 2006; Thomas et al., 2005; Lu et al., 2004; Chen et al., 1999).

Cytokines within the TME have a vast array of different roles, however, some have been categorised as being Th1-like (e.g. IL-2, IL-12), stimulating the cell mediated immune response or Th2-like (e.g. IL-4, IL-10) which will down regulate the Th1 response in favour of the more humoral antibody mediated response, leading to an immunosuppressive environment (Jebreel et al., 2007; Pries and Wollenberg, 2006; Section 1.9.3.1). For example, IL-10 can prevent the anti-tumour action of IL-12 which is involved in stimulating and activating NK cells and cytotoxic T cells and differentiating CD4⁺ lymphocytes (Jebreel et al., 2007; Smyth et al., 2000). IL-12 is
also involved in the Th1/Th2 balance (Smyth et al., 2000) and has been found to induce a shift towards a Th1-like profile creating a more anti-tumour environment (Luckheeram et al., 2012). The Th2 response can also feed itself, with the Th2 cytokine IL-6 stimulating the production of another Th2 cytokine, IL-10, which itself has the capability to reduce the production of the anti-tumour Th1 cytokines from immune cells within the TME (Lathers and Young, 2004).

Greater levels of Th2-like cytokines (IL-4, IL-6, IL-10 and GM-CSF) have been detected in HNSCC patient plasma and serum than Th1-like cytokines (IFN-γ, IL-2, IL-12 and TNF-α; Johnson et al., 2014; Jebreel et al., 2007; Pries and Wollenberg, 2006; Lathers and young, 2004; Sparano et al., 2004; Chen et al., 1999). In some cases some Th1 cytokines were found to be increased, such as IL-2 and GM-CSF, suggesting that not all Th1 cytokines are altered in all types of HNSCC (Lathers and young, 2004). Jebreel et al (2007) used ELISA to measure cytokines in the serum of HNSCC patients (n=57) vs controls (n=40) and found a reduction in the level of IL-12 in patients compared to controls and that IL-10 was more likely to be detectable in the HNSCC serum. Also Th2 cytokines were found to increase with tumour stage, an observation also found by Sparano et al (2004). In addition Sparano et al (2004) also found that the production of Th2-like cytokines was subsite related, with higher levels of IL-10 found in laryngeal carcinomas compared to other HNSCC sub-sites.

Fresh tumour specimens have also been found to release several Th2 cytokines including IL-4, IL-6 and GM-CSF (Pries and Wollenberg, 2006; Lu et al., 2004).

It is likely that the release of some of these immunosuppressive cytokines from the tumour will also influence immune cells to release further tumour promoting cytokines (e.g. IL-10), therefore exacerbating tumour growth and development (Pries and Wollenberg, 2006).

TGF-β is another immunosuppressive cytokine over-expressed in many cancers including HNSCC (Lu et al., 2004). The role of TGF-β1 however, appears to depend on the stage of development of the HNSCC; evidence suggests that during the early stages of tumour development TGF-β acts to hinder epithelial cell proliferation by attracting lymphocytes, but in the later stages it is thought to promote tumour invasion via its
paracrine effects on the tumour stroma (Lu et al., 2004). Lu et al (2004) found that in 43.8% of HNSCC patients (n=32), the adjacent tissue to the tumour over expressed TGF-β1 and that 78.1% of tumour tissue over expressed TGF-β1 when compared to controls. In contrast other studies have found that there was no difference in the plasma levels of TGF-β1 between HNSCC and controls (Lathers and Young, 2004) which may suggest that the increase in TGF-β seen in the tissue remains localised to the tumour microenvironment.

The current chapter focuses on the detection of immunosuppressive factors, Th1, Th2 and Th17-like cytokines released by various cell types found within the HNSCC TME, including tumour epithelial cell lines, primary-derived fibroblasts and whole primary dissociated HNSCC tumour. Cell lines and primary-derived fibroblasts were cultured in both hypoxic and normoxic conditions in order to determine whether the low oxygen levels within the TME cause any changes in the cytokine profile of these cells.

Quantibody® cytokine arrays and ELISAs were used to determine the cytokines present in conditioned medium collected from the above cell types with a view to further understanding the interactions which may be occurring within the TME, what cytokines might be involved in the local immune regulation and which cells are likely to be orchestrators of this.

3.1.1 – Aims and hypothesis

To determine the Th1, Th2, Th17 cytokine profile of UMSCC HNSCC cell lines, primary-derived fibroblasts and overnight dissociated tumour. To determine whether HNSCC cell lines and primary-derived fibroblasts have a differing cytokine profile when grown in hypoxic conditions when compared to those grown in normoxic conditions. To determine whether cells cultured from laryngeal and oropharyngeal tissue subsites have a different cytokine profile.

Hypothesis 1 – HNSCC cell lines, primary-derived fibroblasts and overnight dispersed tumour produce immunosuppressive cytokines such as IL-10 and TGF-β
**Hypothesis 2** - HNSCC cell lines and primary-derived fibroblasts cultured under hypoxic conditions will release more immuno-suppressive factors than those grown under normoxic conditions.

**Hypothesis 3** - HNSCC cell lines, primary-derived fibroblasts and overnight dispersed tumour will produce higher levels of Th2 type cytokines (IL-4 and IL-2) compared to Th1 inducing cytokines (e.g. IFN-γ and IL-12).

### 3.2 - Methods

**3.2.1 - Quantibody® cytokine array**

A Th1, Th2, Th17 Quantibody® cytokine array (section 2.4) was used to quantify the levels of a set of 20 T cell biology relevant cytokines (Table 3.1) in HNSCC-derived culture medium (CM) obtained from a known number of cells (cell lines and primary-derived fibroblasts) cultured under both normoxic and hypoxic culture conditions (Section 2.1.1.4). Detection limits for each cytokine can be located in chapter 2, table 2.2.

**Table 3-1** - List of cytokines analysed by the Th1-Th2-Th17 Quantibody® cytokine array

<table>
<thead>
<tr>
<th>GM-CSF</th>
<th>IFN-γ</th>
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<tr>
<td>IL-1β</td>
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<td>TGF-β1</td>
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<td>TNF-β</td>
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</table>
HNSCC cell lines were kindly provided by Dr Thomas Carey, University of Michigan, USA and consisted of both laryngeal and oropharyngeal-derived epithelial cells (table 3.2) and the fibroblasts were derived from primary explants of dispersed primary tumour or node tissue (table 3.2; table 3.3; section 2.1.1.4). In addition the medium collected from overnight culture of dispersed primary HNSCC tissue or metastatic nodes was also investigated (section 2.2.2, Table 3.4).

Table 3-2 - The origin of CM assayed using the Th1-Th2-Th17 Quantibody®

<table>
<thead>
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<th>CM origin</th>
<th>Normoxic CM</th>
<th>Hypoxic CM</th>
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<td>✓</td>
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<tr>
<td>Oropharyngeal cell lines UMSCC-4 &amp; 47 (n=3)</td>
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<td>✓</td>
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<td>Laryngeal primary fibroblasts (n=2)</td>
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<td>✓</td>
</tr>
<tr>
<td>Oropharyngeal primary fibroblasts (n=2)</td>
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<td>✓</td>
</tr>
<tr>
<td>Laryngeal overnight dispersed tumour (n=6)</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>Oropharyngeal overnight dispersed tumour (n=4)</td>
<td>✓</td>
<td>x</td>
</tr>
</tbody>
</table>

Table 3-3 - Characteristics of the cell lines and primary-derived fibroblasts from which the CM was obtained for the Quantibody® cytokine array.

<table>
<thead>
<tr>
<th>HNSCC cell lines</th>
<th>Gender</th>
<th>Age</th>
<th>Stage</th>
<th>Anatomic site of origin</th>
<th>Specimen site</th>
</tr>
</thead>
<tbody>
<tr>
<td>UM-SCC-11b</td>
<td>Male</td>
<td>65</td>
<td>T2N2aM0</td>
<td>Larynx</td>
<td>Larynx</td>
</tr>
<tr>
<td>UM-SCC-81b</td>
<td>Male</td>
<td>53</td>
<td>T2N0M0</td>
<td>Tonsil (oropharynx)</td>
<td>Tonsillar pillar</td>
</tr>
</tbody>
</table>
Table 3-4 - List of patients characteristics from which overnight dispersed tumour/ node CM was obtained for the use in the Quantibody® cytokine array.

<table>
<thead>
<tr>
<th>Overnight dispersed tumour</th>
<th>Gender</th>
<th>Age</th>
<th>Stage</th>
<th>Anatomic site of origin</th>
<th>Specimen site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laryngeal ON CM 1</td>
<td>Male</td>
<td>41</td>
<td>T4N1</td>
<td>Larynx</td>
<td>Epiglottis tumour</td>
</tr>
<tr>
<td>Laryngeal ON CM 2</td>
<td>Male</td>
<td>41</td>
<td>T4N1</td>
<td>Larynx</td>
<td>Supraglottis node</td>
</tr>
<tr>
<td>Laryngeal ON CM 3</td>
<td>Male</td>
<td>78</td>
<td>T4N3</td>
<td>Larynx</td>
<td>Larynx tumour</td>
</tr>
<tr>
<td>Laryngeal ON CM 4</td>
<td>Male</td>
<td>78</td>
<td>T4N3</td>
<td>Larynx</td>
<td>Larynx node</td>
</tr>
<tr>
<td>Laryngeal ON CM 5</td>
<td>Female</td>
<td>72</td>
<td>T4n2b</td>
<td>Larynx</td>
<td>Larynx right node</td>
</tr>
<tr>
<td>Laryngeal ON CM 5</td>
<td>Female</td>
<td>72</td>
<td>T4n2b</td>
<td>Larynx</td>
<td>Larynx left node</td>
</tr>
<tr>
<td>Oropharyngeal ON CM 1</td>
<td>Female</td>
<td>37</td>
<td>reoccurrence</td>
<td>Oropharynx</td>
<td>Left tonsil/ tongue base</td>
</tr>
<tr>
<td>Oropharyngeal</td>
<td>Male</td>
<td>61</td>
<td>T1N2a</td>
<td>Oropharynx</td>
<td>Tongue base</td>
</tr>
</tbody>
</table>
CM was collected from the epithelial cell lines and the primary-derived fibroblasts following 1, 2, 3 and 4 days of culture (section 2.1.1.4), however, due to limited funds only day 2 and day 3 CM were assayed.

No duplicate readings were possible using the Quantibody® array, again due to cost; however the reason for using the array was to identify differences in levels of cytokines between different cell types which may be of interest. These cytokines were then studied further using less expensive techniques such as ELISA. The Quantibody® cytokine array was performed in two separate runs on four slides using a separate standard curve for each run so the data between runs was comparable.
Using the results of the Quantibody® cytokine array as a guide, two of the cytokines detected (IL-10 and TGF-β) were assayed further using Duoset® ELISAs (R&D systems). They were selected as they gave interesting results from the Quantibody® cytokine array and in addition they are known to be immunosuppressive factors which play an important role in the tumour microenvironment.

Conditioned medium from HNSCC cell lines and primary-derived fibroblasts (section 2.1.1.4; table 3.3), as well as from overnight culture of dispersed primary tumours and nodes, laryngeal (n=11) and oropharyngeal (n=13) were assayed for IL-10 and TGF-β content (Table 3.5). CM collected from overnight dispersed tumour following the depletion of lymphocytes was also assayed (section 2.2.2.1). The ELISAs were carried out as described in section 2.3 and all samples were done in duplicate on at least 2 runs.

In addition to the methods described for the standard ELISA in section 2.3 the CM used in the TGF-β ELISA had to be activated prior to use, to convert the latent TGF-β into its active form for detection. Medium samples were activated using 20µl of 1N HCL per 100µl of samples and incubated for 10 minutes before being neutralised by adding 20µl of 1.2N NaOH/0.5M HEPES per 100µl of sample for a further 10 minutes. Samples were then ready for use in the ELISA.

The absorbance of each well of the completed ELISA plate was measured at 450nm with a wavelength correction of 570nm and a four parameter logistic (4-PL) curve fit was used to generate a standard curve to enable the concentrations of the cytokines in the samples to be evaluated.

Any absorbance readings obtained from the CM alone were subtracted from the final absorbance for each sample and an average taken of the duplicate concentration readings. Paired student T tests were used to determine significant differences between cytokine levels in the CM from cell lines and primary-derived fibroblasts, between cell lines and fibroblasts of different origin, between cells cultured under hypoxic and normoxic conditions and between overnight dispersed tumours of differing origins. Comparisons were also made between CM obtained from tumour and nodes using SPSS.
Table 3-5 - Characteristics of patients from which overnight dispersed tumour node CM was obtained for the use in IL-10 and TGF-β1 ELISAs.

<table>
<thead>
<tr>
<th>Overnight dispersed tumour</th>
<th>Gender</th>
<th>Age</th>
<th>TNM</th>
<th>Specimen site</th>
<th>Node or tumour (N/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oropharyngeal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ON CM 1</td>
<td>Female</td>
<td>37</td>
<td>R</td>
<td>Tonsil/tongue base</td>
<td>T</td>
</tr>
<tr>
<td>ON CM 2</td>
<td>Male</td>
<td>50</td>
<td>T3N2c</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 3</td>
<td>Male</td>
<td>60</td>
<td>T3N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 4</td>
<td>Male</td>
<td>61</td>
<td>T1N2a</td>
<td>Tongue base</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 5</td>
<td>Male</td>
<td>48</td>
<td>T2N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 6</td>
<td>Male</td>
<td>54</td>
<td>T2N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 7</td>
<td>Male</td>
<td>54</td>
<td>T1N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 8</td>
<td>Male</td>
<td>63</td>
<td>T1N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 9</td>
<td>Male</td>
<td>48</td>
<td>T1N2a</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 10</td>
<td>Male</td>
<td>57</td>
<td>T1N2b</td>
<td>Base of tongue</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 11</td>
<td>Male</td>
<td>79</td>
<td>T3N2c</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 12</td>
<td>Male</td>
<td>68</td>
<td>T3N3</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 13</td>
<td>Male</td>
<td>42</td>
<td>T2N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td><strong>Laryngeal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ON CM 14</td>
<td>Male</td>
<td>68</td>
<td>T3N2b/c</td>
<td>Epiglottis</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 15</td>
<td>Male</td>
<td>41</td>
<td>T4N1</td>
<td>Epiglottis/supraglottis</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 16</td>
<td>Male</td>
<td>41</td>
<td>T4N1</td>
<td>Epiglottis/supraglottis</td>
<td>T</td>
</tr>
<tr>
<td>ON CM 17</td>
<td>Male</td>
<td>68</td>
<td>T3/T4N0</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>ON CM 18</td>
<td>Male</td>
<td>78</td>
<td>T4N3</td>
<td>Larynx</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 19</td>
<td>Male</td>
<td>78</td>
<td>T4N3</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>ON CM 20</td>
<td>Female</td>
<td>72</td>
<td>T4N2b</td>
<td>Larynx</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 21</td>
<td>Female</td>
<td>72</td>
<td>T4N2b</td>
<td>Larynx</td>
<td>N</td>
</tr>
<tr>
<td>ON CM 22</td>
<td>Male</td>
<td>?</td>
<td>T4N2c</td>
<td>Piriform fossa</td>
<td>T</td>
</tr>
<tr>
<td>ON CM 23</td>
<td>Male</td>
<td>80</td>
<td>T1aN0</td>
<td>Vocal cord</td>
<td>T</td>
</tr>
<tr>
<td>ON CM 24</td>
<td>Male</td>
<td>71</td>
<td>T4N0</td>
<td>Larynx</td>
<td>T</td>
</tr>
</tbody>
</table>
### 3.3 – Detection of cytokines in conditioned medium from HNSCC cell lines, primary-derived fibroblasts and overnight dispersed tumour

A Th1, Th2, Th17 Quantibody® multiplex cytokine array was used to quantify a range of 20 cytokines potentially involved in the anti-tumour immune response in the conditioned medium (CM) obtained from HNSCC cell lines and primary fibroblasts cultured under both normoxic and hypoxic conditions as well as overnight dispersed tumour CM. The concentration of each cytokine detected in all four HNSCC cell lines was combined and the average compared with the average of the cytokine concentration determined from four primary-derived fibroblasts.

#### 3.3.1 - Comparison of cytokine concentration in conditioned medium from HNSCC cell lines and primary-derived fibroblasts grown under normoxic conditions

CM collected from HNSCC cell lines under normoxic conditions had detectable levels of 16 out of the 20 cytokines measured, whereas 20/20 cytokines were detected in the CM collected from primary-derived fibroblasts (table 3.6). GM-CSF, IL-2, IL-6, IL-21, IL23 and TGF-β1 were found at the highest levels, with well over 1000pg/ml in most of the cell line and primary-derived fibroblast CM obtained under normoxic conditions (Table 3.6).
Table 3-6 - Comparison of cytokine concentrations in CM from HNSCC cell lines and primary-derived fibroblasts cultured under normoxic conditions

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Normoxic cell line CM (n=4)</th>
<th>Normoxic fibroblast CM (n=4)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>627.5 ±427.1</td>
<td>1486.5 ±458.8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>42.1 ±42.1</td>
<td>215.8 ±125.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-1β</td>
<td>11.1 ±11.1</td>
<td>11.6 ±7.8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-2</td>
<td>118.6 ±118.6</td>
<td>1320.7 ±668.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-4</td>
<td>32.9 ±32.9</td>
<td>51.8 ±32.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-5</td>
<td>3.6 ±3.6</td>
<td>41 ±25.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-6</td>
<td>1576 ±421.1</td>
<td>2138.3 ±192.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>4.9 ±4.9</td>
<td>634.5 ±376</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0 ±0</td>
<td>19.7 ±11.9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-13</td>
<td>5.7 ±5.7</td>
<td>612.2 ±350</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-17</td>
<td>33.7 ±33.7</td>
<td>133.1 ±82.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-17F</td>
<td>0 ±0</td>
<td>14.6 ±8.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-21</td>
<td>8423.9 ±4209</td>
<td>18952.3 ±6577.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-22</td>
<td>0 ±0</td>
<td>0.5 ±0.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-23</td>
<td>1391.8 ±520.8</td>
<td>2281.4 ±759.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-28A</td>
<td>0 ±0</td>
<td>1 ±1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>153.8 ±142.5</td>
<td>0.1 ±0.07</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>TFG-β1</td>
<td>1672.4 ±806.2</td>
<td>8972 ±5188.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TNF-α</td>
<td>24.3 ±24.3</td>
<td>253.9 ±191.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TNF-β</td>
<td>6.2 ±3.6</td>
<td>370.2 ±245.1</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

* p<0.05 - Mann-Whitney U tests were used to determine significant differences in cytokine concentration between cell line and fibroblast CM obtained under normoxic conditions. Cell line results were averaged from the data obtained from UM-SCC-11b, 81b, 4 and 47 CM. Primary-derived fibroblast results were averaged from data obtained from four cultures (oropharyngeal n=2 and laryngeal n=2).
A significantly higher concentration of MIP-3α was detected in the CM collected from cell lines under normoxic conditions (153.8 ±142.5 pg/ml) compared with that from CM collected from primary-derived fibroblasts under the same conditions (0.1 ±0.07 pg/ml). MIP-3α concentration ranged from 3.5-584.2 pg/ml in normoxic cell line CM and from 0-0.31pg/ml in normoxic primary-derived fibroblast CM with only 2/4 fibroblasts showing any positivity (1 oropharyngeal and 1 laryngeal fibroblasts).

In 13/20 cases, primary-derived fibroblasts produced higher levels of cytokines when compared to cell line CM, however, there was no significant difference seen for the levels of the 19 other cytokines between cell line CM and primary-derived fibroblast CM collected under normoxic conditions.

3.4.2 - Comparison of cytokine concentrations in conditioned medium from HNSCC cell lines and primary-derived fibroblasts grown under hypoxic conditions.

CM collected from cell lines under hypoxic conditions had detectable levels of 14 out of the 20 cytokines investigated, whereas 17 out of 20 cytokines were detected in the primary-derived fibroblast CM collected under hypoxic conditions. IL-2, IL-6, IL-10, IL-21, IL23 and TGF-β1 were found in the highest levels in both cell line and primary-derived fibroblast CM (Table 3.7).
Table 3-7 - Comparison of cytokine concentrations in CM from HNSCC cell lines and primary-derived fibroblasts cultured under hypoxic conditions

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Hypoxic cell line CM (n=4)</th>
<th>Hypoxic primary FB CM (n=4)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>181 ±181</td>
<td>686.8 ±362.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>49.5 ±49.5</td>
<td>212.4 ±75.9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-1β</td>
<td>12.3 ±12.3</td>
<td>3.1 ±0.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-2</td>
<td>1236.9 ±112.6</td>
<td>1483.6 ±141.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-4</td>
<td>0 ±0</td>
<td>29.4 ±29.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-5</td>
<td>15.3 ±6.5</td>
<td>55.5 ±16</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-6</td>
<td>1227.2 ±279</td>
<td>1788.2 ±1048.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>525.4 ±177.6</td>
<td>851 ±115.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0 ±0</td>
<td>12.5 ±12.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-13</td>
<td>58.6 ±41.9</td>
<td>513.4 ±92</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>IL-17</td>
<td>0 ±0</td>
<td>107.3 ±107.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-17F</td>
<td>0 ±0</td>
<td>3.8 ±3.8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-21</td>
<td>11524.4 ±1580.8</td>
<td>16815.8 ±2317.9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-22</td>
<td>0 ±0</td>
<td>0 ±0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-23</td>
<td>2637.7 ±333.4</td>
<td>1556.6 ±554.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-28A</td>
<td>0 ±0</td>
<td>0 ±0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>17.2 ±11</td>
<td>0 ±0</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>TFG-β1</td>
<td>7438.1 ±1130</td>
<td>10693.6 ±1200.9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TNF-α</td>
<td>90.8 ±30.7</td>
<td>213.3 ±125.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TNF-β</td>
<td>88.2 ±34.6</td>
<td>396.1 ±140</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

* p<0.05 - Mann-Whitney U tests were used to determine significant differences in cytokine concentration between cell line and fibroblast CM obtained under hypoxic conditions. Cell line results were averaged from the data obtained from UM-SCC-11b, 81b, 4 and 47 CM. Primary-derived fibroblast results were averaged from data obtained from four cultures (oropharyngeal n=2 and laryngeal n=2).
There was a significant difference ($p<0.05$) in the concentration of IL-13 and MIP-3α between HNSCC cell line CM and primary-derived fibroblast CM collected under hypoxic conditions, with the cell line CM having higher levels of MIP-3α and fibroblast CM having higher levels of IL-13 (table 3.7).

IL-13 concentration ranged from 0-117.6pg/ml in cell line CM collected under hypoxic conditions with only 2/4 cell lines showing positivity and 296.3-704.7pg/ml in primary-derived fibroblast CM collected under hypoxic conditions. In contrast MIP-3α concentration ranged from 1.9-48.6pg/ml in cell line CM, with no detectable levels in any of the primary-derived fibroblast CM collected under hypoxic conditions.

Although 15 out of the remaining 18 cytokines were detected at higher levels in the primary-derived fibroblasts compared to the cell lines, no significant differences were observed in the CM collected under hypoxic conditions.

3.3.3 - The effect of hypoxia on the secretion of cytokines from HNSCC cell lines

HNSCC cell lines grown under normoxic conditions had detectable levels of 16/20 of the cytokines, whereas 14/20 cytokines were detected in the hypoxic cell line CM (data from table 3.6 and 3.7).

GM-CSF, IL-2, IL-6, IL-21, IL23 and TGF-β1 were found in highest levels in both normoxic and hypoxic cell line CM (table 3.6 and 3.7).

CM collected from cell line cultured under hypoxic conditions demonstrated significantly higher levels of IL-2, TGF-β1 and TNF-β compared to that collected under normoxic conditions (table 3.6 and 3.7; figure 3.1).

IL-2 concentration ranged from 0-474.2pg/ml in normoxic cell line CM with only 1 of 4 cell lines (UM-SCC-11b) showing positivity whereas cell lines cultured under hypoxic conditions had up to three times the concentration of IL-2 in their CM with 4 of 4 showing positivity (range 1015.1-1536 pg/ml, figure 3.1a). TGF-β1 concentration ranged from 0-3870 pg/ml in normoxic cell line CM with 3 of 4 cell lines showing positivity (UM-SCC-81b was negative) whereas hypoxia stimulated the cell lines to secrete up to more than twice as much TGF-β1 into the medium (range 4871-10310
TNF-β concentration was much lower than the IL-2 and TGF-β1 levels and was observed in three out of the four cell lines cultured under normoxic conditions; however, hypoxia still induced a tenfold increase in the secretion of TNF-β from the cell lines compared to cells cultured under normoxic conditions with all cell lines showing positivity with hypoxia (normoxic range 0-14.8 pg/ml vs hypoxic range 15.6-149.2 pg/ml; figure 3.1c).

![Bar chart showing the difference in a) IL-2 b) TGF-β1 and c) TNF-β concentration (pg/ml) secreted by one laryngeal and three oropharyngeal HNSCC cell lines cultured under normoxic and hypoxic conditions (mean ±SEM; *significant difference p<0.05; Mann Whitney U test).](image)

Cell lines cultured in hypoxia did not generate any significant differences between the levels of the remaining 17 cytokines compared with cell lines cultured under normoxic conditions.
3.3.4 - The effect of hypoxia on the secretion of cytokines from HNSCC primary-derived fibroblasts

All twenty cytokines investigated were present in detectable levels in the CM collected from primary-derived fibroblasts cultured under normoxic condition compared to 17 of 20 cytokines in CM collected from primary-derived fibroblast CM collected under hypoxic conditions (table 3.6 and 3.7). GM-CSF, IL-2, IL-6, IL-10, IL-13, IL-21, IL-23 and TGF-β1 were found at the highest levels with most going over 1000pg/ml in both normoxic and hypoxic primary-derived fibroblast CM (table 3.6 and 3.7).

No significant differences were induced by culturing the primary-derived fibroblasts under hypoxic conditions compared to normoxic conditions for any of the twenty cytokines investigated.

3.3.5 - Measurement of cytokine concentrations in conditioned medium from dispersed primary HNSCC tumour and node tissue from both laryngeal and oropharyngeal subsites

The dispersal of the whole tumour or node samples was achieved using enzymatic digestion of the whole sample of tissue obtained from resection surgery (section 2.2.2), meaning that each sample was a different starting weight. The cytokine concentration measured in the CM following overnight incubation with the dispersed tumour/node tissue was therefore corrected for each tumour sample to give levels from 1mg of starting material.

The conditioned medium collected from laryngeal dispersed tumour samples following overnight incubation (n=6) had detectable levels of 14 out of 20 cytokines whereas 15 out of 20 cytokines were detected in the oropharyngeal dispersed overnight CM (n=5; table 3.8). Although the levels of cytokines observed appeared to be substantially lower than those seen in either of the cell lines or the fibroblasts, it must be noted that the values are not directly comparable as exact number of cells were unknown for the dispersed tumour. IL-21 was found to be the cytokine with the highest expression in both laryngeal and oropharyngeal samples showing at least a 10 fold higher expression compared to other cytokines (Table 3.8).
Table 3-8 - Cytokine concentration in Overnight dispersed tumour CM of laryngeal and oropharyngeal origin

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Laryngeal overnight CM (n=6)</th>
<th>Oropharyngeal overnight CM (n=5)</th>
<th>Signif.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>4.5 ±2.1</td>
<td>2 ±0.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.4 ±0.1</td>
<td>0.2 ±0.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.9 ±0.2</td>
<td>0 ±0</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.3 ±0.5</td>
<td>0.7 ±0.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-4</td>
<td>0 ±0</td>
<td>0 ±0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.05 ±0.02</td>
<td>0.02 ±0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-6</td>
<td>6.6 ±0.9</td>
<td>4.6 ±0.8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>9.5 ±1.4</td>
<td>9 ±2.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0 ±0</td>
<td>0 ±0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.1 ±0.1</td>
<td>0.1 ±0.02</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-17</td>
<td>0 ±0</td>
<td>0.02 ±0.02</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-17F</td>
<td>0 ±0</td>
<td>0 ±0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-21</td>
<td>64.8 ±23</td>
<td>34.2 ±16.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-22</td>
<td>0 ±0</td>
<td>0 ±0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-23</td>
<td>4.6 ±1.5</td>
<td>3.7 ±1.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-28A</td>
<td>0 ±0</td>
<td>0.01 ±0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>1.6 ±0.2</td>
<td>1.4 ±0.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TFG-β1</td>
<td>9 ±3.2</td>
<td>6.9 ±2.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.3 ±0.5</td>
<td>1.8 ±1.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TNF-β</td>
<td>0.1 ±0.01</td>
<td>0.03 ±0.01</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

*p<0.05 - Mann-Whitney U tests were used to determine significant differences in cytokine concentration between overnight dispersed tumour CM of laryngeal and oropharyngeal origin (table 3.8). Results from laryngeal (n=6) or oropharyngeal (n=5) were averaged to determine concentration.
The only significant difference observed between laryngeal and the oropharyngeal overnight dispersed tumour CM samples was with IL-1β where it was detectable in the laryngeal subsites (range 0.39-1.93 pg/ml) but undetectable in the oropharyngeal samples (table 3.8).
3.4 - ELISA results

3.4.1 - IL-10 concentration in conditioned medium from HNSCC cell lines, primary fibroblasts and overnight dispersed tumour

IL-10 was detectable in all CM collected from overnight dispersed primary tumour and node tissue (figure 3.2).

To determine if there was a difference in IL-10 levels in the overnight dispersed tumour/node CM from different primary sites, results were combined into CM collected from laryngeal (n=10) and oropharyngeal tumours (n=13). The concentration of IL-10 in overnight CM of oropharyngeal origin ranged from 297 to 2121 pg/ml and that of laryngeal origin ranged from 196 to 2564 pg/ml. No significant difference in IL-10 levels was observed between the two sites of origin (p>0.05).

In contrast IL-10 was below the detectable limit (31.2 pg/ml) in all CM collected from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under both normoxic and hypoxic conditions.
Lymphocytes were depleted in five of the 23 dispersed tumour/node samples (Orpharyngeal n=3, laryngeal n=2; section 2.2.2.1) before CM was collected and a comparison was made between these lymphocyte depleted samples and their non-depleted counterparts (figure 3.3).

The IL-10 concentration in non-lymphocyte depleted overnight dispersed tumour/node CM ranged from 372 to 1983 pg/ml and was significantly higher \((p<0.05)\) than the lymphocyte depleted samples where it ranged from 0 to 471 pg/ml.

The CM samples obtained from tissue dispersed from patients with laryngeal tumours (n=10) were a combination of tissues from metastatic nodes (n=5) and primary tumour (n=5). Therefore the results were divided into tissue from nodal and tumour sites to determine whether there was a difference in IL-10 production between tissue from these locations (figure 3.4).

**Figure 3.3** - Bar chart showing the IL-10 concentration (pg/ml) in non-lymphocyte depleted and lymphocyte depleted overnight dispersed tumour/node CM (Mean; ±SEM). * Significant difference \(P<0.05\). Paired student t test.
Figure 3-4 - Bar chart showing IL-10 concentration in CM collected from dispersed laryngeal nodal tissue and laryngeal tumour tissues. (Mean; ±SEM). * Significant difference $P<0.05$. Paired student t tests.

The IL-10 concentration in the CM derived from laryngeal nodal tissue was significantly greater ($p<0.05$; range 205 to 2564pg/ml), than that in the CM derived from the primary tumour tissue (253 to 2144pg/ml).

Within the oropharyngeal subgroup, all but one of the samples was derived from nodal tissue and so similar comparison could not be made.
3.4.2 – TGF-β concentration in conditioned medium from HNSCC cell lines, primary-derived fibroblast cultures and overnight dispersed primary tumour

In contrast to IL-10, TGF-β1 was detectable in all cell lines, primary-derived fibroblasts and overnight dispersed tumour CM.

3.4.2.1 – TGF-β concentration in conditioned medium from HNSCC cell lines and primary-derived fibroblasts cultured under both hypoxic and normoxic conditions

In order to determine the effect of oxygen concentration on the secretion of TGF-β1 from cell lines (n=4) and primary-derived fibroblasts (n=4) the results were divided into those cultured under either normoxic or hypoxic conditions. TGF-β1 was detectable in 4/4 cell lines obtained from normoxic and hypoxic culture conditions and in the CM collected from primary-derived fibroblasts 4/4 from normoxic culture and 3/4 from hypoxic. In all cell lines and fibroblast cultured under normoxic and hypoxic conditions there was an overall increase in TGF-β release (pg/ml) in CM collected following 1 day of culture through to that collected following 4 days of culture (figure 3.5a & b). However, there was only a significant increase observed between cell line CM cultured under normoxic conditions between day 1 and day 2, cell line CM culture under hypoxic conditions between day 3 and day 4 and primary-derived fibroblast CM cultured under normoxic conditions between day 1 and day 2.

No significant difference was seen in TGF-β1 release from both cell lines and primary-derived fibroblast between cells cultured under normoxic and hypoxic CM irrespective of the number of days the CM was cultured with the cells for.

Primary-derived fibroblasts cultured under normoxic conditions released a greater amount of TGF-β into the CM compared with HNSCC cell lines cultured under the same conditions regardless of the length of culture, however the differences were not significant (Figure 3.6a). In contrast HNSCC cell lines cultured under hypoxic conditions released more TGF-β1 into the CM than fibroblasts cultured under the same conditions but was only significant following 4 days of culture (figure 3.6b).
Figure 3.5 - Bar charts showing TGF-β1 concentration (pg/ml) in a) HNSCC cell lines (n=4) and b) primary-derived fibroblasts (n=4) following culture under normoxic and hypoxic conditions. D1-D4 represents the number of days the cells were cultured before CM was collected. (Mean; ±SEM). * Significant difference \( P<0.05 \). Paired student t tests.
Figure 3-6 - Bar charts showing TGF-β concentration (pg/ml) in CM collected from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) following a) normoxic and b) hypoxic culture (Mean; ±SEM). * Significant difference P<0.05. Paired student t tests.
3.4.2.2 – TGF-β concentrations in CM collected from overnight dispersed HNSCC tumours/nodes obtained from laryngeal and oropharyngeal sites

The release of TGF-β1 from primary tumours and nodes, following dispersal and overnight incubation, from tissue of both oropharyngeal (n=14) and laryngeal (n=12) origin was compared (figure 3.7).

Figure 3-7 - Bar chart showing the difference in TGF-β concentration (pg/ml) in overnight conditioned medium (ON CM) collected from dispersed oropharyngeal and laryngeal tumours/nodes (Mean; ±SEM). * Significant difference P<0.05. Paired student t tests.

The concentration of TGF-β in overnight CM of oropharyngeal origin ranged from 0 to 1054.06 pg/ml and was significantly greater than that found in overnight dispersed tumour CM of laryngeal origin (range 0 to 457.45 pg/ml)

Overnight dispersed tumour was also depleted of lymphocytes prior to the collection of CM from six of the tumours and the release of TGF-β into the CM compared to the corresponding non-depleted samples (section 2.2.2.1; figure 3.8).
The concentration of TGF-β1 in overnight CM without lymphocyte depletion ranged from 0 to 348.73 pg/ml and was greater than that seen in the lymphocyte depleted samples (range 0 to 173.49 pg/ml) however the difference was not significant.

The overnight CM samples obtained from laryngeal subsites were a combination of tissue from nodes (n=5) and from tumour (n=6). The results for CM from all dispersed nodal and all dispersed tumour tissue were combined to determine whether there was a difference in TGF-β1 production from tissue from these different sites (figure 3.9).

**Figure 3-8** - Bar chart showing the TGF-β1 concentration (pg/ml) in non-depleted and lymphocyte depleted overnight dispersed tumour/ node CM (Mean; ±SEM). Paired student t test.
The TGF-β1 concentration in the CM derived from nodal tissue of patients with laryngeal cancer was significantly greater ($p<0.05$; range 0 to 457.45 pg/ml), than that in the CM derived from the primary laryngeal tumour tissue (0 to 188.16 pg/ml).

Similar comparisons could not be done for the oropharyngeal derived tissue because the majority (all but one) was from nodal tissue.

**Figure 3-9** - Bar chart showing TGF-β1 concentration in CM collected from laryngeal nodal tissue and laryngeal tumour tissues. (Mean; ±SEM). * Significant difference $P<0.05$. Paired student t test.
3.5 – Discussion

A myriad of different cytokines are known to be present within the tumour microenvironment (TME). All have different roles to play, most of which have yet to be elucidated. Cytokines within the TME, released by cancer cells and associated stroma are implicated in the ability of tumour to avoid detection and destruction by the immune system.

The aim of this chapter was to determine which cytokines were produced and by which cell types within the TME, with a plan to link their presence with various functions which could lead to tumour development and progression.

The detection of cytokines in HNSCC cell lines was used as a model for the epithelial cells within the tumour microenvironment as a precursor to clinical samples. In addition fibroblasts derived from either the primary HNSCC tissue or metastatic nodes were cultured to see what role the tumour associated fibroblasts may play in controlling the tumour microenvironment. Finally, the presence of cytokines was investigated in the CM collected from enzymatically dispersed tumour and node tissue following overnight incubation in the hope that this would generate the most in vivo-like condition having all cell types present and fresh from tumour extraction.

The TME is predominantly hypoxic (Kim et al., 2009) so an attempt was made to imitate the conditions the cells might encounter within the TME with the understanding that these conditions may induce the different cell types (epithelial and fibroblast) to produce different cytokines than those seen under standard culture conditions.

Initially a TH1, TH2 and TH17 Quantibody® array was carried out to screen for the presence of 20 cytokines relevant to T cell biology. This method was selected as a way of assaying a broader range of cytokines within a few samples to get a better idea of the cytokine profile of different cell types within the TME. From these results specific cytokine ELISAs were selected (IL-10 and TGF-β1) to expand the data obtained on a larger dataset. ELISA offers a more sensitive approach to assaying single cytokines and also allows more samples to be assayed at once, so was used to confirm Quanitobdy® results. IL-10 and TGF-β1 were selected for ELISAs due to their relevance to the immunosuppressive TME.
3.5.1 - Cytokines detected in HNSCC cell line and primary-derived fibroblast culture medium

Both epithelial cancer cells and cancer-associated fibroblasts release cytokines into the TME. One hypothesis was that both HNSCC cell lines and primary-derived fibroblasts would release a wide array of cytokines and that they would produce relatively high levels of immunosuppressive factors such as IL-10 and TGF-β. It was also hypothesised that both cell types would release more Th2 cell inducing cytokines (IL-4 and IL-2) compared to Th1 inducing cytokines (e.g. IFN-γ and IL-12) which would confer a more immunosuppressive environment.

Quantibody® data found that CM from HNSCC cell lines cultured under both normoxic and hypoxic conditions contained GM-CSF, IFN-γ, IL-1β, IL-2, IL-5, IL-6, IL-10, IL-13, IL-21, IL-23, MIP-3α, TGF-β1, TNF-α and TNF-β. CM from primary-derived fibroblasts cultured in normoxic and hypoxic conditions contained GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, IL-17F, IL-21, IL-23, MIP-3α, TGF-β1, TNF-α and TNF-β. These data show that cell lines and primary-derived fibroblasts are capable of releasing a wide array of different cytokines, some of which are pro-tumour and others that are anti-tumour. This suggests a network of different factors may be involved in tumour development. It might be the balance of these factors, rather than their presence which leads to tumour progression.

The Quantibody® found both IL-10 and TGF-β in CM from cell lines and primary-derived fibroblasts cultured in both normoxic and hypoxic conditions. However, ELISA data found that IL-10 was not present in detectable levels within the CM from both HNSCC cell lines and primary-derived fibroblasts, though ELISA data agreed with the Quantibody® data regarding the presence of TGF-β. The CM used in both assays was from the same batch, so differing culture conditions could be ruled out as a cause for this disparity. Also IL-10 concentrations were over 500pg/ml in the Quantibody® suggesting that the detection limit of the ELISA kit was also not a factor. The ELISA was repeated several times (n=4) all of which gave negative results for IL-10, whereas the Quantibody was only carried out once due to cost. It may be that a repeat with the Quantibody® would have yielded different results. The original hypothesis regarding cell lines and primary-fibroblasts ability to secrete immunosuppressive factors can be
accepted, as in the majority of cases, TGF-β was released and the Quantibody® found evidence of IL-10 release.

Other studies have also looked at the release of cytokines by HNSCC cell lines. Chen et al (1999) collected supernatant from UMSCC HNSCC cell lines cultured under normoxic conditions (different lines to the ones used in this study) and used ELISA to identify cytokines. They found HNSCC cell lines released IL-6, IL-8 and GM-CSF but did not detect IL-1β, TNF-α, TGF-β, IL-2, IL-12, IFN-γ, IL-4 and IL-10 in the supernatant. The data from this study both agree and disagree with the finding in this chapter as TGF-β and IL-10 were both found in cell line CM. However some studies did find TGF-β in cancer cell lines (Desai et al., 2013). IL-6 and GM-CSF were a common find in HNSCC supernatant (Pries and Wollenberg, 2006; Chen et al., 1999). IL-6 is a pro-inflammatory molecules which can stimulate CD4+ effector T cell differentiation into the Th17 subtypes and is thought to be anti-tumour (Kishimoto, 2010). GM-CSF is also an anti-tumour cytokine as it is able to enhance the function of various T effector populations. The data obtained in this chapter is in disagreement with some results from Chen et al (1999) who failed to detect IL-1β, TNF-α, IL-2, IL-4, IL-10 and TGF-β1 in cell line CM. This could be due to the use of different cell lines or a difference in cell culture techniques. Woods et al (1998) also failed to detect IL-2 and IFN-γ, which again disagrees with the data obtained in this chapter. IL-2 has several functions including activating NK cells and stimulating CD4+ T cells to differentiate into Th2 cells which have pro-tumour activity (Zhu et al., 2010). IL-2 is also involved in the generation and function of Treg cells (Lippitz, 2013; Bergmann et al., 2011; Kryczek et al., 2007). Though IL-2 induces pro-tumour T cell subsets, it is reported that reduced levels of IL-2 in the TME correlates with a poor prognosis for cancer patients (Lippitz, 2013), partly due to its ability to increase cytotoxic activities. However, the primary function of IL-2 in the TME may be dependent on the types of immune cells present in the tumour.

There appears to be a lot of variation between different research in terms of cytokines identified in the culture medium of cancer cell lines. Desai et al (2013) took a selection of different cancer cell lines and looked at their cytokine profiles using ELISA. They found that TGF-β, TNFα and IL-1β was expressed by all cell lines, but certain cytokines were cell line specific. This highlights how different each cancer type can be and
strengthens the need to research each individually. A possible reason for the differences seen may be due to the cells producing the cytokines but not releasing them. TGF-β is a known immunosuppressant that is involved in aiding the tumour in evading the immune system. It is possible that the cancer cells need a stimulus from other absent cells (e.g. immune cells) in order to release the TGF-β into the tumour microenvironment. Again this highlights the possible importance of cell to cell interactions within the tumour microenvironment. TGF-β is thought to drive a pro-tumour environment in later stages of tumour development and will affect epithelial cell phenotypes and may also effect the surrounding fibroblast cells (Pickup et al., 2013; Yang et al., 2010).

Studies have also focused on the cytokine profiles of cancer-associated fibroblasts (CAFs). TGF-β has been found to be released by CAFs in several types of cancer including breast cancer (Yu et al., 2014) and HNSCC (Takahashi et al., 2015; Rosenthal et al., 2004). Yu et al (2014) isolated stromal fibroblasts from invasive breast cancer tissue and collected CM after 48 hours of culture. They analysed TGF-β levels by cytokine antibody array and found that they produce TGF-β. This agrees with the data found in this chapter and strongly suggests a role for this immunosuppressive factor in cancer progression. This also agrees with the original hypothesis that primary-derived fibroblasts are able to secrete immunosuppressive factors.

Other factors such as TNFs (Takahashi et al., 2015; Calon et al., 2014), IL-6 (Augsten, 2014) and VEGF (Takahashi et al., 2015) have also been identified in the CM of CAFs. Takahashi et al (2015) compared the levels of IL-6, IL-8, TNF, TGF-β and VEGF levels in CAFs compared to normal fibroblast. CAFs were isolated from HNSCC tissue from six patients. They found that all five cytokines were expressed at higher levels in CAFs compared to normal fibroblasts. This suggests that the TME is able to up-regulate the secretion of certain cytokines by the CAFs, some of which will aid in the progression of the tumour (e.g. TGF-β).

One of the original hypothesis was that both HNSCC cell lines and primary-derived fibroblasts would release a greater level of Th2 type cytokines (IL-2 and IL-4) compared to Th1 (IFN-γ and IL-12), leading to the pro-tumour TME. HNSCC cell lines cultured under normoxic and hypoxic conditions released the Th2 inducing cytokine IL-2, though only cell lines cultured in normoxia produced IL-4. Primary-derived
fibroblasts produced both IL-2 and IL-4 when cultured in both normoxic and hypoxic conditions. HNSCC cell lines cultured in both normoxic and hypoxic conditions produced the Th1 inducing cytokine IFN-γ but not IL-12, whereas primary-derived fibroblasts cultured in normoxia and hypoxia produced both. IL-2 appeared to be the predominant cytokine produced by all cells with levels over 1000pg/ml whereas IL-4, IFN-γ and IL-12 were all found at levels below 216pg/ml. Other research has found evidence showing a tilt towards a more Th2 response in cancer patients (Sparano et al., 2015; Monte et al., 2011; Schreck et al., 2009). However, in this case there appeared to be no predominant tilt towards either Th1 or Th2 which leads to the rejection of the original hypothesis. It may be that the production of a predominantly Th2 pro-tumour environment requires the presence of other cell types, such as immune cells.

To determine the differing roles of epithelial cancer cells and associated fibroblasts, the levels of cytokines were compared. A significantly higher level of MIP-3α was observed in HNSCC cell line CM compared to primary-derived fibroblast CM collected under both normoxic and hypoxic conditions. MIP-3α (CCL20) is a strong chemottractant for lymphocytes, including immunosuppressive Tregs (Chen et al., 2011; Akahoshi et al., 2003). This increased abundance of Tregs within the TME leads to increased suppression of T effector cells, aiding the progression of the tumour (Chen et al., 2011). This suggests that cancer cells are more involved in the recruitment of T lymphocytes through the expression of CCL20.

In addition, HNSCC derived fibroblasts grown under hypoxic conditions secreted greater levels of IL-13 compared to fibroblasts grown in normoxia. IL-13 is an anti-inflammatory cytokine and can reduce the production of pro-inflammatory molecules (Wynn, 2003). Therefore it acts to suppress the immune response against the cancerous cells allowing it to progress. The higher levels seen in hypoxic fibroblast CM may suggest the involvement of the stromal cells in the pro-tumour TME.

The array of cytokines released by both cell lines and associated fibroblasts was startlingly similar. It seems that both cell types are involved in the immunosuppressive microenvironment and potentially able to influence other cells through soluble factors.
3.5.2 - The effect of oxygen levels on cytokine release from HNSCC cell lines and primary-derived fibroblasts

Hypoxia is thought to play a role in the immunosuppressive microenvironment (Kumar and Gabrilovich, 2014; Janssen et al., 2005). By culturing both HNSCC cell lines and primary-derived fibroblasts in a hypoxic chamber it was possible to try and imitate the low oxygen levels they might experience within the solid tumour. Cytokine profiles from cells cultured in hypoxia and normoxia could then be compared to see if there were any differences to give an indication to whether hypoxia plays a role in the immunosuppressive TME. The main hypothesis was that hypoxia would induce both cell lines and primary-derived fibroblasts to release greater levels of pro-tumour cytokines (e.g. IL-10 and TGF-β) compared to those grown in normoxia.

The only difference observed between cell lines cultured in normoxia compared to hypoxia was the presence of IL-4 in the CM collected under normoxia and IL-17 in the CM collected under hypoxia. There was a significant difference between the concentration of IL-2, TGF-β1 and TNF-β when comparing normoxic and hypoxic cell line CM, with hypoxic CM having the higher levels of all three cytokines.

The detection of IL-4 in CM collected from HNSCC cell lines cultured under normoxic conditions is interesting, as IL-4 is involved in the differentiation of CD4⁺ T cells into Th2 cells, which has been shown to be pro-tumour (Zhu et al., 2010). Conversely, IL-17 was detected in the CM from cell lines cultured under hypoxic conditions. IL-17 is a cytokine predominantly released by Th17 T effector cells, though appears in this case to have been released by HNSCC cell lines. It is a pro-inflammatory molecule that can induce other pro-inflammatory molecules and is thought to be pro-tumour (Luckheeram et al., 2012). Knowing that hypoxia is a well known characteristic of tumours, it was thought that HNSCC cells cultured under hypoxic conditions would cause the release of more pro-tumour cytokines when compared to cells cultured under normoxic conditions. It is possible that in the absence of immune cells, the range of cytokines released into the TME is altered, suggesting a more pivotal role for immune cells in the pro-tumour environment.

TNF-β was found in significantly higher concentration in the CM obtained from cell lines cultured in hypoxia compared to normoxia. TNF-β is a pro-inflammatory cytokine.
which can inhibit tumour genesis (Pfeffer, 2003). It is itself inhibited by the presence of the cytokine IL-10 which is known to be increased in the tumour microenvironment. But from the results of this study IL-10 appears to be produced predominantly by immune cells which are obviously not present in cell line culture. The production of TGF-β and IL-2 by the HNSCC epithelial cells, especially under hypoxic conditions, suggests that the tumour is attempting to generate an environment in which it is able to proliferate and spread, however, whether it will be able to do this is likely to be dependent on the other cells present within the TME and therefore the balance of these factors.

Hypoxia did not induce any significant changes in the level of cytokine production from primary-derived fibroblasts, which may be attributed to the fact that these cells have been cultured in isolation and any effect may need the presence of other cell types to influence the changes in cytokine production. Cell to cell interaction can play a big role in the cytokine profile of a cell. So without the presence of the other cells, the hypoxic culture conditions may have had little/no effect. Further work to determine the fibroblasts exact role within the TME is required.

Though culturing primary-derived fibroblasts in hypoxia caused no significant difference compared to normoxic culture, cell lines cultured in hypoxia produced higher levels of three cytokines (TNF-β, TGF-β and IL-2). In particular the higher levels of TGF-β suggest that hypoxia can lead to a more immunosuppressive microenvironment. The original hypothesis can be partly accepted, as cell lines cultured under hypoxia did produce higher levels of some cytokines involved in the pro-tumour TME.

One study looked at cytokine profiles of human prostate cancer fibroblasts cultured in both normoxic and hypoxic (1% O₂) conditions. They found that TGF-β levels were higher in the CM from fibroblasts grown in hypoxia and that levels increased over time compared to fibroblasts grown in normoxia (Berger et al., 2003). This disagrees with the data obtained in this chapter which showed no significant differences in TGF-β levels between primary-derived fibroblasts cultured in normoxia vs. hypoxia. It is possible that if the primary-derived fibroblast had been cultured in hypoxia for longer, they would have started to produce higher levels of TGF-β, so this needs further investigation. It has also been suggested that up-regulation of growth factors secretion
(e.g. TGF-β) is a mechanism activated to aid cells in overcoming harsh hypoxic environments (Vaupel, 2004). Through an extensive literature search, it became clear that few people had looked at the differences in cytokines profiles of cancer cell lines and primary-derived fibroblasts cultured in different oxygen levels. This study looks at an extensive array of cytokines offering more information about the effect of hypoxia on the cytokine profile of tumour cells.

3.5.3 - Cytokines detected in overnight dispersed tumour culture medium

To understand the cytokine profile of the TME, it was important to assess what the tumour as a whole was producing and not just selected cell types. Within the tumour there are various cell types, including epithelial cell, fibroblasts and immune cells all of which can interact with each other through cell to cell contact and through the release of soluble factors. It is possible that the cytokine profile of each individual cell type is different when in isolation (e.g. epithelial cell lines) when compared to being within the tumour mass. In order to get an idea of what the tumour was producing as a whole, fresh tissue biopsies were enzymatically dispersed to single cells and incubated overnight to capture a snap shot of what cytokines were being released. It was hypothesised that tumour would release a range of cytokines and that there would be high levels of immunosuppressive factors such as IL-10 and TGF-β. It was also hypothesised that there would be higher levels of Th2 inducing cytokines compared to Th1 type cytokines.

Due to the nature of the dispersed tumour, it was impractical to count the cells cultured overnight before the CM was collected. This was due to the cells being clumped together despite dispersion. This means a direct comparison between the levels of cytokines seen in cell line/ fibroblast CM and dispersed tumour CM was not possible. However, samples were weighed prior to dispersal so cytokine levels could be corrected to concentration per mg of tumour to allow comparison between tumour subsites. Even though a comparison between cytokine levels was not possible, it was possible to look at the difference in cytokine profile between cell line and fibroblast CM and dispersed tumour CM.
The following cytokines were detectable in 1 or more overnight dispersed tumour CMs; GM-CSF, IFN-γ, IL-2, IL-5, IL-6, IL-10, IL-13, IL-21, IL-23, MIP-3α, TGF-β1, TNF-α and TNF-β.

IL-10 was detected by ELISA in high levels in the majority of CM from overnight dispersed tumour from both laryngeal and oropharyngeal origin which agrees with data obtained from the Quantibody® array. There was no significant difference seen in IL-10 release between the two subsites which again agrees with the Quantibody® results. Lymphocyte depletion of whole tissue resulted in a significant reduction by over ¾ in the levels of IL-10, suggesting that lymphocytes are responsible for the release of the majority of the IL-10 measured in the overnight dispersed CM. The depletion of lymphocytes was not complete leaving a small number present in the sample therefore it is possible that any remaining IL-10 was coming from these cells. This firstly shows that there are lymphocytes within the tumour which agrees with other research (Uppaluri et al., 2008). The presence of certain sub-sets of lymphocytes (e.g. CD8⁺ T cells) can be a positive prognosis for cancer patients (Webb et al., 2014) however they become altered by the TME over time. Tumour-derived lymphocytes from HNSCC produce IL-10, which will lead to immune-suppression and subsequent progression of the tumour, suggesting a role for them in the pro-tumour milieu.

TGF-β1 was detected by ELISA in overnight dispersed tumour CM from both laryngeal and oropharyngeal origins. There was a significant difference in TGF-β1 concentration between laryngeal and oropharyngeal CM with oropharyngeal having higher levels. This is in contrast to the Quantibody® results which showed no significant difference between the two subsites. The oropharyngeal samples used in the study are largely nodal, possibly suggesting that the nodal tissue secretes more of the immunosuppressive factor TGF-β1, though this could also be down to the sub-site. The ELISA data agree with several studies which have found primary tumour to release TGF-β1 (Lu et al., 2004). There was no significant difference seen between the lymphocyte depleted samples and the non-depleted tumour samples. This doesn’t necessarily mean that the lymphocytes don’t produce any TGF-β1, but it may mean that other cells in the TME such as the cancer epithelial cells and cancer associated fibroblasts are producing TGF-β1.
Chen et al (1999) found that HNSCC tumour specimens released IL-6, GM-CSF and other well known cytokines within the TME are IL-4 and IL-10 (Pries and Wollenberg, 2006).

Overnight dispersed tumour CM could be divided into those originating from laryngeal and oropharyngeal subsites. The majority of oropharyngeal tissue was from nodes, whereas the majority of laryngeal tissue from tumour. The difference observed between CM obtained from different sub-sites was the release of IL-1β from laryngeal CM and IL-17 and IL-28A from oropharyngeal origin.

IL-1β is a pro-inflammatory cytokine which can be released by many cells including the immune cells within the TME (Colotta et al., 2009; Apte et al., 2006) and can contribute to tumour growth and progression (Leibovich-Rivkin et al., 2013). Though both the epithelial cell lines and primary-derived fibroblasts produced IL-1β it is likely that immune cells within the tumour cells influencing its release or releasing it themselves. This difference seen between laryngeal and oropharyngeal subsites may be attributed to differing levels of tumour infiltrating lymphocytes. Green et al (2013) found that infiltration by lymphocytes is sub-site dependant and that oropharyngeal tumour had the greatest number of infiltrating lymphocytes in both the stroma and tumour. An increase level of lymphocytes can confer a positive prognosis, and with IL-1β acting as a pro-tumour cytokines, it would be interesting to investigate the link between IL-1β and oropharyngeal tissue further.

Though it was not possible to compare the actual concentrations of cytokines in cell lines/primary fibroblasts to overnight dispersed tumour, it is possible to compare their presence. Fibroblasts alone produced IL-4, IL-12p70, IL-17 and IL-17F while dispersed overnight tumour CM did not. This may have been due to a few factors;

- Cell to cell interactions within the whole dispersed tumour caused the cytokine profile of the cancer associated fibroblasts to change, so they would produce less/hone of the cytokines produced when they were grown in isolation.
- The fibroblasts may have been producing the cytokines but other cells may have been using it up.
• The dispersed tumour is a mixture of cells, so there are likely to be less fibroblasts in the culture and the levels of those cytokines may therefore have been lower than the detectable limit of the assay, even though they may still have been produced at low levels.

3.6 - Conclusion

HNSCC cell lines, primary-derived fibroblasts and overnight dispersed tumour can release immunosuppressive factors such as IL-10 and TGF-β. It is clear that there is a complex network of cytokines all of which play their own part within the TME. In the subsequent chapters, the CM obtained will be used to assess the effect of soluble factors on the proliferation and function of immune cells.
Chapter 4 -
The effect of HNSCC-derived CM on the proliferation of immune cells from healthy and patient PBMC
4.1 - Introduction

After determining the range of cytokines released by HNSCC cell lines, primary-derived fibroblasts and overnight dispersed tumour, the CM collected was used to assess their effect on the proliferation of PBMC from normal donors and HNSCC patients.

PBMC from normal and HNSCC patients were cultured before CM from HNSCC cell lines, primary-derived fibroblasts and overnight dispersed tumour was added. The effect on cell activity and proliferation was assayed using the MTS assay. The MTS assay utilises the fact that metabolically active cells change a compound into a coloured product. The intensity of colour is directly proportional to the number of viable cells.

It was decided to initially look at the effect of HNSCC-derived CM on whole PBMC in order to get an idea how the populations act as a whole. However, any increase/decrease in proliferation/activity gave no indication as to which cell types were being influenced. The PBMC were subsequently sorted into T effector cells, Treg cells and CD8+ cytotoxic T cells to assess the effect of the CM on individual populations. As further validation, the same assay was carried out on whole PBMC and the FACS Aria was used to determine whether the percentage of cell types was altered.

It has been found that cancer patients have increased numbers of Treg cells (CD4+CD25+) in their peripheral blood when compared to normal’s, leading to a protumour environment. This includes some of the following: non-small cell lung cancer (Woo et al., 2001), ovarian cancer (Woo et al., 2001), hepatocellular carcinoma (Ormandy et al., 2005), gastric and esophageal cancer (Ichihara et al., 2003) and HNSCC (Wolf et al., 2003).

Wolf et al (2003) found CD4+ effector cells in the peripheral blood of HNSCC patient had impaired proliferation and that patients had increased numbers of T regulatory cells when compared to controls. Other studies, such as that of Lim et al (2014) found that as well as an increase in T regulatory cells there was a decrease in CD8+ T cells. This suggests that tumours increase their ability to evade the immune system by utilising the Treg cells to suppress other immune cells at the same time as reducing the number of CD8+.
Other studies investigated whether the percentage of CD4$^+$ and CD8$^+$ T cells change in the peripheral blood of patients when compared to normals. Kuss et al (2004) found that HNSCC patients had overall lower absolute numbers of CD4$^+$ and CD8$^+$ T cells compared to normal’s and patients with re-occurrence tended to have the lowest CD4$^+$ cell counts.

4.1.2 – Aim and hypothesis

The aim of this chapter was to determine the effect that CM from HNSCC derived cell lines, primary-derived fibroblasts and overnight dispersed tumour had on the proliferation of whole PBMC from both healthy volunteers and HNSCC patients and individual immune cell populations from healthy PBMC. Also, to determine the effect of HNSCC-derived CM on the relative numbers of immune cells within the whole PBMC from healthy donors. It was hypothesised that the addition of HNSCC CM would cause an overall reduction in the proliferation of PBMC from both healthy and patient donors.

It was hypothesised that patient PBMC will be affected to a lesser extent than healthy PBMC due to possibility of pre-priming by the local soluble factor tumour microenvironment. It was also hypothesised that HNSCC-derived CM would cause an increase in the proliferation and number of Tregs and a decrease in T effectors and CD8$^+$ T cells.

The soluble factors released by both HNSCC cell lines and primary-derived fibroblasts were analysed, so by adding CM from both cell types to PBMC, it was possible to determine if there was any differential effect between cell lines and primary-derived fibroblast soluble factors on the proliferation and relative numbers of immune cells.

The cell lines and primary-derived fibroblasts were cultured in both normoxia and hypoxia to determine whether cells cultured in hypoxia produced any different soluble factors and whether they affected the proliferation and relative number of immune cells differently. It was hypothesised that CM obtained from cells cultured under hypoxia would create a more immunosuppressive profile compared to normoxic CM, with greater levels of Treg cells and lower levels of T effector cells and CD8$^+$ T cells.
4.2 – Methods

4.2.1 - MTS assays on normal and HNSCC patient PBMC samples

MTS assays were used to assess the effect of HNSCC-derived CM on the proliferation/activity of immune cells in circulation from both healthy and HNSCC patients. CM from HNSCC cell lines (n=4), primary-derived fibroblasts (n=4) and overnight dispersed tumour tissue (n=16) were used (table 3.3, table 4.2). The MTS assays were carried out as described in section 2.7, and the cell sorting as described in section 2.6.

Initially MTS assays were carried out on whole healthy and patient PBMC samples (table 4.1). PBMC samples were collected and processed as described in section 2.2.1. The normal PBMC samples were then sorted into T effector cells, Treg cells and CD8+ T cells before the same tests were carried out. It was not possible to carry out the MTS assays on individual populations from HNSCC patient PBMC due to the low numbers of PBMC samples and also the very low yield of individual immune cells due to the likely immune-suppressed state of the patients.

Each HNSCC-derived CM was added to four whole PBMC cultures; however in patient PBMC the yield of PBMC was not high enough to ensure all the CM could be tested on one sample. Therefore multiple samples were used to obtain a full data set.

Four healthy and six patients PBMC samples were used in the MTS assays on whole PBMC, and a different four healthy PBMC samples were used for MTS assays on isolated immune cell populations.
Table 4-1 - Table showing the information for HNSCC-patients PBMC used for culture

<table>
<thead>
<tr>
<th>Patient PBMC</th>
<th>Gender</th>
<th>Age</th>
<th>TNM</th>
<th>Specimen site</th>
<th>Node or Tumour (N/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laryngeal origin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>Male</td>
<td>78</td>
<td>T4N3</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Sample 2</td>
<td>Male</td>
<td>?</td>
<td>T4N2c</td>
<td>Piriform fossa</td>
<td>T</td>
</tr>
<tr>
<td>Sample 3</td>
<td>Male</td>
<td>58</td>
<td>T4N2bM0</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Sample 4</td>
<td>Male</td>
<td>80</td>
<td>T1aN0</td>
<td>Vocal cord</td>
<td>T</td>
</tr>
<tr>
<td>Oropharyngeal origin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 5</td>
<td>Female</td>
<td>38</td>
<td>T2N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>Sample 6</td>
<td>Male</td>
<td>42</td>
<td>T2N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>Sample 7</td>
<td>Male</td>
<td>52</td>
<td>T1/T2N?M0</td>
<td>Tonsil</td>
<td>N</td>
</tr>
</tbody>
</table>
Table 4-2 - Table showing the source of HNSCC patient tissue used to collect the overnight dispersed tumour CM

<table>
<thead>
<tr>
<th>Overnight dispersed CM</th>
<th>Gender</th>
<th>Age</th>
<th>TNM</th>
<th>Specimen site</th>
<th>Node or Tumour (N/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Laryngeal origin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>Male</td>
<td>68</td>
<td>T3N2b/c</td>
<td>Epiglottis</td>
<td>N</td>
</tr>
<tr>
<td>Sample 2</td>
<td>Male</td>
<td>68</td>
<td>T3/T4N0</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Sample 3</td>
<td>Male</td>
<td>78</td>
<td>T4N3</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Sample 4</td>
<td>Male</td>
<td>78</td>
<td>T4N3</td>
<td>Larynx</td>
<td>N</td>
</tr>
<tr>
<td>Sample 5</td>
<td>Female</td>
<td>72</td>
<td>T4N2b</td>
<td>Larynx</td>
<td>N</td>
</tr>
<tr>
<td>Sample 6</td>
<td>Male</td>
<td>73</td>
<td>T1aN0</td>
<td>Vocal cord</td>
<td>T</td>
</tr>
<tr>
<td>Sample 7</td>
<td>Male</td>
<td>?</td>
<td>T4N2c</td>
<td>Piriform Fossa</td>
<td>T</td>
</tr>
<tr>
<td>Sample 8</td>
<td>Male</td>
<td>80</td>
<td>T1aN0M0</td>
<td>Vocal cord</td>
<td>T</td>
</tr>
<tr>
<td>Sample 9</td>
<td>Male</td>
<td>71</td>
<td>T4N0</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td><strong>Oropharyngeal origin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 10</td>
<td>Male</td>
<td>54</td>
<td>T1N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>Sample 11</td>
<td>Male</td>
<td>60</td>
<td>T3N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>Sample 12</td>
<td>Male</td>
<td>61</td>
<td>T1N2a</td>
<td>Tongue base</td>
<td>N</td>
</tr>
<tr>
<td>Sample 13</td>
<td>Male</td>
<td>48</td>
<td>T2N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>Sample 14</td>
<td>Male</td>
<td>63</td>
<td>T1N2b</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>Sample 15</td>
<td>Male</td>
<td>48</td>
<td>T1N2a</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>Sample 16</td>
<td>Male</td>
<td>57</td>
<td>T1N2b</td>
<td>Tongue base</td>
<td>T1</td>
</tr>
</tbody>
</table>

Whole healthy and HNSCC patient PBMC and isolated immune cell populations from healthy PBMC were plated out at 5 x 10⁴ cells/ wells before being left to incubate for 24 hours. Dynabeads® Human T-activator CD3/CD28 (Gibco, Life Technologies Ltd) using a ratio of 3 beads/ cell and IL-2 were added to each well in order to stimulate the cells to proliferate. One set of wells was left without stimulation in order to see the cells base activity.
HNSCC cell line, primary-derived fibroblasts and overnight dispersed tumour CM was added to each well in duplicate at a ratio of 1:3 with the media. Cells were left to incubate for 24, 48 and 72 hours before the MTS assays was performed as described in section 2.7. Initial optimisation experiments found that changes in proliferation occurred after 24 hours and continued until the cells became unviable after around 72 hours.

In order to determine whether the PBMC were reacting to the cytokines contained with the HNSCC-derived CM, and not the medium itself, some wells had extra medium added instead of the CM. This allowed the effect of the cytokines to become evident. All graphs used the same control data obtained from adding medium to patient and normal PBMC and assessing the proliferative activity.

Once raw data had been obtained from the plate reader they were processed by subtracting the media only results from all other well values so the colour of the MTS reagent was not included in the results.

Once the data had been processed, T tests were used to determine whether there were any significant differences in proliferation upon the addition of HNSCC-derived CM, between healthy/patient PBMC and between different incubation times.

4.2.2 - Recording immune cell population % change in whole PBMC upon the addition of HNSCC derived CM

Though showing a change in proliferative activity in individual immune cell populations can tell us some useful information, it is not necessarily the way they would react in a whole PBMC sample under the influence of other cell types. An experiment was devised to assess the change in the % of T effector cells CD4^+CD25^-, Treg cells CD4^+CD25^{int}CD127^{low} / CD4^+CD25^{hi}CD127^{low} and CD8^+ T cells within whole heathy PBMC upon the addition of HNSCC-derived CM.

Whole PBMC samples from healthy donors (n=4) were stained with CD4, C25 and CD127 and the % of T effector cells, Treg cells and CD8^+ T cells was recorded using the FACS Aria before the addition of HNSCC-derived CM. Cells were plated out and HNSCC-derived CM added in duplicate before being incubated for 48 hours. There
were not enough cells in patient samples to carry out this experiment, so it was decided to perform it on healthy PBMC alone.

Treated healthy PBMC were re-stained with CD4, CD25 and CD127 antibodies and analysed on the FACS Aria to record the % of T effector cells, Treg cells and CD8\(^+\) T cells post addition of HNSCC-derived CM. T tests were then used to assess whether there was any significant change in the percentage of T effector cells CD4\(^+\)CD25\(^-\), Treg cells CD4\(^+\)CD25\(^{int}\)CD127\(^{low}\) / CD4\(^+\)CD25\(^{hi}\)CD127\(^{low}\) and CD8\(^+\) T cells post HNSCC-derived CM treatment.

Results obtained were expressed as a percentage of the parent population. Treg cells and T effector cell are expressed as a percentage of the parent CD4\(^+\) population, whereas CD8\(^+\) T cells are expressed as a percentage of all lymphocytes in the sample (figure 4.1).

![Figure 4-1](image)

**Figure 4-1** - Example of the data received from the FACS Aria. The number Treg and T effector cells are taken as a percentage of the parent which consists of all CD4\(^+\) T cells which can be seen on the hierarchy. CD8\(^+\) T cells are taken as a percentage of all lymphocytes excluding all doublets. P1 is all lymphocytes and P2/P3 are plots drawn to exclude the doublets.
4.3 – Results

4.3.1 - The effect of HNSCC cell line and primary-derived fibroblast CM on the proliferation of whole PBMC from healthy controls and HNSCC patients

Conditioned medium (CM) from HNSCC cell lines and primary-derived fibroblasts cultured under both normoxic and hypoxic condition for 1 to 4 days was added to whole PBMC isolated from both healthy controls and HNSCC patients and the effect on the proliferative activity was assessed using the MTS assay.

4.3.1.1 - Does CM (from cell line and fibroblasts) cultured for a longer period of time produce a significant difference in proliferation of healthy/patient PBMC

The length of time which the cell lines or primary-derived fibroblasts were cultured for before collection of CM had no significant effect on the proliferative activity of either healthy or patients PBMC. Figure 4.2 shows representative data highlighting this.

![Figure 4.2](image-url)

**Figure 4.2** - Bar chart showing the effect of CM obtained from 4 different cell lines cultured under normoxic condition for 1 to 4 days on the proliferation of healthy PBMC. It is representative data showing that there is no significant difference seen from CM that has been cultured with cells for longer. T tests were used to determine whether there were any significant differences between data points.
As the length of cell line and primary-derived fibroblasts culture made no significant difference to PBMC proliferation, it was decided to display all results obtained from the addition of CM collected following 3 days of culture only, to correspond with data in previous and subsequent chapters.

4.3.1.2 - The effect of HNSCC-derived CM on the proliferation of healthy and patient PBMC

The effect of CM collected from cell lines and primary-derived fibroblasts cultured under both hypoxic and normoxic conditions was assessed using the MTS assay following 24, 48 and 72 hours of incubation with both healthy and HNSCC patient-derived PBMC. Following 48 and 72 hours of incubation significant increases were observed, compared to the medium only control, following the addition of CM to both healthy and patient-derived PBMC regardless of the origin of the CM or the oxygen tension they were cultured under (figure 4.3b & c). Similar results were found following 24 hours of incubation of the CM with healthy PBMC, except the increase seen in proliferation following incubation with fibroblast medium collected under normoxic conditions was not significantly different compared to the medium only control (figure 4.3a) and the only significant increase seen in proliferation following incubation of patient PBMC with CM for 24 hours was with the CM collected from HNSCC-derived fibroblasts under hypoxic conditions (figure 4.3a).
Bar charts showing the effect of CM obtained from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under hypoxic and normoxic conditions on the proliferative activity of healthy (n=4) and patient (n=4) PBMC after a) 24 hours, b) 48 hours and c) 72 hours of incubation. (Mean; ±SEM). * Significant difference \( p<0.05 \) when compared to the addition of medium alone.

**Figure 4-3** - Bar charts showing the effect of CM obtained from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under hypoxic and normoxic conditions on the proliferative activity of healthy (n=4) and patient (n=4) PBMC after a) 24 hours, b) 48 hours and c) 72 hours of incubation. (Mean; ±SEM). * Significant difference \( p<0.05 \) when compared to the addition of medium alone.
4.3.1.3 - Effect of MTS assay incubation length

PBMC were incubated with four independent cell line (n=4) and four different primary-derived fibroblast (n=4) CMs and medium only as a control. The cell lines and fibroblasts were cultured in both hypoxic and normoxic conditions for 24, 48 and 72 hours to determine whether length of exposure effected the level of proliferative activity of the cells from healthy (n=4) and patient (n=4) PBMC. T tests were used to determine whether there was a significant difference in proliferation after varying lengths of exposure (figure 4.4).

To ensure that the change in proliferative activity of PBMC over time wasn’t just the natural increase in cell number, a comparison was made between the activity of PBMC plus medium alone over 24, 48 and 72 hours (figure 4.4).

Upon the addition of medium alone, there was no change in activity in either healthy or HNSCC patient PBMC over time. A significant increase in proliferative activity was seen in healthy PBMC after 48 hour culture with CM obtained from cell lines cultured under normoxic and hypoxic conditions and HNSCC-derived fibroblasts cultured under normoxic conditions when compared to 24 hour incubation (figure 4.4a). In patient PBMC, a significant increase was seen upon the addition of CM from cell lines cultured under hypoxic conditions and fibroblasts cultured under normoxic conditions for 48 hours compared to those incubated for 24 hours (figure 4.4a & 4.4b). Following 72 hours of incubation, all CM from all origins caused a significant increase in proliferative activity of both healthy and patient PBMC when compared to the 24 hour incubation (figure 4.4e & 4.4f).

There was no significant difference seen in proliferative activity of patient PBMC between 48 and 72 hours upon the addition of any CM and only CM from HNSCC-derived fibroblasts cultured in both hypoxic and normoxic conditions caused a significant increase in healthy PBMC proliferation (figure 4.4c & 4.4d). For further sections, MTS assays were carried out after 48 and 72 hours as there was more stimulation after this time.
Figure 4-4 - Bar chart showing the change in proliferative activity of healthy (n=4) and patient (n=4) PBMC after the addition of CM from cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under hypoxic and normoxic conditions, between a) & b) 24 – 48 hours, c) & d) 48 – 72 hours and e) & f) 24 to 72 hours (mean: ±SEM). * Significant difference \( p<0.05 \) students paired t test.
4.3.1.4 - The effect of hypoxic culture conditions during the collection of HNSCC-derived CM on the proliferation of PBMC

The data was compared in order to determine whether hypoxia resulted in changes to the cell lines (n=4) and primary-derived fibroblasts (n=4) secretome which then goes on to influence the proliferation of PBMC upon co-culture. Paired student T tests were used to determine whether there was a significant difference.

The proliferative activity of healthy PBMC was significantly reduced following 48 hours of incubation with CM obtained from cell lines cultured under hypoxic conditions compared to those cultured in CM collected under normoxic conditions (figure 4.5a). The opposite was true upon the addition of CM obtained from primary-derived fibroblasts, where hypoxia derived CM caused a significantly greater increase in proliferative activity of healthy PBMC compared to the CM collected under normoxia. No further significant differences were observed in the proliferation of healthy PBMC following culture in CM collected from either cell lines or primary-derived fibroblasts under normoxic or hypoxic conditions. No significant differences were seen in the proliferative activity of HNSCC patient PBMC upon the addition of CM obtained from cell lines and primary-derived fibroblasts cultured under hypoxic condition when compared to CM obtained from normoxic conditions (figure 4.5b).

4.3.1.5 – Differences in the proliferative response of healthy and HNSCC patient PBMC between the addition of cell line and primary-derived fibroblasts CM

The only difference observed between the effect of cell line and primary-derived fibroblast CM on proliferation of healthy PBMC was a significant increase in the proliferation of healthy PBMC following 48 hours of incubation in CM obtained from cell lines cultured under normoxic conditions compared to the PBMC cultured in primary-fibroblast CM cultured under normoxic conditions (figure 4.5). No significant differences were observed between the addition of CM from cell line and primary-derived fibroblasts after 72 hours of incubation with healthy or HNSCC patients PBMC.
Figure 4-5 - Bar chart showing the effect of CM obtained from HNSCC cell lines and primary-derived fibroblasts cultured under normoxic and hypoxic conditions on the proliferative activity of a) healthy and b) patients PBMC after 48 and 72 hours of incubation. (Mean; ±SEM). *Significant difference $p<0.05$; paired student t test. Control used were the same for both patient and normal PBMC graphs.
4.3.1.6 - Differences between the proliferation of healthy vs patient PBMC following the addition of HNSCC cell line and primary-derived primary fibroblast CM

This section looks at whether healthy and patient PBMC proliferation is effected differently upon the addition of CM obtained from HNSCC cell lines and primary-derived fibroblasts cultured under both hypoxic and normoxic conditions.

Healthy PBMC had significantly greater levels of proliferation than HNSCC patient-derived PBMC after the addition of CM obtained from both HNSCC cell lines and primary-derived fibroblasts cultured under normoxic and hypoxic conditions (figure 4.6a & b). Referring back to Figure 4.3 which showed the basal proliferation levels of healthy and patient PBMC with medium alone after 48 and 72 hours, it can be seen that healthy PBMC had a slightly higher base level of proliferation. However, this has been taken into account when comparing proliferation of healthy and patient PBMC figures using the T test.
Figure 4-6 - Bar chart showing a comparison between the proliferative activity of healthy (n=4) and patient (n=4) PBMC incubated for a) 48 hours and b) 72 hours, after the addition of CM obtained from cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under normoxic and hypoxic conditions. (Mean; ±SEM). *Significant difference p<0.05; paired student t test.
4.3.2 - The effect of overnight dispersed HNSCC CM on the proliferation of whole PBMC from healthy controls and HNSCC patients

CM from overnight dispersed HNSCC was added to whole PBMC, harvested from the blood of both healthy controls (n=4) and HNSCC patients with either laryngeal (n=3) or oropharyngeal (n=3) tumours and the effect on proliferation was determined using the MTS assay following 24, 48 and 72 hours of incubation.

A significant increase in proliferation was seen between 24 and 48 hour incubation of both healthy PBMC and patient PBMC of laryngeal and oropharyngeal origin (figure 4.7 a, b & c) with all overnight CM tested.

There was a significant increase in proliferation of healthy PBMC (Figure 4.7a) and patient PBMC of laryngeal origin (figure 4.7b) between 24 and 72 hours of incubation upon the addition of all overnight CM and in patient PBMC of oropharyngeal origin (figure 4.7c) between 24 and 72 hours after the addition of oropharyngeal derived overnight CM but not laryngeal derived overnight CM.

There was no significant increase in proliferation of healthy PBMC and patient PBMC in between 48 and 72 hours of incubation after the addition of any overnight CM (figure 4.7a, b & c).
Figure 4-7 - Bar charts showing the change in proliferative activity of a) healthy PBMC (n=4) and patient PBMC of b) laryngeal (n=3) and c) oropharyngeal (n=3) origin, after the addition of CM from overnight CM dispersed tumour from laryngeal origin (n=12) and oropharyngeal origin (n=11) between 24 – 48 hours, 48 – 72 hours and 24 to 72 hours (mean: ±SEM). * Significant difference p<0.05. Student t test.
The difference in proliferation between PBMC treated with medium only and those treated with overnight dispersed tumour CM for 48 and 72 hours was determined using a paired student t test.

There was a significant increase in proliferation in both healthy and patient PBMC of laryngeal and oropharyngeal origin upon the addition of all overnight CM after 48 and 72 hours of incubation (figure 4.8a & b).

Figure 4-8 - Bar charts showing the effect of the addition of CM obtained from overnight dispersed tumour on the proliferative activity of healthy and patient PBMC after a) 48 hours and b) 72 hours of incubation. (Mean; ±SEM). * Significant difference *p<0.05 when compared to medium alone using students t test. ^ Significant difference between data sets.

4.3.3 - The effect of laryngeal vs oropharyngeal overnight CM on proliferative activity of healthy and patient derived PBMC

Overnight dispersed tumour CM was collected from tissue of laryngeal and oropharyngeal origin. To determine whether the overnight CM from different HNSCC
sites of origin has a differential effect on the proliferative activity of healthy and patient PBMC, data sets were compared and unpaired student t tests were used to assess significance.

Overnight CM of laryngeal and oropharyngeal origin had no differential effect on proliferative activity of healthy and patient PBMC after 48 hours of incubation (figure 4.8a) and only laryngeal derived overnight CM caused a significant increase in proliferative activity of laryngeal patient PBMC when compared to the effect of oropharyngeal derived overnight CM on the same patient samples after 72 hours of incubation (figure 4.8b).

4.3.4 - Effect of overnight dispersed tumour CM on the proliferative activity of healthy PBMC vs patient PBMC of oropharyngeal and laryngeal origin

To determine whether healthy (n=3) and patient PBMC reacted differently upon the addition of overnight dispersed tumour CM, data sets were compared and t tests were used to access significance. Patient PBMC was also divided into those collected from patients with laryngeal (n=3) and oropharyngeal (n=3) tumours to determine whether site of origin had any impact on the proliferative reaction of the PBMC to overnight CM.

Referring back to figure 4.8, the addition of all overnight CM caused a significant increase in proliferation of PBMC from both healthy and patients PBMC, so medium alone was not included on the following figure.

After 48 hours of incubation there was a significant difference in proliferation when comparing healthy PBMC and patient PBMC of oropharyngeal origin upon the addition of all overnight CM (figure 4.9a) with healthy PBMC having the greater levels of proliferation. There was also a significant difference between proliferative activity of patient PBMC of laryngeal and oropharyngeal origin upon the addition of all overnight CM with laryngeal derived PBMC having the greatest level of proliferation. No significant difference in the number of cells present after 48 hours was observed between healthy PBMC and patient PBMC of laryngeal origin upon the addition of overnight CM.
The same significant difference were observed following the addition of CM from oropharyngeal origin after 72 hours of incubation but no significant differences in proliferation were seen between PBMC of different origins following the addition of laryngeal derived CM (figure 4.9b).
4.3.5 – Lymphocyte depletion prior to collection of CM from dissociated tumours of both laryngeal and oropharyngeal origin and the effect on proliferation of healthy and patient PBMC

To determine the influence of lymphocyte secretions on the proliferation of both healthy and patient PBMC, four tumour samples were divided into two pieces with half being depleted of lymphocytes and the other half cultured as normal (section 2.2.2.1). The CM collected from both halves were added to healthy PBMC (n=4) and patient PBMC of laryngeal (n=3) and oropharyngeal (n=3) origin and incubated for 48 and 72 hours and an MTS assay performed.

Figure 4-9 - Bar chart showing the proliferative activity of healthy PBMC (n=4) vs patient PBMC of laryngeal (n=3) and oropharyngeal (n=3) origin after the addition of overnight CM of laryngeal (n=12) and oropharyngeal (n=11) origin after a) 48 and b) 72 hours of incubation. (Mean ±SEM). * Significant difference $p<0.05$; students unpaired t test.
In all cases the cells metabolic activity following the incubation of lymphocyte depleted CM with PBMC, regardless of origin, appeared to be lower, at the end of the incubation period than that of PBMC incubated with non-lymphocyte depleted CM, however the difference seen were not significant.

4.4 - Results

4.4.1 - The effect of HNSCC-derived CM on the proliferative activity of CD8+ T cells, T regulatory cells and T effector cells sorted from healthy PBMC

Healthy PBMC were sorted into CD8+ T cells, Treg cells and T effector cells (CD4+CD25−) before being cultured for 48 hours with CM from HNSCC cell lines and primary-derived fibroblasts collected following 3 days of culture under both normoxic and hypoxic conditions as well as overnight dispersed tumour CM before an MTS assay was used to determine metabolic activity as a surrogate for proliferation.

Due to the low cell numbers obtained from sorting patient PBMC (particularly T regulatory cells) it was decided to only work with healthy PBMC. This was also the reason for co-culturing with CM obtained from cell cultured over 48 hours only (excluding 24 and 72 hour cultures).

4.4.1.1 - The effect of HNSCC cell line and HNSCC fibroblast-derived CM on CD8+ T cell, T regulatory cell and T effector cell proliferation

CM was added to each sorted cell population isolated from 4 different healthy PBMC samples, however due to the large variation in metabolic activity observed between the sorted cells, samples were represented separately.

A lot of variation was observed in the proliferative activity of the sorted cells from different healthy PBMC samples, with the cells from healthy PBMC 2 and 3 having naturally higher levels of activity, particularly in the T effector cells when compared to samples 1 and 4 (figure 4.10). It was also evident on looking at the level of absorbance produced from CD8+ cells that some of these had up to a 2 fold lower level of proliferation (number of cells) following the 48 hour incubation period compared with that of the T effectors (CD4+CD25−).
In 1 of 4 healthy PBMC samples after the addition of medium alone, CD8⁺ levels were zero, possibly due to cell death. However after the addition of HNSCC derived CM, the cells appear to be alive and proliferating. Although the absorbance measured in the other three cones was higher than that seen from the CD8⁺ cells from PBMC 1 there were more cells present in the medium only controls as well so no significant differences were observed (figure 4.10a).

T regulatory cells from 2 of the 4 healthy PBMC samples had a greater number of cells present following the 48 hour incubation with CM from HNSCC cell lines and fibroblasts cultured under both normoxic and hypoxic conditions when compared to addition of medium alone, however the increase was not significant following the addition of the primary-derived fibroblast CM collected under normoxia.

A significant different was seen in the proliferation of T effector cells in 2 of 4 healthy PBMC samples upon the addition of CM from cell lines and primary fibroblasts cultured under hypoxic conditions (but not normoxic) when compared to addition of medium alone. There was also a significant difference in the proliferation of T effector cells in 1 of 4 healthy PBMC samples upon the addition of all HNSCC. No significant difference was seen in T effector cell proliferation in 2 of 4 sample upon the addition of all CM when compare to medium alone.

4.4.1.2 – The effect of CM origin and culture conditions on the proliferation of immune cells sorted from healthy PBMC

Neither the origin of the CM (HNSCC cell line or fibroblast) or hypoxia had any influence on the proliferative activity of sorted T cells from healthy PBMC.
Figure 4-10 - Bar charts showing the effect of CM obtained from HNSCC cell lines (n=4) and HNSCC primary-derived fibroblasts (n=4) cultured under normoxic and hypoxic conditions on the proliferation of a) CD8$^+$ T cells, b) T regulatory cells and c) T effector cells (sorted from healthy PBMC; n=4; Mean ± SEM). * Significant difference p<0.05 when compared to medium alone; students paired test.
4.4.1.3 - The effect of CM collected from overnight dispersed HNSCC samples on the proliferation of CD8\(^+\) T cells, T regulatory cell and T effector cell from healthy PBMC

CM obtained from overnight dispersed tumour culture, was cultured with CD8\(^+\) T cells, T regulatory cells and T effector cells from healthy PBMC for 48 hours before an MTS assays was performed to assess immune cell metabolic activity. Due to limited stocks of overnight dispersed tumour CM, only one healthy PBMC sample (PBMC 1) could be investigated. Overnight dispersed tumour CM was divided into that harvested from tumours of laryngeal origin (n=3) and those harvested from oropharyngeal origin (n=5).

The addition of CM, obtained from the overnight dispersal of laryngeal and oropharyngeal tumours, to CD8\(^+\) T cells and T effector cells (CD4\(^+\)CD25\(^-\)) resulted in a significant increase in cell number/proliferation when compared to the addition of medium alone (figure 4.11). There was no significant difference in proliferation of Treg cells upon the addition of overnight dispersed tumour CM when compared to medium alone.

The origin of overnight dispersed tumour CM (laryngeal and oropharyngeal) had no differential effect on the proliferative activity of CD8\(^+\) T cells, Treg cells or T effector cells from healthy PBMC (figure 4.11).

![Figure 4-11](image.png)

**Figure 4-11** - Bar chart showing the effect of CM collected from overnight dispersal of laryngeal (n=3) and oropharyngeal (n=5) tumours on the proliferative activity of CD8\(^+\) T cells, T regulatory cells and T effector cells from healthy PBMC (n=1) after 48 hours of incubation. (Mean ±SEM). * Significant difference p<0.05 when compared to medium alone; students paired t test.
4.5 - The effect of HNSCC derived cell line and primary fibroblasts CM on the levels of immune cell in healthy PBMC samples

In section 4.4, Treg cells, T effector cells and CD8+ T cells were physically separated from each other before the effects of HNSCC derived CM on their proliferation was assessed. However, this may not be the way the immune cells would react within a whole PBMC population, surrounded by other cell types. The percentage of immune cells was assessed pre-incubation to get an idea of the initial composition of the healthy PBMC. Healthy whole PBMC was then incubated alongside HNSCC CM and the percentage of Treg cells, T effector cells and CD8+ T cells was assessed using FACS. Medium only was also added to the PBMC in order to ensure any differences seen were due to the cytokine released by the cells rather than the contents of the medium.

CM from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under both normoxic and hypoxic condition was added to whole PBMC to assess their impact on the percentage of Treg cells, T effector cells and CD8+ T cells within healthy PBMC. Each healthy PBMC sample was kept as an individual data set due to their very different initial compositions, so giving the best idea of how each sample reacts to the CM. T tests were used to assess whether there was significant difference between data sets.

4.5.1 - The effect of HNSCC cell line and primary fibroblasts CM on CD8+ T cell percentage in healthy PBMC

CD8+ T cell percentages were assessed pre-incubation and post-incubation with medium only and CM from HNSCC cell line and primary-fibroblast cultured under both normoxic and hypoxic conditions.

Pre-incubation percentages of CD8+ T cells ranged from 11-20.8%. There was a significant difference between the percentages of CD8+ T cells between pre-incubation and post-incubation with medium in all four PBMC samples. Two of four PBMC samples had a decrease in the percentage of CD8+ T cells (figure 4.12b & c), whereas the other two samples had an increase in the percentage of CD8+ T cells (figure 4.12a & d). Post-incubation of CD8+ T cells with medium ranged from 7.45-18.9%.
Upon comparing data from pre-incubation and that of the addition of HNSCC derived CM, all CM caused a significant difference. This followed the same pattern as seen with the medium alone with two of four PBMC samples showing a decrease in the percentage of CD8+ T cells (figure 4.12b, c), and the other two samples had an increase in the percentage of CD8+ T cells (figure 4.12a, d).

However, when comparing the difference between the addition of medium and HNSCC CM, there was only significant difference in three occasions. There was a significant increase in the percent of CD8+ T cells upon the addition of CM obtained from primary-derived fibroblasts cultured under normoxic (from 16.4% to 17.63%) and hypoxic (from 16.4% to 18.23%) conditions when compared to the addition of medium alone in one out of four PBMC samples (figure 4.12b).

The addition of CM obtained from primary-derived fibroblasts cultured under normoxic condition caused a significant increase in CD8+ T cell percentage when compared to medium alone (from 7.5% to 8.83%). In both cases the % of CD8+ T cells was still significantly less than pre-incubation percentages (figure 4.12c). In all other cases there was no significant difference in CD8+ T cell percentage upon the addition of HNSCC CM when compared to medium alone.

In one PBMC sample there was a significant difference in CD8+ T cell percentage between the addition of CM from cell line and primary-derived fibroblasts cultured under normoxic conditions (figure 4.12c) with primary-derived fibroblast CM causing the greater % of cells at 8.83% compared to 7.33% from cell line CM.

No other significance was seen between cell line and primary-derived fibroblasts CM and no significance difference was seen between CM obtained from cells cultured under normoxic and hypoxic conditions.
Figure 4-12 – Bar chart showing the effect of CM from cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under normoxic and hypoxic conditions on the percentage of CD8+ T cells from healthy PBMC samples 1, 2, 3 & 4 (a, b, c & d). Each CM was added to each PBMC sample in singlecate.

*significant difference when comparing the addition of HNSCC CM to medium alone

^ Significant difference when comparing the addition of HNSCC and fibroblast CM and medium alone to pre-incubation results

*significant difference between highlighted data
4.5.2 - The effect of HNSCC cell line and primary fibroblasts CM on T regulatory cell percentage in healthy PBMC

Treg cell percentages were assessed pre-incubation and post-incubation with medium and cell line and primary-derived fibroblast CM.

Pre-incubation percentages of Treg cells ranged from 8.15-17.7% and post-incubation upon the addition of medium ranged from 10.6-18.35%. There was a significant difference in % of Treg cells between pre-incubation and post-incubation with the addition of medium in two out of four healthy PBMC samples. In both cases there was an increase in the % of Treg cells with around a 2% increase (figure 4.13b & c).

There was a significant increase in Treg cell % in four out of four PBMC samples when comparing the pre-incubation levels and post-incubation with all HNSCC derived CMs (figures 4.13a, b, c & d). There was up to a two-fold increase in Treg cell % when compared to pre-incubation upon the addition of some CM, in particular CM obtained from primary-derived fibroblasts. When comparing the difference in Treg cell % post-incubation upon the addition of medium and HNSCC derived CM there was in general an increase. There was a significant increase in Treg cell % in four out of four PBMC samples upon the addition of CM from primary-derived fibroblasts cultured under both normoxic (average increase of 15%) and hypoxic conditions (average increase of 15.1%; figure 4.13a, b, c & d). There was also a significant increase in Treg cell % upon the addition of CM from cell lines cultured under hypoxic condition in four out of four samples (average increase of 8.8%) and cultured under normoxic conditions in two out of four PBMC samples (average increase of 7.4%; figure 4.13a, b, c & d). There was a significant difference between the % of Treg cells upon the addition of CM from cell lines and primary-derived fibroblasts cultured under normoxic conditions in three out of four PBMC samples (figure 4.13a, c & d). The addition of normoxic primary-derived fibroblast CM caused on average an 11.2% increase in Tregs when compared to the addition of normoxic cell line CM. There was also a significant increase in Treg cell % upon the addition of hypoxic primary-derived fibroblasts CM (8.4% higher on average) when compared to the addition of hypoxic cell line CM in two out of four PBMC samples (figure 4.13a & d).
No significant difference was seen between CM obtained from cell lines and primary-derived fibroblasts cultured under normoxic and hypoxic conditions.
Figure 4-13 - Bar chart showing the effect of CM from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under normoxic and hypoxic conditions on the percentage of Treg cells from healthy PBMC samples 1, 2, 3 & 4 (a, b, c & d). Each CM was added to each PBMC sample in singlecate.

*significant difference when comparing the addition of HNSCC CM to medium alone

^ Significant difference when comparing the addition of HNSCC and fibroblast CM and medium alone to pre-incubation results

"significant difference between highlighted data
4.5.3 - The effect of HNSCC derived cell line and primary-derived fibroblasts CM on T Effector cell percentage in healthy PBMC

T effector cell percentage was assessed pre-incubation and post-incubation with medium only and CM from HNSCC cell lines and primary-derived fibroblasts.

Pre-incubation percentages of T effector cells ranged from 30.35-47.6% and post-incubation upon the addition of medium ranged from 32.33-53.05%. There was a significant difference in % of T effector cells between pre-incubation and post-incubation with the addition of medium in three out of four healthy PBMC samples. There was an average 9% increase of T effector cells after incubation with medium compared to pre-incubation (figure 4.14b & c).

There was a significant increase in T effector cell % in three out of four PBMC samples when comparing the pre-incubation levels and post-incubation with all HNSCC derived CMs (figures 4.14a, b & d). In the fourth PBMC sample only CM obtained from primary-derived fibroblasts cultured under hypoxic conditions caused a significant increase in T effector cell % when compared to pre-incubation (figure 4.14c).

Next, the difference in T effector cell % post-incubation upon the addition of medium and HNSCC derived CM there was assessed. There was a significant increase in T effector cell % in three out of four healthy PBMC samples upon the addition of CM from primary-derived fibroblasts cultured under both normoxic (average increase of 6.7%) and hypoxic conditions (average increase of 8.3%; figure 4.14a, b & d). There was also a significant increase in T effector cell % upon the addition of CM from cell lines cultured under hypoxic condition in one out of four samples (increase of 5.4%) and cultured under normoxic conditions in two out of four PBMC samples (average increase of 5.6%; figure 4.14b).

No significant difference was seen in % of T effector cells when comparing the results obtained from the addition of cell line and fibroblast CM.

No significant difference was seen between CM obtained from cell lines and primary-derived fibroblasts cultured under normoxic vs hypoxic conditions.
Figure 4-14 - Bar chart showing the effect of CM from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under normoxic and hypoxic conditions on the percentage of T effector cells from healthy PBMC samples 1, 2, 3 & 4 (a, b, c & d). Each CM was added to each PBMC sample in singlecate.

* significant difference when comparing the addition of HNSCC CM to medium alone

^ Significant difference when comparing the addition of HNSCC and fibroblast CM and medium alone to pre-incubation results

* significant difference between highlighted data
4.5.4 – The effect of HNSCC-derived overnight dispersed tumour CM on the percentage of immune cells in healthy PBMC

The percentage of CD8⁺ T cells, Treg cells and T effector cells within healthy PBMC was assessed pre-incubation and post-incubation with medium only and CM from overnight dispersed tumour. Due to lack of samples, it was not possible to use the same samples as in previous sections. A small selection of the remaining samples was used to ensure that each cell type was exposed to at least 5 different CMs from overnight dispersed tumour. There were not enough samples to compare the effect of sub-site in these examples so all data was collated and averaged. All samples were still of just oropharyngeal and laryngeal origin. Samples are listed in table 4.3.

Table 4.3 - Showing the patients information for the overnight dispersed tumour CM used in this section

<table>
<thead>
<tr>
<th>Overnight dispersed tumour sample</th>
<th>Gender</th>
<th>Age</th>
<th>TNM</th>
<th>Specimen site</th>
<th>Node or Tumour (N/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>Female</td>
<td>70</td>
<td>T3N1</td>
<td>Tonsil</td>
<td>N</td>
</tr>
<tr>
<td>Sample 2</td>
<td>Male</td>
<td>54</td>
<td>?</td>
<td>Tonsil</td>
<td>T</td>
</tr>
<tr>
<td>Sample 3</td>
<td>Male</td>
<td>84</td>
<td>T2N2c</td>
<td>Tongue base</td>
<td>N</td>
</tr>
<tr>
<td>Sample 4</td>
<td>Male</td>
<td>62</td>
<td>T1bN0M0</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Sample 5</td>
<td>Female</td>
<td>65</td>
<td>?</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Sample 6</td>
<td>Male</td>
<td>70</td>
<td>T4N0</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Sample 7</td>
<td>Male</td>
<td>73</td>
<td>T1N0</td>
<td>Glottis</td>
<td>T</td>
</tr>
<tr>
<td>Sample 8</td>
<td>Female</td>
<td>79</td>
<td>T4N0</td>
<td>Larynx</td>
<td>T</td>
</tr>
</tbody>
</table>

4.5.4.1 – The effect of CM from overnight dispersed tumour on the percentage of CD8⁺ T cells in PBMC obtained from healthy donors

There was a significant decrease in the % of CD8⁺ T cells in the lymphocyte fraction of all four healthy PBMC upon the addition of overnight dispersed tumour CM when compared to pre-treatment levels and the post-treatment with medium alone (figure 4.15). The percentage fell by 1/2 in two samples and by 3/4 in the other two, with one
sample going from 16.5% CD8\(^+\) T cells post-treatment with medium to 4.9% upon treatment with overnight dispersed tumour CM (figure 4.15).

**Figure 4-15** – Bar chart showing the effect of CM from overnight dispersed tumour CM (n=5) on the percentage of CD8\(^+\) T cells from healthy PBMC samples (n=4). Each individual CM was added to the cells in singleate.

*significant difference when comparing the addition of overnight dispersed tumour CM to medium alone.

^ Significant difference when comparing the addition of overnight dispersed tumour CM to pre-incubation.

**4.5.4.2 – The effect of CM from overnight dispersed tumour on the percentage of T regulatory cells in PBMC obtained from healthy donors**

There was a significant increase in the % of Treg cells in the CD4\(^+\) T cell fraction of healthy PBMC upon the addition of overnight dispersed tumour CM in three out of four samples when compared to pre-treatment levels and the post-treatment with medium alone (figure 4.16). In one out of four samples (Healthy PBMC 3) there was significant increase in the % of Treg cells upon the addition of overnight dispersed tumour CM compared to the addition of medium alone but not compared to pre-incubation levels.
The percentage of Treg cells in healthy PBMC 1 and 4 doubled, going from 18% to 40% of all CD4\(^+\) T cells in one and 11.4% to 23% in the other.
4.5.4.3 – The effect of CM from overnight dispersed tumour on the percentage of T effector cells in PBMC obtained from healthy donors

There was a significant increase in the % of T effector cells in the CD4+ T cell fraction of healthy PBMC upon the addition of overnight dispersed tumour CM in all four samples when compared to pre-treatment levels and the post-treatment with medium alone (figure 4.17). The increase in T effector cells upon the addition of overnight dispersed tumour CM was not as pronounced as with Treg cells with an increase in percentage of between 7 and 12.2% as levels of T effector cells were at a higher parentage to begin with.
Figure 4-17 - Bar chart showing the effect of CM from overnight dispersed tumour CM (n=5) on the percentage of T effector cells from healthy PBMC samples (n=4). Each individual CM was added to the cells in singlecate.

*significant difference when comparing the addition of overnight dispersed tumour CM to medium alone

^ Significant difference when comparing the addition of overnight dispersed tumour CM to pre-incubation
4.6 – Discussion

Immune cells are known to infiltrate solid tumours from the periphery where they will come into contact with soluble factors released by cells within the TME such as cancerous epithelial cells, cancer-associated fibroblasts and other immune cells (Fridman et al., 2012). The presence and number of different immune cells within the TME can be different between different types of cancer and even between patients with the same disease (Fridman et al., 2013). With this in mind, the presence of immune cells within the TME has been used in an attempt to predict patient outcome, but due to such variability has not been transferred to a clinical setting. Although T effector cells and CD8+ T cells are present in the TME, there appears to be an inability to prevent tumour progression suggesting that these infiltrated cells may have an impaired functionality or that their numbers are reduced relative to those of suppressive T cells. It is thought that an increased number of Tregs and immunosuppressive factors within the TME are responsible for suppression, causing CD4+ T effectors and CD8+ T cells to become un-reactive (Strauss et al., 2007).

There are many different reactions occurring within the TME and it is an ongoing topic as to the exact mechanisms driving immune-suppression. In the current chapter, the effect of HNSCC derived soluble factors on the proliferation and relative numbers of immune cells was assessed. Immune cells from peripheral blood of both HNSCC patients and from healthy donors were utilised to determine if those from patients had been “pre-conditioned” by the presence of tumour.

4.6.1 – The effect of HNSCC-derived CM on the proliferation of whole PBCMC and isolated immune cell populations

Whole PBMC from both healthy individuals and HNSCC patients were exposed to HNSCC-derived CM in order to determine whether the soluble factors within the CM had the ability to alter the proliferative activity of PBMC as a whole. The hypothesis was that soluble factors released from HNSCC derived cells would inhibit the proliferation of immune cells with less inhibition being observed in the patient PBMC which had already been pre-primed by the tumour. Following this, the effect of the CM on the proliferation and relative numbers of individual immune cell populations was investigated with the hypothesis that in order to induce immune suppression the soluble
factors released by the tumour differentially affected the immune cell populations by inhibiting the proliferation of CD4\(^+\) T effector cells and CD8\(^+\) cytotoxic cells whilst concurrently enhancing the proliferation/expansion of Treg cells. CM collected from different cellular fractions within the tumour microenvironment was compared for their effect on proliferation to determine whether a specific cell type might be responsible for the effects seen.

### 4.6.1.1 – The effect of HNSCC-derived CM on the proliferation of whole PBCMC

The current project found surprisingly an increase in the proliferation of whole PBMC from both healthy and patient donors upon culture for 48 and 72 hours with HNSCC-derived CM from all sources (HNSCC cell lines, fibroblasts and overnight dispersed tumour) compared to addition of medium alone. This is in disagreement with the original hypothesis which predicted a reduction in PBMC proliferation. As this was a mixture of immune cells, it could have been that some populations were decreasing but this is masked by an increase in other populations.

In all instances the HNSCC-derived CM (from all sources) when cultured with PBMC for 48 and 72 hours induced a significantly higher level, up to twice as much in some cases, of proliferation in healthy PBMC when compared to that of patient PBMC. This suggests that the patient PBMC had already been influenced by the presence of tumour, proving part of the hypothesis to be true. Both patient and healthy PBMC were plated out at the same concentration and had very similar baseline levels of proliferation, therefore the difference in proliferation observed upon the addition of HNSCC-derived CM can be attributed to the soluble factors within the CM.

Previous studies have found that certain immune cell populations are found at higher levels in the periphery of cancer patients, such as Treg cells compared to healthy individuals (Ormandy et al., 2005; Wolf et al., 2003; Woo et al., 2001), which might be as a result of exposure to tumour derived circulating factors.

Although an overall increase in PBMC proliferation was observed following the addition of all HNSCC CM, it could not be assumed that all populations within the PBMC were increasing at the same rate if at all. Past work has shown that CD4\(^+\) T effector cells and CD8\(^+\) T cells within the tumour microenvironment of various cancers
are present in lower numbers compared to normal tissue and have reduced function compared to Tregs which have expanded to further suppress the effector populations (Wolf et al., 2003). With this in mind, it was decided to isolate three immune cell populations (CD4$^+$ T effector cells, CD4$^+$/CD25$^+$CD127$^{lo}$ Treg cells and CD8$^+$ T cells) and repeat the experiment with the hope of finding out whether the different immune cells within the PBMC were affected differentially by the HNSCC derived CM. It was hypothesised that T effectors and CD8$^+$ T cell would show lower levels of proliferation upon culture with HNSCC-derived CM compared to the addition of medium alone and that Treg cells would show increased levels of proliferation. In this case the individual immune cell populations were only obtained from healthy PBMC. Unfortunately there were not enough cells within HNSCC patient PBMC samples to conduct a comparison as with the previous section.

4.6.1.2 – The effect of HNSCC-derived CM on the proliferation of CD8$^+$ T cells

Upon the addition of all CM from HNSCC cell lines and primary-derived fibroblasts cultured under both normoxic and hypoxic conditions to CD8$^+$ T cells, there was a significant increase in proliferation in only one out of four samples compared to the addition of medium alone. However, the sample that showed an increase in proliferation, following the addition of CM, showed no activity upon the addition of medium alone therefore it is suspected that the increase seen may have been an artefact of this. In contrast the addition of CM from overnight dispersed tumour did cause an increase in CD8$^+$ T cells, suggesting that other cell types, apart from the epithelial cells and the fibroblasts, such as TILs within the TME may be contributing to the proliferative activity of CD8$^+$ T cells. Although this data indicates there is not a change in the number of isolated CD8$^+$ T cells in response to HNSCC CM it was thought that there could be a change in the ratio of CD8$^+$ T cells compared to other populations within whole PBMC. In addition the cells within the PBMC population could influence the activity of the other cells and for this reason it was decided to look at the effect of HNSCC-derived CM on the percentage of CD8$^+$ T cells within the PBMC. This way the cell to cell communications within the PBMC remain intact, whilst being able to look at how the ratios of cells change. A comparison of data sets for the effect of HNSCC CM on CD8$^+$ T cells measured using both methods can be observed in Table 4.4.
One out of four CD8\(^+\) T cell populations showed a decrease in percentage within healthy PBMC upon the addition of CM from primary-fibroblasts cultured in hypoxia and two of four showed a decrease in percentage upon the addition of CM from fibroblasts cultured in normoxia. The addition of all overnight dispersed tumour CM resulted in significant decrease in the percentage of CD8\(^+\) T cell in all four samples. This conflicts with data obtained from the proliferation assays where when an effect was seen it was an increase in proliferation rather than a decrease. These data suggest that either the presence of other immune cells within the PBMC may be influencing the levels of CD8\(^+\) T cells in response to the CM or that the CM is stimulating the proliferation of all the immune cells but some more than others so it appears that the percentage of the CD8\(^+\) T cells are reduced. The presence of Tregs in whole PBMC may go some way to explaining the difference as they are capable of suppressing CD8\(^+\) T cells (Vignali et al., 2008). It is interesting that CM from overnight dispersed HNSCC tumour caused a decrease in the percentage of CD8\(^+\) T cells in all four samples as opposed to cell line and fibroblast CM which only effected one out of four samples. It may be that other cells within the tumour such as TILs are releasing soluble factors which can affect the proportion of CD8\(^+\) T cells within PBMC.
Table 4-4 - Summary of the differences in data obtained from the addition of HNSCC-derived CM on the proliferation of CD8⁺ T cells in isolation and percentage change of CD8⁺ T cells within whole PBMC

<table>
<thead>
<tr>
<th>HNSCC derived CM origin</th>
<th>Isolated CD8⁺ T cell proliferation</th>
<th>CD8⁺ T cell % in healthy PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines cultured under normoxia</td>
<td>¼ samples showed increase in proliferation and ¾ had no change</td>
<td>4/4 samples showed no difference in %</td>
</tr>
<tr>
<td>Cell lines cultured under hypoxia</td>
<td>¼ samples showed increase in proliferation and ¾ had no change</td>
<td>4/4 samples showed no difference in %</td>
</tr>
<tr>
<td>Primary-derived fibroblasts cultured under normoxia</td>
<td>¼ samples showed increase in proliferation and ¾ had no change</td>
<td>2/4 samples showed decrease in % and 2/4 had no change</td>
</tr>
<tr>
<td>Primary-derived fibroblasts cultured under hypoxia</td>
<td>¼ samples showed increase in proliferation and ¾ had no change</td>
<td>1/4 samples showed decrease in % and 3/4 had no change</td>
</tr>
<tr>
<td>Laryngeal and oropharyngeal overnight dispersed tumour</td>
<td>Increase in proliferation</td>
<td>4/4 samples showed decrease in %</td>
</tr>
</tbody>
</table>

These data suggest that the effect of the tumour-derived CM within the TME may depend on the interaction of immune cells. In isolation the CD8⁺ T cells were able to proliferate freely which would have conferred a more anti-tumour environment. However, when in contact with other immune cells within the PBMC, the proportion of CD8⁺ T cells decreased conferring a more pro-tumour environment. As the effect of the HNSCC-derived CM on CD8⁺ T cells was compared to the addition of medium alone in
both experiments, it was shown that soluble factors alone and cell to cell contact alone could affect CD8$^+$ T cells, but it was only in tandem that they were able to create the more immunosuppressive reduction of CD8$^+$ T cells.

CD8$^+$ T cells have been found within various tumours, specifically at the invasive margin and in the core (Fridman et al., 2012). They are able to mount an anti-tumour response in the presence of CD4$^+$ T effector cells, however, within the TME they fail to prevent the progression of cancer (Gooden et al., 2011). One study looked at the number of CD8$^+$ T cells in the periphery and tumours of breast cancer patients and found that there was no difference in the number of CD8$^+$ T cells in the peripheral blood compared to healthy controls, but there was a significant increase in the number of CD8$^+$ T cells within the tumour compared to adjacent tissue (Rad et al., 2015).

CD8$^+$ T cells have been found at varying levels within the TME of different cancers. Balermpas et al (2016) looked at CD8$^+$ T cell levels in the TME of HNSCC and found that 59.6% of patients had low levels and 40.4% had high levels, with the high levels being found mainly in patients with earlier stage disease with the number decreasing as the tumour progressed. The study also found that high numbers of CD8$^+$ T cells were related to an improved survival. This is in agreement with other cancer types in which better survival has been correlated with a higher number of CD8$^+$ T cells within the tumour nest (Naito et al., 1998 – colorectal cancer; Schumacher et al., 2001 – Esophageal carcinoma). This suggests that as the CD8$^+$ T cells are exposed to the tumour environment for longer, they proliferate and/or migrate to a lesser extent. In this chapter the percentage of CD8$^+$ T cells decreased in all samples following the addition of overnight dispersed tumour CM suggesting that there is something secreted from the tumour microenvironment which can reduce the proportion of CD8$^+$ T cells.

Many papers stress that the absolute numbers of CD8$^+$ T cells alone are not as important as the ratio of CD8$^+$ T cells to the number of Tregs (Gooden et al., 2011). In the current study the ratio of CD8$^+$ T cells reduces whilst Tregs percentages increased within whole PBMC. Fu et al (2007) found that with increasing numbers of Tregs there was a concomitant reduction in CD8$^+$ T cells infiltrating hepatocellular carcinoma suggesting that Tregs are able to reduce the activity of CD8$^+$ T cells within the TME, but not necessarily the proliferation. Unitt et al (2005) did discover that Tregs were able to
suppress the proliferation of autologous CD8\(^+\) T cells from hepatocellular carcinoma patients. As this study found increased numbers of Tregs and lower levels of CD8\(^+\) T cells upon the addition of overnight dispersed tumour CM it might be that the Tregs present in the whole PBMC population could reduce the proliferation of the CD8\(^+\) cells however further studies would be needed to confirm this.

4.6.1.3 – The effect of HNSCC-derived CM on the proliferation of T regulatory cells

Results are summarised in table 4.5. Two out of four Treg populations from healthy PBMC showed an increased level of proliferation upon the addition of CM from cell lines cultured under normoxia and hypoxia and primary-derived fibroblasts cultured under hypoxia compared to the addition of medium alone. In both samples, the activity of Tregs went up two fold compared to medium alone. CM obtained from fibroblasts cultured under normoxia failed to increase proliferation of any Treg populations and two Treg populations were not stimulated to proliferate by any CM. In contrast to the effect on the CD8\(^+\) cells CM from overnight dispersed tumour CM caused no significant difference in proliferation of Tregs compared to the addition of medium alone.

The percentage of Tregs within the whole PBMC reflected the proliferation data upon the addition of HNSCC-derived CM in that both caused an increase in Treg. However, the CM from cell lines and primary-derived fibroblasts cultured in hypoxia were able to increase the percentage of Tregs in all PBMC samples rather than two out of four seen when Tregs were in isolation. The addition of CM from overnight dispersed tumour caused an increase in the percentage of Tregs in whole PBMC whereas no difference in proliferation was seen when Tregs were in isolation.

This suggests that the cell line and fibroblast derived soluble factors are able to increase the proliferation of Tregs in isolation but when other immune cells are present, the levels of Tregs are further increased.
Table 4-5 - Summary of the differences in data obtained from the addition of HNSCC-derived CM on the proliferation of Tregs in isolation and percentage change of Tregs within whole PBMC

<table>
<thead>
<tr>
<th>HNSCC derived CM origin</th>
<th>Isolated T regulatory cell proliferation</th>
<th>T regulatory cell % in healthy PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines cultured under normoxia</td>
<td>2/4 samples showed increase in proliferation and 2/4 had no change</td>
<td>2/4 samples showed increase in % and 2/4 had no change</td>
</tr>
<tr>
<td>Cell lines cultured under hypoxia</td>
<td>2/4 samples showed increase in proliferation and 2/4 had no change</td>
<td>4/4 samples showed increase in %</td>
</tr>
<tr>
<td>Primary-derived fibroblasts cultured under normoxia</td>
<td>4/4 samples showed no change</td>
<td>4/4 samples showed increase in %</td>
</tr>
<tr>
<td>Primary-derived fibroblasts cultured under hypoxia</td>
<td>2/4 samples showed a significant increase in proliferation and 2/4 showed no change</td>
<td>4/4 samples showed increase in %</td>
</tr>
<tr>
<td>Laryngeal and oropharyngeal overnight dispersed tumour</td>
<td>No change in proliferation</td>
<td>4/4 samples showed an increase in %</td>
</tr>
</tbody>
</table>

Tregs have been found at increased levels in both the periphery and TME of cancer patients compared to healthy controls (Unit et al., 2005; Sasada et al., 2003; Liyange et al., 2002). Both Liyange et al (2002) and Sasada et al (2003) found that the level of Tregs in the peripheral circulation of pancreatic, breast and gastric carcinoma was significantly greater in patients than in healthy controls. It was also found that levels of Tregs in the TME was significantly increased compared to non-cancer tissue in pancreas, breast and hepatocellular carcinoma (Unit et al., 2005; Liyange et al., 2002). An increased level of Tregs within the TME is thought to suggest a more immune-
suppressive environment which leads to a poor prognosis in cancers such as breast, gastric and ovarian (Facciabene et al., 2011; Sasada et al., 2003). However, in some cancers the increase in Tregs appears to be correlated to better survival. This may be due to the concomitant increase in T effector cells seen as Treg number rise (Facciabene et al., 2011), however, it is widely accepted that Tregs play a suppressive role within the TME. The exact mechanisms behind this increase in Tregs has yet to be pinpointed, but it is thought to be a combination of Tregs infiltrating tumour in large numbers and/or the expansion of Tregs within the tumour (Facciabene et al., 2011). The current study found that the majority of HNSCC-derived soluble factors were able to increase the numbers and proliferation of Tregs which suggests that the TME is able to directly alter Treg numbers as well as attract them from surrounding tissues. CCL22 is up-regulated within tumour masses and has been found to be a chemoattractant for Treg cells into the TME, though it has also been found that the TME continues to cause the expansion of Tregs once within the confines of the tumour (Facciabene et al., 2011). The main cytokines thought to be responsible for the expansion of Tregs within the TME are IL-10 and TGF-β both of which were identified within the CM obtained from HNSCC sources (Facciabene et al., 2011; Valzasina et al., 2006).

In this chapter, the aim was to try and take healthy Tregs and expose them to the soluble factors they might encounter within the TME. Although not consistent, in general there was an increase in proliferation of Tregs upon the addition of HNSCC soluble factors, suggesting that the tumour can induce the development of an immunosuppressive environment via the expansion of Tregs or by the inhibition of the other anti-tumour cell types, either directly or via the activation of the Treg cells.

4.6.1.4 – The effect of HNSCC-derived CM on the proliferation of T effector cells

In one out of four T effector cell populations from healthy PBMC, there was an increase in proliferation upon the addition of CM from cell lines and primary-fibroblasts cultured under normoxic conditions compared to medium alone. In two out of four T effector samples, there was an increase in proliferation upon the addition of CM from HNSCC cell lines and primary-derived fibroblasts cultured under hypoxic conditions compared to the addition of medium alone. The addition of overnight dispersed tumour CM
caused a massive increase in the proliferation of T effector cells compared to medium alone with the activity going up more than four fold.

The proportion of T effectors within healthy PBMC was assessed and compared with the proliferation data (table 4.6).

**Table 4-6** - Summary of the differences in data obtained from the addition of HNSCC-derived CM on the proliferation of T effector cells in isolation and percentage change of T effector cells within whole PBMC

| Significant observations upon the addition of HNSCC-derived CM compared to medium alone |
|---------------------------------|------------------|------------------|
| **HNSCC derived CM origin**     | **Isolated T effector cell proliferation** | **T effector cell % in healthy PBMC** |
| Cell lines cultured under normoxia | 1/4 samples showed an increase in proliferation and 3/4 showed no change | 1/4 samples showed an increase in % and 3/4 showed no change |
| Cell lines cultured under hypoxia | 2/4 samples showed an increase in proliferation and 2/4 showed no change | 1/4 samples showed an increase in % and 3/4 showed no change |
| Primary-derived fibroblasts cultured under normoxia | 1/4 samples showed an increase in proliferation and 3/4 showed no change | 3/4 samples showed an increase in % and 1/4 showed no change |
| Primary-derived fibroblasts cultured under hypoxia | 2/4 samples showed an increase in proliferation and 2/4 showed no change | 3/4 samples showed an increase in % and 1/4 showed no change |
| Laryngeal and oropharyngeal overnight dispersed tumour | Increase in proliferation in 4/4 samples | 4/4 samples showed an increase in % |

The percentage of T effector cells increased in more samples when compared to the proliferation of isolated T effector cells upon the addition of HNSCC-derived CM. For
example, upon the addition of CM from primary-derived fibroblasts cultured in normoxia, there was an increase in proliferation in only one out of four samples, yet the same CM caused an increase in T effector cell percentage within whole PBMC in three out of four samples. It was hypothesised that the addition of HNSCC-derived CM would cause a decrease in the number of T effector cells. However, it appears that the addition of HNSCC-derived CM either caused no significant differences in T effectors cell numbers or an increase. In fact, overnight dispersed tumour CM caused an increase in both the proliferation and percentage of T effector cells in four out of four PBMC samples. Therefore the hypothesis can be rejected. It might be that the T effector cells are stimulated to expand but are not functional; this is investigated further in subsequent chapters.

It seems surprising that despite finding an increased proportion of Tregs following the addition of CM to PBMC, which are known to suppress the proliferation of T effector cells (Gooden et al., 2011), the CD4+ effectors were still able to expand. It is possible that Tregs are in fact preventing proliferation of T effector cells at the same time as the T effector cells being stimulated by the soluble factors, therefore masking any visible change.

Increased levels of CD4+ T effector cells have been found in the TME of breast cancer compared to non-cancer tissue but are not significantly increased in the periphery of these patients compared to healthy donors (Rad et al., 2015). The fact that the peripheral T effectors are not expanded may be due to the soluble factors produced by the tumour in vivo not being released into the periphery. However, immune cells which come into contact with the soluble factors within the close proximity of the TME due to being recruited into the tumour may be more affected and this is what these experiments are aiming to simulate. The current work found that when peripheral T effector cells come into contact with the soluble factors of the TME they are able to expand in numbers.

These data suggest that the soluble factors within the TME are capable of increasing the numbers of T effector cells within the tumour. It would be assumed that an increase in T effector cells would create a more anti-tumour microenvironment which does not explain why the tumours are allowed to progress. Therefore, this suggests that a reduction in the proportion of T effector cells may not be the main contributor to the
immune-suppressive environment, but it may be an alteration in their function that leads to the progression of cancer.

4.6.1.1 – The effect of hypoxia on the ability of CM to alter immune cell proliferation/proportion

Hypoxia is a well documented characteristic of solid tumours (Barsoum et al., 2014; Palazon et al., 2012; section 1.11). The low levels of oxygen found at the centre of tumours leads to adaptations in the surrounding cancer cells which have been well documented, however, the effect of a hypoxic environment on immune cells has had little attention in comparison.

As immune cells infiltrate tumours, they will encounter regions with lower levels of oxygen which may alter their function and numbers within the TME (Palazon et al., 2012). However, the current project did not investigate the direct effects of hypoxia on immune cells but it looked at how changes in the secretome of the TME cells might influence different sets of immune cells. For example the hypoxic TME is known to up-regulate molecules such as HIF-1α and CCL28 which can have both direct and indirect effects on the functions of immune cells.

In the current project, HNSCC cell lines and primary-derived fibroblast were cultured under both normoxic and hypoxic conditions. Their cytokine profiles were compared (chapter 3) and the effect of their secretome on the proliferation of immune cells was assessed. It was hypothesised that the CM from cells cultured under hypoxic conditions would cause a greater increase in Treg populations with a concomitant decrease in T effector cells and CD8\(^+\) T cell populations compared to the CM collected under normoxic conditions, conferring an overall more immnosuppressive environment. However, the data in this chapter rejects this hypothesis. There was no significant difference seen in the proliferation of isolated populations of CD8\(^+\) T cells, T effector cells or Treg or the proportions of these cells within the whole PBMC, upon the addition of CM from HNSCC cell lines and primary-derived fibroblasts cultured under hypoxia compared to the CM collected under normoxia. Also in the majority of cases there was no change in the proliferation of whole PBMC from both HNSCC patients and healthy donors. There was one case where after 48 hours of culture, healthy PBMC proliferation was significantly greater upon the addition of CM from HNSCC cell lines.
cultured under normoxia compared to hypoxia. However, this was an isolated case and was not representative of the majority of data.

Based on the results found in Chapter 3 it is not surprising that hypoxia had no effect on the proliferative influence of the CM collected from primary derived fibroblasts because there were no significant differences in the cytokine profile of primary-derived fibroblasts cultured under hypoxic and normoxic conditions. In contrast, HNSCC cell lines cultured under hypoxia had significantly higher levels of IL-2 and TGF-β compared to those cultured under normoxia. TGF-β is a known immunosuppressive factor which is capable of driving the conversion of naïve T cell into Tregs which will go on to suppress both T effector cells and CD8$^+$ T cells (Pickup et al., 2013). IL-2 on the other hand is a cytokine known to promote the expansion of many antigen activated T lymphocytes and is also involved in the expansion and function of Tregs (Malek, 2003). It was therefore surprising that hypoxia did not encourage the CM to be more suppressive against the CD4$^+$ and CD8$^+$ cells and more stimulatory towards the Tregs. In other words, high levels of Treg cell proliferation and lower levels of T effector and CD8$^+$ T cell proliferation. This was not what the data reflected suggesting that hypoxia induced soluble factors produced by cells within the TME are not the main drivers of immune-suppression.

One explanation for the observations is that the immunosuppressive function of hypoxia may require the presence of the tumour/stoma cells and therefore cell to cell contact driven processes alongside soluble factors. One known method by which hypoxia can effect T cells within the TME is hypoxia induced death of cells which causes the release of ATP into the surrounding area. ATP is metabolised into adenosine which can bind to receptors on the surface of T cells, causing their suppression (Barosum et al., 2014; Palazon et al., 2012). This requires the cells to be present and also for hypoxia to be present at the same time. In these experiments the HNSCC cell lines and primary-derived fibroblasts were not present with the immune cells as the aim of this thesis was to assess the function of soluble factors alone.

Tumour derived cells cultured in hypoxia are also known to release other molecules not assayed in this thesis, such as HIF-1α (Barsoum et al., 2014; Facciabene et al., 2011). HIF-1α is thought to suppress T cells and reduce the ability of CTLs to kill targets.
(Palazon et al., 2011; Lukashev et al., 2006) as well as up regulating FOXP3+ Tregs (Barsoum et al., 2014). Though we did not look for HIF-1α it is reasonable to assume that it was present within the CM from cell lines and primary-derived fibroblasts. If it was present, it had no effect on the ability of T effectors, CD8+ T cells and Tregs to proliferate.

It is also conceivable that the soluble factors produced by cell lines and primary-derived fibroblasts cultured in hypoxia do not affect the proliferation of immune cells, but instead, target their function. To investigate this further, the effect of hypoxia derived CM on Treg, T effector and CD8+ function is investigated further in the following chapters.

In conclusion, the data obtained in this chapter strongly suggests that soluble factors obtained from HNSCC cell lines and primary-derived fibroblasts cultured under hypoxia are not the main driver of immune-suppression and that other mechanisms are involved. This requires further investigation into the involvement of cell to cell contact, which is beyond the scope of this thesis which is focused on the function of soluble factors within the TME.

4.6.1.2 - Differential effects of the HNSCC secretome on immune cell proliferation relative to cell type

Both cancerous epithelial cells and stromal fibroblasts release soluble factors into the TME which can influence the function of immune cells (Ostman and Augsten, 2009; Rosenthal et al., 2004). The current chapter set out not only to determine whether soluble factors released by the TME could alter the proliferation/proportion of immune cells but also to discover which of the cell types within the TME were responsible. As both the HNSCC cell lines and primary-derived fibroblasts cultured under hypoxia and normoxia were all found to release immunosuppressive factors including IL10 and TGF-β (Chapter 3), it was hypothesised that both would confer a more immunosuppressive environment by increasing Tregs proliferation/proportion and to reduce the proliferation/proportion of T effectors and CD8+ T cells compared with cells incubated in medium alone. In contrast however, data from chapter 3 found that the only cytokine which was significantly different between cell lines and primary-fibroblasts CM was MIP-3α with cell lines secreting more. MIP-3α (CCL20) is a molecule
produced by cancer epithelial cells in the tumour microenvironment and acts as an anti-tumour molecule by attracting various immune cells into the tumour microenvironment including dendritic cells which can activate CD8$^+$ T cells (Zou and Restifo, 2010; Balkwill, 2003). Though a pro-tumour molecule it seemed unlikely that any direct effect on the proliferation of immune cells would be seen in the absence of other cells such as dendritic cells. In general, primary-derived fibroblasts appeared to produce higher levels of immunosuppressive factors such as IL-10 and TGF-β albeit not significantly, providing the expectation that CM from primary-derived fibroblasts may produce a more immunosuppressive tumour microenvironment compared to cell line CM.

The results found no difference between the ability of CM obtained from cell lines and primary-derived fibroblasts to alter the proliferation of whole PBMC from HNSCC patients and healthy donors or any isolated immune cells. However, there was a difference seen in the percentage of T effectors, Tregs and CD8$^+$ T cells when cultured as whole PBMC: one out of four CD8$^+$ T cell populations from healthy PBMC increase in proportion upon the addition of CM from primary-derived fibroblasts compared to the addition of CM from cell lines (both cultured in normoxic conditions) which might have suggested that the cancer associated fibroblasts can in some cases promote anti-tumour mechanisms, however the concurrent increase in Treg percentage in three of four healthy PBMC suggests that an immunosuppressive environment might be overriding. T effector percentage showed no change when compared the addition of CM from cell lines vs primary-derived fibroblasts.

This suggests the soluble factors within the fibroblasts CM are able to increase the levels of Tregs but only in the presence of the rest of the PBMC. It is possible that the CM alters the ratio of cell types rather than increasing/decreasing the individual immune cell populations.

This leads us to accept the hypothesis that CM from primary-derived fibroblasts conferred the most immunosuppressive environment, with an increase in the percentage of Tregs and a mostly unchanged level of anti-tumour T effectors and CD8$^+$ T cells.
4.7 – Conclusion

Soluble factors released by HNSCC cell lines, primary-derived fibroblasts and overnight dispersed tumour have the ability to effect the proliferation and percentage of immune cells within healthy PBMC samples. In some samples there was a reduction in CD8+ T cells and an increase in Treg cells which could result in an immunosuppressive environment, favouring the development of cancer. However, alterations in the proliferation of immune cells does not mean that the cells in question are functioning properly. In the subsequent chapters the effect of HNSCC-derived CM on the function of Tregs, CD8+ T cells and T effector cells will be assessed to determine whether TME soluble factors can have any functional effect.
Chapter 5

The effect of HNSCC-derived CM on the function of T regulatory cells from healthy donors
5.1 - Introduction

T regulatory cells (Tregs) are found naturally in healthy individuals where they are involved in immune-regulation (Facciabene et al., 2012; Curiel et al., 2004; Dieckmann et al., 2001). They are able to utilise the release of immune-suppressive cytokines to suppress components of the immune system, including CD4\(^+\) T effector cells to prevent autoimmune reaction and maintain immune homeostasis (Vignal et al., 2008; Wing et al., 2006; Lan et al., 2005; Fontenot and Rudensky, 2004). Tregs are essential to a normal functioning human body, and in their absence, autoimmune diseases can develop (Duhen et al., 2012). Tregs can be classified as nTregs, which are naturally occurring, originating from the thymus and iTregs which are in induced from naive CD4\(^+\) T cells upon activation (Facciabene et al., 2012; section 1.9.2).

Tregs have been found in increased numbers in the periphery and also in the tumour microenvironment of cancer patients where they can account for up to 11% (mean \(\pm\)SEM) of infiltrating CD4\(^+\) T cells (Curiel et al., 2004). Though Tregs are essential for maintaining a healthy immune system, their presence in cancer can be detrimental. For example, T effector cells (CD4\(^+\)CD25\(^-\)) that are able to recognise tumour-associated-antigen (TAA) and CD8\(^+\) T cells are suppressed by Treg in the TME which can lead to tumour progression (Chaput et al., 2009; Shevach, 2002).

Tregs have been found in elevated numbers in the tumour microenvironment of HNSCC patients with an accompanied reduction of T effector cells within the peripheral blood (Schaefer et al., 2005). There is an ongoing debate as to whether the presence of Tregs within the solid tumour is a positive or negative prognostic indicator for the patient (Facciabene et al., 2012; Curiel et al., 2004) with some believing that their infiltration into the solid tumour is negative due to their effect on the function of T effector cells.

The ability to identify, isolate and culture Tregs has been well documented by many research groups. Tregs can either be isolated using magnetic activated cell sorting (MACS), which uses antibody-coated magnetic beads to separate the population of interest from the rest of a culture (Dieckmann et al., 2001). Fluorescence activated cell sorting (FACS) following labelling of a range of surface markers with fluorescently conjugated antibodies. Throughout the current project, Tregs were isolated using a
series of characteristic surface markers; CD4^+CD25^+CD127^{low} which were sorted from PBMC obtained from healthy individuals using the FACS Aria II™ (section 2.6). These cells were found to be FOXP3^+ by colleagues (Drennan PhD thesis), indicating the likelihood that they were in fact Tregs.

Following isolation and culture of Tregs for subsequent analysis requires stimulation, due to their anergic state when cultured alone. Even upon stimulation their proliferative activity is low (Curiel et al., 2004; Levings et al., 2002; Dieckmann et al., 2001) though they still remain active and able to suppress other immune cells. Many research groups have used CD3 coated 96 well plates and soluble CD28 for stimulation of Treg cells along with IL-2 (Curiel et al., 2004; Dieckmann et al., 2001), however the current study employed CD3/CD28 coated beads and IL-2 to induce stimulation.

Other studies have looked at whether the Tregs need cell to cell contact with T effector cells to suppress them, or whether they can do it via soluble messengers using a transmembrane well. Dieckmann et al (2001) found that cell to cell contact was essential and that neutralisation with antibodies against anti-IL-10 and anti-TGF-β had no effect on the ability of the Tregs to suppress T cell proliferation.

The current study looks at the effect of HNSCC derived CM on the function of Tregs. As Tregs can suppress CD4^+ T effector cells, they were co-cultured and the ability of Tregs to suppress the CD4^+ T effector cells was analysed in the presence and absence of HNSCC derived soluble factors. The proliferation of T effector cells was tracked using a fluorescent dye; CFSE, whose fluorescence halves in each daughter cell population and can be monitored using flow cytometry.

5.1.1 – Aims and hypothesis

The aim of this chapter was to determine the effect of different HNSCC-derived CM on the function of Tregs from healthy donors by utilising the CFSE assay as a means of measuring their suppressive effect against autologous T effector cells. The effect of CM from different cell types and culture conditions (normoxia and hypoxia) were also compared.
It was hypothesised that the HNSCC-derived CM would cause an increase in the ability of Tregs to suppress the proliferation of T effector cells and therefore create a more immunosuppressive environment. It was also hypothesised that CM obtained from cell lines and primary-derived fibroblasts cultured in hypoxic conditions would lead to a greater level of Treg suppressive activity compared to the addition of CM obtained in normoxic conditions.

5.2 – Methods

To determine whether there was a change in the function of Treg from healthy PBMC following culture with HNSCC derived CM, CFSE assays were used to assess the changes in proliferation of autologous CD4⁺CD25⁻ effector T cells (section 2.8). Briefly, Tregs and T effector cells were sorted from healthy PBMC samples (n=5; section 2.6), and the T effectors were stained with CFSE before being co-cultured in 96 well plates with the Tregs. Following 24 hours of culture, HNSCC derived CM from cell lines (n=4), primary-derived fibroblasts (n=4; section 2.1.1.4) and overnight dispersed tumour CM (n=10; table 3.2; table 5.1) was added at a 1 in 3 ratio with medium already present in the wells. Wells with the addition of medium alone (n=2) were included as a control. Further control wells were also included which contained just the T effectors and HNSCC derived CM to ensure any effect seen on the proliferation of T effectors was coming from the Tregs alone and not directly from the CM. Due to the limited number of Tregs isolated, each conditions could only be assayed in a single well. The results for all of the assays treated with the same type of CM (e.g. CM obtained from all cell lines cultured under hypoxic conditions) were grouped together and the mean taken. Data was grouped in this way to enable a comparison to be made between the effects of the secretome from different cells types found within the TME cultured under different conditions.
### Table 5-1 - List of patients from which overnight dispersed tumour CM was obtained

<table>
<thead>
<tr>
<th>Overnight (ON) dispersed tumour</th>
<th>Gender</th>
<th>Age</th>
<th>TNM</th>
<th>Specimen site</th>
<th>Node or Tumour (N/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laryngeal ON CM 1</td>
<td>Male</td>
<td>56</td>
<td>T1/T2N0</td>
<td>Laryngeal</td>
<td>T</td>
</tr>
<tr>
<td>Laryngeal ON CM 2</td>
<td>Female</td>
<td>65</td>
<td>?</td>
<td>Laryngeal</td>
<td>N</td>
</tr>
<tr>
<td>Laryngeal ON CM 3</td>
<td>Male</td>
<td>62</td>
<td>T1b</td>
<td>Laryngeal</td>
<td>T</td>
</tr>
<tr>
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Results from each of the five healthy PBMC sample were kept separate due to the differences in their baseline suppression values from each PBMC. Following a further 48 hours of co-culture the cells were acquired using the FACS Aria the proliferation index and suppression analysed using Modfit software (section 2.8.3). Unpaired student T tests were used to determine whether there were any significant differences seen in Treg function following the addition of different types of HNSCC derived CM.
5.3 - Results

5.3.1 - The effect of HNSCC cell line and primary-derived fibroblast CM on the function of T regulatory cells sorted from healthy PBMC samples

The following data shows the effect of the addition of CM from HNSCC cell lines and primary-derived fibroblasts cultured under both normoxic and hypoxic conditions on the function of Tregs obtained from normal PBMC (figure 5.1).

T effector cells stained with CFSE in isolation were classed as having zero suppression in all five healthy PBMC samples (not shown on graph). When T effectors cells were co-cultured with Tregs plus medium alone, there was a range of between 10 and 34% suppression depending on which healthy PBMC sample the cells were harvested from (figure 5.1a-e).

The addition of CM obtained from cell lines cultured under normoxic and hypoxic conditions caused a significant increase in the percentage suppression of T effector cells by Tregs in one out of five PBMC samples when compared to the addition of medium alone. The percentage average suppression increased from 10 to 23.5% upon the addition of CM from cell lines cultured under normoxic conditions and from 10 to 22.8% following the addition of CM obtained from cell lines cultured under hypoxic conditions (figure 5.1c).

The addition of CM obtained from primary-derived fibroblasts cultured under normoxic conditions caused a significant increase in the average percentage suppression of T effectors by Tregs, from 25.7 to 56.5% in one out of five samples compared to the addition of medium alone. (figure 5.1e). The addition of CM from primary-derived fibroblast cultured under hypoxic conditions caused a significant increase in suppression in two out of five healthy PBMC samples increasing from an average of 10 to 22.5% in one sample and 25.7 to 58.2% in the other (figure 5.1c & e).

In three out of five healthy PBMC samples, the addition of HNSCC derived CM from all sources made no difference to the Tregs ability to suppress T effector cells (figure 5.1a,b & d).
Figure 5-1 – Bar charts showing the effect of HNSCC derived CM on the ability of Tregs to suppress autologous T effector cells from five different healthy PBMC samples (a, b, c, d & e; Mean±SEM). Unpaired student T tests were used to compare data sets. *significant difference when comparing the addition of HNSCC CM to medium alone ^ Significant difference between data sets (p<0.05)
In one out of five PBMC samples, CM from primary-derived fibroblasts cultured under normoxic conditions (56% suppression) caused a significant increase in suppression compared to the addition of CM from cell lines cultured under normoxic conditions (18%). The addition of CM from primary-derived fibroblasts cultured under hypoxic conditions (58% suppression) also caused significantly greater suppression in Tregs compared to the addition of CM from cell lines cultured under hypoxic conditions (18.4% suppression). Example Modfit plots show the reduced number of T effector cell daughter populations due to suppression by Tregs upon the addition of CM from primary-derived fibroblasts cultured under both normoxic and hypoxic conditions, compared the addition of cell line CM (figure 5.2 a & b).

The addition of CM collected from HNSCC cell lines or primary-derived fibroblasts cultured under different oxygen levels (normoxia and hypoxia) had no differential effect on Treg suppressive activity.
Figure 5-2 – Modfit plots showing the effect of the addition of CM from HNSCC cell lines (a; n=1) and primary-derived fibroblasts (b; n=1) both cultured under normoxic conditions on the ability of Tregs to suppress the proliferation of autologous T effector cells. Plot shows the data from a single point as an example of where the results obtained originated. Proliferation index (PI) was used to calculate % suppression.
5.3.2 - The effect of HNSCC-derived overnight dispersed tumour CM on the function of T regulatory cells sorted from healthy PBMC samples.

CM from overnight dispersed tumour of laryngeal (n=5) and oropharyngeal (n=5) origin were added to the Treg/ T effector cell co-culture.

The following data shows the effect of the addition of CM from HNSCC-derived overnight dispersed tumour on the function of Tregs obtained from normal PBMC (figure 5.3).

The addition of CM from overnight dispersed tumour of laryngeal origin caused a significant two-fold increase in suppression of T effectors by Tregs in two out of five PBMC samples and a significant 10% decrease in suppression in one out of five samples (figure 5.3c, d & e) compared to the addition of medium only.

In the same two PBMC samples, the addition of CM from overnight dispersed tumour of oropharyngeal origin caused a significant 2.5-fold increase in % suppression compared to medium alone (figure 5.3c & e).

An example of the Modfit data obtained shows the proliferation of T effector cells co-cultured with Treg in the presence of medium alone compared to the addition of CM from overnight dispersed tumour CM from both laryngeal and oropharyngeal origin (figure 5.4). Data shown is representative and from single data points.

The anatomical origin (laryngeal or oropharyngeal) of overnight dispersed tumour CM made no significant difference to the ability of Tregs to suppress T effector cells.
Figure 5.3 – Bar charts showing the effect of CM from overnight dispersed tumour of laryngeal (n=5) and oropharyngeal (n=5) origin on Tregs ability to suppress the proliferation of T effector cells from five different healthy PBMC samples (a,b,c,d & e; Mean±SEM). Unpaired student T tests were used to compare the data sets.

*significant difference when comparing the addition of HNSCC CM to medium alone (p<0.05).
Figure 5-4 – Modfit plots showing the effect of the addition of medium only (a; n=1) and CM from HNSCC overnight dispersed tumour of laryngeal (b; n=1) and oropharyngeal (c; n=1) origin on the ability of Tregs to suppress the proliferation of autologous T effector cells. Plot shows the data from a single point as an example of where the results obtained originated. Proliferation index (PI) was used to calculate % suppression.
5.4 – Discussion

The main role of Tregs in the healthy individual is to regulate immune response whereby they maintain self-tolerance by suppressing T effector cells (Roychoudhuri et al., 2015; Noack and Miossec, 2014; Nishikawa and Sakaguchi, 2014). The absence of Tregs can lead to autoimmune disease, as T effector cells are unchecked and will begin to attack self-antigen, highlighting their importance in the normal functioning immune system (Nishikawa and Sakaguchi, 2010; Shevach, 2002). However, in cancer patients, Tregs are implicated in the inability of the host immune system to eradicate tumour cells (Nishikawa and Sakaguchi, 2014). In cancer patients, there is an increase in the numbers of Tregs within the tumour itself (Nishikawa and Sakaguchi, 2010; Whiteside, 2012) and in the peripheral blood (Whiteside, 2012) which leads to increased suppression of T effector cells, which will contribute to the overall immune-suppressive TME (Roychoudhuri et al., 2015). This increase in Treg is thought to be a poor prognostic marker for many cancer patients, particularly when there is a concomitant decrease in CD8+ T cells within the TME (Nishikawa and Sakaguchi, 2014). Conversely, in some cancers, including HNSCC, an increase in Tregs has been found to confer a positive prognosis (Badoual et al., 2006). This conflicting data may in part be due to the differing composition of FOXP3+ T cells with some not having regulatory mechanisms but still retaining the ability to release pro-inflammatory molecules (Nishikawa and Sakaguchi, 2014).

It has been shown that Treg infiltrate the solid tumour (Nishikawa and Sakaguchi, 2014). The production of CCL22 within the TME by cancer cells and other immune cells is thought to be one way by which Tregs are attracted (Faget et al., 2011). Once within the TME, Tregs are exposed to various cytokines and cells, all of which exert their force in one way or another. This study wanted to look at the effect of TME-derived cytokines on the suppressive activity of Tregs. Even though it is clear that the TME affects the Tregs, it remains unclear what the exact role of cell to cell interaction and cytokines are, therefore more work must be carried out.

The current chapter describes the effect of the soluble factors released by HNSCC derived cell lines, primary-derived fibroblasts and overnight dispersed tumour on the ability of Tregs to suppress T effector cells to try and determine which cells might be
involved in the immunosuppression. The Tregs and T effector cells used in the study were harvested from the PBMC of healthy individuals so that they had not undergone any “preconditioning” by the tumour. Some research has looked at the suppressive capabilities of Tregs isolated from TILs and the periphery of patients. For example Drennan et al (2014) looked at the suppressive activity of Tregs from the periphery and TIL from HNSCC patients, however these cells have already come into contact with the TME. The aim was to determine whether Tregs from healthy donors become more immunosuppressive towards CD4+ T effector cells upon culture with HNSCC-derived CMs as those taken directly from the tumour microenvironment appear to, and if so which cells are inducing this effect.

All five Treg populations from healthy donors were able to suppress autologous T effector cells. This agrees with other work, such as Shen et al (2009) who co-cultured Tregs and T effectors from normal’s and used CFSE assays to track proliferation. Normal Tregs were able to suppress the proliferation of T effectors in a dose dependant manner. It is also worth pointing out that they gained proliferation index values of between 2 and 2.5, which reflects the data gained in this chapter. Earle et al (2005) who utilised [3H] thymidine incorporation also showed that Tregs isolated from healthy individuals and cultured were able to suppress whole PBMC in a dose dependant manner. Interestingly, although all five Tregs samples were able to suppress the proliferation of T effector cells there was a substantial difference in baseline suppression ranging from 10-35%. In the studies mentioned above, it is possible to see that their healthy patients also showed variability in the Tregs ability to suppress T effector cells by observing the degree of error in their data (Shen et al., 2009; Earle et al., 2005). The immune system in a healthy individual is not a static entity, and can fluctuate depending on many different factors such as environment and genetic traits. Brodin et al (2015) studied and compared the immune systems of twins and found that the differences were too great to be attributed to genetics alone and that environmental factors played an important role. They concluded that this may be down to repeated infection by different strains of bacteria/ virus. This reflects the data obtained in this chapter, with each healthy donor showing variability in their Tregs populations. The anonymous source of the healthy blood meant that no clinical details were available for these samples with regard to age or health status.
In this study, two out of five Treg samples showed enhanced suppressive capabilities towards autologous CD4⁺ T effector cells upon the addition of HNSCC-derived CM compared to the addition of medium alone. One sample showed increased suppressive activity upon the addition of CM from cell lines cultured in both normoxic and hypoxic conditions and CM from fibroblasts cultured under hypoxic conditions. The second sample showed increased suppression upon the addition of CM from fibroblasts cultured under both normoxic and hypoxic conditions. The same two Treg samples also showed increased suppression upon the addition of overnight dispersed tumour CM of both laryngeal and oropharyngeal origin. Three out of the five Treg samples showed no significant change in their ability to suppress T effector cells upon the addition of HNSCC derived CM, regardless of origin, compared to medium alone. It was hypothesised that HNSCC-derived CM would enhance the ability of Tregs to suppress T effector cells. The ability to accept or reject this hypothesis would require further work into the mechanisms behind the increased levels of suppression seen in some samples.

The data suggests that soluble factors derived from the HNSCC TME are able to enhance the suppressive activity of Treg towards autologous CD4⁺ T effector cells in some samples. Again, as mentioned previously, this goes back to the variability seen within each sample. It may be that some individuals have Tregs that will react to soluble factors to a greater extent compared to the rest of the population. With a larger cohort of normal samples, it would be interesting to see what percentage of the population has this more reactive Treg population. This also may give an indication as to how susceptible a person is to developing cancer. If some individuals Treg cells are more sensitive to the cytokines found within the TME and either gain enhanced suppressive abilities or are just more susceptible to expansion, this could lead to increased immune-suppression and a higher chance of tumour progression. Though just speculation, it would be interesting to investigate this susceptibility of healthy individuals more in the hope of being able to identify those who are at higher risk of cancer.

Some papers have shown that certain cytokines can affect the suppressive function of Tregs. TNFα/β is pro-inflammatory cytokine released by many cells including tumour cells and immune cells such as macrophages and T/B lymphocytes (Chen and
Opeenheim, 2011; Chen et al., 2007). It was found at varying concentrations within all HNSCC-derived CM cultured alongside the T effectors and Tregs in this chapter (chapter 3). TNF works through its interaction with various receptors, such as TNFR2 which can be found on the surface of Treg and T effector cells (Chen and Opeenheim, 2011). Chen et al (2007) co-cultured peripheral murine Tregs and T effector cells with the addition of TNF. They found that initially the T effectors were able to proliferate in the presence of Tregs, however, upon extended exposure, Tregs began to suppress the T effector cells again, suggesting that Tregs take longer to react to the presence of TNF compared to T effector cells. They found that adding IL-2 further enhanced this effect, and in addition they found up regulation of FOXP3 and increased suppressive activity of Tregs. Chen et al (2007) also added the cytokines IL-6 and IL-1β and found no difference in the ability of Tregs to suppress T effector cells.

TGF-β is a well known immunosuppressive cytokine that is released by Tregs (Pickup et al., 2013). TGF-β was found in all HNSCC-derived CM showing that cancer cells and fibroblasts also release this factor (chapter 3). TGF-β may be able to convert naïve CD4⁺CD25⁻ T cells into Tregs, and is also one way in which Tregs exert their immunosuppressive function (Pickup et al., 2013). Chen et al (2003) found that TGF-β up-regulated the FOXP3 gene on CD4⁺CD25⁻ T cells and could suppress other T cells in vitro. This points to an increased number of Tregs as opposed to increased function.

IL-2 is a well known cytokine involved in the activation and expansion of Tregs with deficiency leading the lower number and function of Tregs (Rochman et al., 2009). It is commonly used during the culture of T cells in order to activate them and induce proliferation, including in this project. IL-2 was another cytokines that was present with all HNSCC-derived CM. There is also research into the potential use of IL-2 therapy for patients with autoimmune disease. A common theme appears to be that cytokines effect Treg number as opposed to their function.

Literature appears to come to the conclusion that it is the expansion of Tregs and not an increase in suppressive activity that causes the immunosuppressive environment and that Treg work in a dose dependant manner with a greater number of Tregs resulting in greater suppression (Oleinika et al., 2013; Curiel et al., 2004; Woo et al., 2001). If this is the case then an increase in Tregs within the culture well could shift the ratio of Tregs.
to T effectors causing a greater level of suppression. In chapter 4, it was found that the proliferation of isolated Tregs and the percentage within PBMC was increased in a lot of samples upon the addition of HNSCC-derived CM, which further supports the idea that it’s an increased number of Tregs that increase the immunosuppressive activity within the TME.

This suggests further work needs to be applied to the current data to determine whether the increased suppressive activity of Tregs was down to an increase in number or actual function. The current study has shown that the addition of HNSCC-derived CM does increase the suppressive activity of Treg towards T effectors, however, it is not clear whether this is an artefact of an increased population of Tregs as opposed to increase in function.

Studies have shown that the suppressive activity of Tregs taken from the tumour microenvironment was not significantly different to those taken from the periphery of the same patients, which suggests that soluble factors within the TME do not enhance the Tregs suppressive ability (Drennan et al., 2014; Chi et al., 2010; Zou, 2006).

Drennan et al (2014) co-cultured Treg and T effectors from the TME and Treg and T effectors from the periphery of HNSCC patients and CFSE assays were used to track the proliferation of T effector cells. They found no significant difference in the suppressive activity of Tregs from the periphery and the TME, and came to the conclusion that it may be the number of Tregs rather than their function that causes the contribution to the immune-suppressive environment. Chi et al (2010) came to a similar conclusion, whereby they showed that Treg from the periphery and tumour of bladder carcinoma patients were equally able to suppress T effector cells and their cytokine secretion.

5.4.1 – Hypoxia derived soluble factors and their effect of the function of T regulatory cells

The hypoxic tumour microenvironment is thought to promote immune suppression and it is thought to do this through the release of cytokines such as HIF-1α and adenosine by cancer cells and associated cells (Sitkovsky, 2009; Kumar and Gabrilovich, 2014).
It was hypothesised that the addition of CM obtained from cell lines and primary-derived fibroblasts cultured under hypoxia to Tregs would enhance their suppressive activity to a greater extent than CM obtained in normoxic conditions. However, in this study there was no differential effect seen so the hypothesis can be provisionally rejected, however, it is important to point out the small healthy sample cohort. As previously mentioned, there seems to be a large variation between Treg population from different individuals, and it may be that these particular five donors did not react to hypoxia derived soluble factors. A larger cohort may reveal individuals with Treg populations that do react to the hypoxia derived soluble factors.

The failure of hypoxia-derived CM to enhance Treg suppressive activity was surprising considering the significantly higher levels of TGF-β and IL-2 in cell line and primary-fibroblast derived CM collected under hypoxia. Both TGF-β and IL-2 are needed for the proliferation and function of Tregs, so it was thought the higher levels would induce greater levels of suppressive activity.

One way by which hypoxia can effect Tregs is through the accumulation of adenosine, which is released by tumours cells when experiencing low levels of oxygen (Sitkovsky, 2009). Adenosine acts through the A2A receptor which can be found on the surface of activated T cells including Tregs (Lee et al., 2010). It is thought signalling through this receptor may lead to increased transcription of immunosuppressive molecules and up-regulation of CD39 and CD73 which can lead to further accumulation of adenosine which will decrease T effector cell functions (Lee et al., 2010).

Another hypoxia related soluble factor is HIF-1α which can influence various types of T cells. HIF-1α is released by cells within the TME which are enduring low oxygen levels (Kumar and Gabrilovich, 2014). HIF-1α is thought to increase the expression of CCL-28 which is involved in the recruitment of Tregs into the TME. However, when Tregs come into direct contact with HIF-1α it will bind with FOXP3 leading to its degradation (Alcantara-Hernandez et al., 2014). HIF-1α may also be involved in the differentiation of Th17 over Tregs (Kumar and Gabrilovich, 2014).

It is clear that the effect of soluble factors derived from the hypoxic tumour environment on the function Tregs is poorly understood. Though there were a few
studies suggesting that HIF-1α and adenosine could directly influence Tregs, the mechanisms behind this are not so well understood. More work on Treg function in a hypoxic microenvironment is needed in order to determine its effect on their function. It may be that that Tregs need to be in hypoxia themselves before any change in suppressive activity will be seen.

5.4.2 – The effect of primary-derived fibroblasts CM on the function of T regulatory cells

In one out of five Treg samples, there was a significant increase in the ability of Tregs to suppress autologous T effector cells upon the addition of CM from primary-derived fibroblasts cultured under both normoxic and hypoxic conditions, compared to the addition of cell line CM cultured under both normoxic and hypoxic conditions. This suggests that in this case, soluble factors within the primary-fibroblast CM were able to stimulate the Tregs to a greater extent than cell line CM.

Looking back to chapter 3, the only significant difference between primary-fibroblast and cell line CM was that cell line CM had greater levels of MIP-3α (aka CCL20). CCL20 is thought to be involved in the migration of Tregs into the TME of different cancers, including hepatocellular carcinoma (Chen et al., 2011) and oesophageal squamous cell carcinoma (Liu et al., 2015). There was no reference to CCL20 being involved in the proliferation or function of Tregs. This study looked at a limited selection of cytokines, so it is possible that there are other factors present in the fibroblasts CM and not in the cell line CM that could impact on Tregs.

Soluble factors released by cancer associated fibroblasts have been found to stimulate the infiltration of immune cells including Tregs (Polanska and Orimo, 2013). Takahashi et al (2015) collected the CM from HNSCC-derived cancer-associated fibroblasts and normal fibroblast and cultured it alongside PBMC before analysing the Tregs using flow cytometry. They found that the proportion of Tregs increased upon the addition of CAF CM compared to normal fibroblast CM. They also found that the expression of IL-10 and TGF-β was higher. This suggests that CAF CM can promote the cytokine driven immunosuppression by Tregs within whole PBMC.
In chapter 4 the percentage of Tregs within whole PBMC increased more upon the addition of CM from fibroblasts compared to cell line CM on several occasions. With this in mind, it seems likely that the levels of Tregs increased more upon the addition of primary-fibroblasts CM compared to cell line CM causing an increase ratio of Tregs to T effector cells.

5.5 - Conclusion

To the best of the author’s knowledge; this is the first study to look at the effect of HNSCC-derived CM on the suppressive activity of Tregs from healthy donors. Though others have added individual cytokines to co-cultures of Tregs and T effector cells, this study has added the milieu of TME derived cytokines, keeping cytokine signal pathways intact. This is important as the cytokine network may need to stay intact in order for their full potential to be realised.

In conclusion, it can be said that in some cases, HNSCC-derived soluble factors can enhance the suppressive ability of Tregs towards T effector cells. Though whether this is down to a mechanism that effects function or to do with increased Treg proliferation is unknown.
Chapter 6

The effect of HNSCC derived CM on the function of T effector cells obtained from healthy subjects
6.1 - Introduction

T effector cells originate from the thymus (Szabo et al., 2003) where they undergo selection to become mature CD4+ before being released into the bloodstream, where they play a pivotal role in host immunity. Mature CD4+ T cells can further differentiate into various subsets depending on the local environment (Chang et al., 2014; Harrington et al., 2005; section 1.9.3; figure 1.9). The two main subsets are Th1 and Th2, which have differing roles in the immune system, with Th1 being involved in the destruction of intracellular pathogens and Th2 linked with the removal of parasitic infection (Harrington et al., 2005; Szabo et al., 2003). A third major subset is Th17 whose function is to orchestrate an immune response against extracellular bacteria and fungi and release cytokines such as IL-17 and IL-22 (Luckheeram et al., 2012; Nakayamada et al., 2012; Zhu et al., 2010; section 1.9.3.1.3). However, this chapter focuses on the Th1/Th2 balance due to their significance in tumour progression. Th1 and Th2 cells can be distinguished by their differing cytokine profiles with Th1 cells releasing IFN-γ, TGF-β, TNF-α and IL-2 and Th2 cells releasing IL-4, IL-5, IL-6, IL-10 and IL-13 (DeNardo and Coussens, 2007; Harrington et al., 2005; Szabo et al., 2003) The decision as to whether the naïve T cells becomes Th1 or Th2 can depend on many different things, including the type of APC presenting an antigen, the concentration of the antigen and the local array of cytokines (Szabo et al., 2003). Two of the most important cytokines in the induction of differentiation are IL-12 for Th1 and IL-4 for Th2 (Szabo et al., 2003; Nishimura et al., 1999). Once the CD4+ T cell has differentiated into Th1 and Th2, they are able to self propagate by using positive feedback loops. For example Th2 cells have the ability to suppress the activity and differentiation of Th1 cells by blocking the IL-12 receptor (Harrington et al., 2005). Though both Th1 and Th2 cells have anti-tumour activity, Th1 cells cause the stronger response, therefore if there are a greater number of Th2 cells, then the chances of tumour progression increases (DeNardo and Coussens, 2007; Nishimura et al., 1999).

Mouse models such as the one described in Ruffell et al (2010) have shown that mutations in genes that control IL-4 signalling cause an increase in Th1 type cells and induces the destruction of the tumour. If the mice had a block in their ability to produce
IFN-γ, so increasing the levels of Th2 compared to Th1, they became more susceptible to tumours. This highlights the importance of the Th1/Th2 balance in the body.

Evidence of this Th1/Th2 imbalance has been found in the TME and periphery of multiple types of cancer such as Hodgkin’s lymphoma (Schreck et al., 2009 - tumour biopsies), pancreatic cancer (Monte et al., 2011 - tumour stroma), bladder and colorectal cancer (Johansson et al., 2008 - peripheral blood) and HNSCC (Sparano et al., 2015 - plasma samples). All found increased levels of Th2 type cytokines when compared with levels of Th1 cytokines. In many cases the increased levels of Th2 compared to Th1 cytokines correlated with a poor prognosis for the patients (Monte et al., 2011; Schreck et al., 2009).

There may be several reasons that this imbalance occurs. Cancer cells are known to release a milieu of cytokines in the local environment to influence immune cells and avoid destruction. Cancer cells release TNF and IL-1β which can induce cancer associated fibroblasts (CAFS) to release thymic stromal lymphopoietin (TSLP) amongst many other factors. TSLP then activates and influences mDCs which can cause the Th2 polarisation by inducing their differentiation from naïve T cells (Monte et al., 2011). The local TME will also release Th2 inducing cytokines whilst trying to reduce levels of Th1 cytokines.

As stated previously, evidence of the Th1/Th2 imbalance has been found in HNSCC patients. Sparano et al (2015) harvested plasma samples from 58 HNSCC patients and assessed levels of Th2 related cytokines IL-4, IL-6 and IL-10 and Th1 related cytokines IL-12 by ELISA. They found a predominantly Th2 environment. They compared results from patients with early and late stage disease and found that late stage disease had lower levels of IL-12 (Th1) and higher levels of IL-10 and IL-6 (Th2). This implies that as levels of Th2 cells increases, the tumour progresses, possibly implicating the Th2 cells in the growth and advancement of HNSCC. Gaur et al (2014) took serum samples from 78 patients with oral squamous cell carcinoma (OSCC) and 39 controls and used ELISA to look at the relative levels of Th1 cytokines (IL-2 and IFN-γ) and Th2 cytokines (IL-4 and IL-10). They found an increase in Th2 cytokines and suppression of
Th1 cytokines when comparing OSCC patients with controls, again revealing a shift towards the pro-tumour Th2 cytokines profile.

In this chapter HNSCC patient derived CD4+ T effector cells were cultured and CM collected to determine the levels of Th1 and Th2 cytokines they secreted. The CM obtained from HNSCC cell lines, primary-derived fibroblasts and overnight dispersed HNSCC tissue was previously assayed to determine which cytokines they release (chapter 3). By adding the CM collected from the HNSCC cell lines or other tumour associated tissue to sorted T effector cells it was possible to assess the impact such soluble interactions had on the cytokine profiles produced by the T effector cells. The question is, do epithelial cancer cells or fibroblasts have the ability to alter the balance of Th1/Th2 cytokines and therefore T effector cell function, or does there need to be the full complement of cells (overnight dispersed tumour) for there to be an effect.

6.1.1 – Aims and hypothesis

The aim of this chapter was determine whether HNSCC-derived CM affected the function of T effector cells obtained from the PBMC of healthy donors by utilising ELISAs to assess the Th1/Th2 cytokine balance. The affect of CM from both HNSCC cell lines and primary-derived fibroblasts were compared as was as the effect of CM obtained in hypoxic conditions compared to normoxic conditions.

It was hypothesised that HNSCC-derived CM would cause T effector cells to release more Th2 cytokines with a concurrent decrease in the production of Th1 type cytokines therefore leading to a more pro-tumour microenvironment.

It was also hypothesised that CM collected from cells cultured in hypoxia would cause an even greater shift towards Th2 cytokine release compared to the addition of CM collected in normoxic conditions.
6.2 - Methods

T effector cells were sorted from healthy PBMC samples (n=4) as described in section 2.6. Cells were plated out at a concentration of 5 x 10^4 in medium and incubated for 48 hours with stimulation (IL-2 and CD3/CD28 beads) and CM obtained from HNSCC cell line (n=4) and HNSCC primary-derived fibroblast (n=4) cultured under both normoxic and hypoxic conditions (table 3.2) and overnight dispersed tumour (n=10; table 6.1). Medium only with stimulation was added to some wells with T effector cells as a control to ensure that if there was a change in cytokine secretion it was from the CM alone and not the medium. Wells with medium only and no T effector cells were used to ensure there was no cytokine present in the medium, and any values were subtracted from the final results.

After the 48 hours of incubation, the CM from each well was then harvested and four different ELISAs were carried out in duplicate to determine changes in the Th1/Th2 balance (section 2.3). Some samples required a dilution to get in range of the ELISAs detection limits; this was taken into account when processing by multiplying the result by the dilution factor. Further to this, ELISA (IFN-γ and IL-2 were selected for the identification of Th1 and IL-4 and IL-10 were selected to identify Th2 cells) were also carried out on the culture medium from HNSCC cell lines, primary-derived fibroblast and overnight dispersed tumour in order to determine the concentration of cytokines added to the wells containing T effector cells. This way cytokine concentrations reported are down to the T effectors alone and not from the addition of the CM.

The results obtained from the CM collected from the co-culture of CM obtained from cell lines, primary-derived fibroblast and overnight dispersed tumour and T effector cells were adjusted to take into account dilution factors, the concentration of cytokines present in CM added at the start of the experiment and any reading obtained from medium alone wells. The four T effector cell populations were obtained from four different healthy donors. As in previous chapters the results for each sample were kept separate, due to the wide variation in results obtained.
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<td>Tongue base/ oropharynx</td>
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6.3 - The effect of HNSCC cell line and primary-derived fibroblast CM on the function of T effector cells obtained from healthy PBMC

CM from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) was added to cultures of T effectors cells before the CM was harvested. IFN-γ, IL-2, IL-4 and IL-10 ELISAs were carried out on the resultant CM.

6.3.1 - Release of the Th1-like cytokine IFN-γ

After 48 hours of incubation with CM obtained from cell line and primary-derived fibroblast cultured under normoxic and hypoxic conditions with T effector cells obtained from healthy PBMC an IFN-γ ELISA was performed on the resulting CM. The final results were then compared to the effect of medium alone (figure 6.1).

Each sample of T effector cells had different base levels of IFN-γ production. T effector cells from healthy PBMC sample 3 produced more than twice the amount of IFN-γ with the addition of medium alone compared to the other three samples (figure 6.1).
There was a significant increase in T effector cells IFN-γ production upon the addition of CM from cell lines cultured under normoxic and hypoxic conditions compared to medium alone in three out of four samples. Two out of four samples showed a two-fold increase in IFN-γ production upon the addition of cell line CM when compared to medium alone (figure 6.1). CM from fibroblasts cultured under hypoxic conditions caused a significant increase in the production of IFN-γ in one out of four samples, though the increase was marginal (figure 6.1). Healthy PBMC 3 showed no increase in IFN-γ production upon the addition of any CM. HNSCC primary-derived fibroblast CM from both normoxic and hypoxic culture conditions caused no increase in IFN-γ production in three out of four T effector cell populations (figure 6.1).

![Figure 6-1](image-url)

*Figure 6-1* – Bar chart showing the release of IFN-γ (pg/ml) from T effector cells obtained from healthy PBMC (n=4) after incubation with CM from cell lines (n=4), fibroblasts (n=4) cultured under normoxic and hypoxic conditions and medium (mean ±SEM).

*significant when compared to relevant medium only sample; paired student t tests.

CM was collected from HNSCC cell lines and primary-derived fibroblast which were cultured in both normoxic and hypoxic conditions. A comparison was made between the addition of normoxic and hypoxic CM on the cytokine profile of T effector cells from
healthy patients to determine whether oxygen levels have an effect on the ability of cancer cells and fibroblast to alter the Th1/Th2 balance.

CM obtained from HNSCC cell lines and primary-derived fibroblasts cultured under hypoxic conditions caused no significant difference in T effector cell IFN-γ production when compared to the addition of normoxia derived CM.

Significantly greater levels of IFN-γ was released by 1 of 4 T effector cell populations after the addition of CM from HNSCC cell lines CM cultured under hypoxic conditions compared to the addition of primary-derived fibroblast cultured under hypoxic conditions. The same was observed in a different T effector population, though this involved CM obtained from cell lines and primary-derived fibroblast cultured under normoxic conditions. In both cases the CM from HNSCC cell lines induced a 2 fold increase in IFN-γ production when compared with the addition of primary-derived fibroblast CM.

No significant difference in IFN-γ production by any other T effector cell population was seen on the addition of cell line or primary-derived fibroblast CM, of either normoxic or hypoxic status.

6.3.2 - Release of Th1-like cytokine IL-2

Each T effector population produced a different base amount of IL-2, with PBMC 1 giving the lowest, which followed a similar pattern as IFN-γ production and healthy PBMC 2 had the highest base levels of IL-2 production. Levels of IL-2 were much greater (~18000-70000 pg/ml) when compared to IFN-γ levels (~200-900 pg/ml; figure 6.2).

There was a significant decrease in T effector cells IL-2 production upon the addition of CM from HNSCC cell lines cultured under normoxic conditions compared to medium alone in 3 out of 4 samples. Whereas, two out of four samples had reduced IL-2 production upon the addition of CM from HNSCC cell lines cultured under hypoxic conditions (figure 6.2).
The addition of CM from primary-derived fibroblasts cultured under both normoxic and hypoxic conditions to T effectors cells also caused them to release significantly reduced level of IL-2 compared to the addition of medium alone in two out of four samples (figure 6.2).

T effectors cells from healthy PBMC sample 2 saw the greatest decrease in IL-2 production upon the addition of all HNSCC derived CMs when compared to the addition of medium alone, with around a 3-fold reduction. The opposite was true with T effector cells from healthy PBMC 3 which had no significant change in IL-2 production upon the addition of all HNSCC derived CMs (figure 6.2).
In one of four T effector cell populations (sample 1) from healthy PBMC, the addition of CM obtained from HNSCC cell lines cultured under normoxic conditions caused a greater production of IL-2 compared to the effect of CM from hypoxic conditions. No similar effect was seen in the other three T effector cell populations (figure 6.2).

In one of four T effector cell populations (sample 2), the addition of CM obtained from primary-derived fibroblasts cultured under normoxic conditions caused a greater production of IL-2 compared to the effect of CM from hypoxic conditions. Again, no effect was seen in the other three T effector cell populations (figure 6.2).

There was a significant difference in IL-2 release from two of four T effector samples upon the addition of CM obtained from HNSCC cell lines and primary-derived fibroblasts cultured under hypoxic conditions (figure 6.3a). In one sample the hypoxic primary-derived fibroblast CM caused a significant increase in IL-2 production.

**Figure 6.2** – Bar chart showing the release of IL-2 (pg/ml) from T effector cells obtained from healthy PBMC (n=4) after incubation with CM from HNSCC cell lines (n=4), primary-derived fibroblasts (n=4) cultured under both normoxic and hypoxic conditions and medium (mean ±SEM).

* significant when compared to medium only; paired student t test.

^ Significant difference between the two data sets
compared to hypoxic cell line CM, however in T effector cells from healthy PBMC 4 the opposite was true, with hypoxic cell line CM causing a significant increase in IL-2 production compared to hypoxic fibroblast CM.

There was a significant difference in IL-2 release from two T effectors samples upon the addition of CM obtained from HNSCC cell lines and primary-derived fibroblast cultured under normoxic conditions (figure 6.3b). In one sample the normoxic fibroblast CM caused a significant increase in IL-2 production compared to normoxic cell line CM. Again, T effectors from healthy PBMC 4 caused the opposite effect with normoxic cell line CM causing a significant increase in IL-2 production compared to normoxic fibroblast CM (figure 6.3b).

No significant difference in IL-2 production was seen upon the addition of HNSCC cell line and primary-derived fibroblast CM of either normoxic or hypoxic culture condition in any other T effector cell population.
Figure 6-3 – Bar chart showing the effect of the addition of CM from a) HNSCC cell lines and primary-derived fibroblasts cultured under hypoxic conditions and b) cell lines and fibroblasts cultured under normoxic conditions; on the production of IL-2 from T effector cells from healthy PBMC (mean ±SEM).

* Significant difference; Paired student T tests
6.3.3 - Release of the Th2-like cytokine IL-4

Three of four T effector cell populations released less than the detectable limit of IL-4 upon the addition of medium alone. One out of four T effector populations released very low levels of IL-4 (figure 6.4).

One T effector cell population out of four showed a significant increase in IL-4 production upon the addition of CM obtained from HNSCC cell lines and primary-derived fibroblasts cultured under both normoxic and hypoxic conditions compared to the addition of medium only (figure 6.4). The levels released were still relatively low (60-120pg/ml) compared to the release of IFN-γ and IL-2 however, one out of four of the T effector cell populations showed a decrease in IL-4 production upon the addition of CM from HNSCC cell lines and primary-derived fibroblasts cultured under both normoxic and hypoxic conditions when compared to the addition of medium alone.

Two out of four T effector populations showed IL-4 production that was below the detectable limit upon the addition of culture medium from both HNSCC cell lines and primary-derived fibroblasts culture under both normoxic and hypoxic conditions (figure 6.4).
There was a significant difference in the IL-4 production from T effector cells when comparing the effect of the addition of CM from HNSCC cell lines cultured under normoxic with hypoxic conditions. Normoxic cell line CM caused the production of higher levels of IL-4 compared to cell line CM collected under hypoxia (figure 6.4). No significant difference in IL-4 production was seen in all T effector cell population when comparing the addition of primary-derived fibroblast CM of normoxic and hypoxic origin (figure 6.4).

There was no significant difference in the production of IL-4 from T effector cells when comparing the addition of HNSCC cell line CM vs primary-derived fibroblast CM.

**Figure 6-4** – Bar chart showing the release of IL-4 (pg/ml) from T effector cells obtained from healthy PBMC (n=4) after incubation with CM from HNSCC cell lines (n=4), primary-derived fibroblasts (n=4) cultured under both normoxic and hypoxic conditions and medium (mean ±SEM).

*Significant when compared to medium only; paired student t tests.

^ Significant difference between the two data sets.
6.3.4 - Release of the Th2-like cytokine IL-10

Three of four T effector cell populations had IL-10 levels at below the detectable limit upon the addition of medium only and one out of four samples produced IL-10 at relatively low levels. The PBMC sample that produced low levels of IL-10 was the same sample that produced the only IL-4 upon the addition of medium alone. These were two separate experiments where the T effectors from this sample released a cytokine upon the addition of medium.

The addition of CM from HNSCC cell lines cultured under both normoxic and hypoxic condition caused a significant reduction in the production of IL-10 when compared to medium in one out of four T effector cell populations (figure 6.5).

CM from primary-derived fibroblasts cultured under normoxic conditions caused a significant increase in IL-10 production compared the addition of medium online in two out of four samples. The increase was relatively small compared to all other cytokines tested and may only be significant due to there being no reaction upon the addition of medium alone. One out of four T effector cell populations showed no significant change in IL-10 production upon the addition of any CM when compared to the addition of medium alone.
No significant difference in IL-10 production by T effector cells was found when comparing the addition of CM from HNSCC cell line and primary-derived fibroblasts cultured under normoxic vs hypoxic conditions.

There was a significant difference in IL-10 release from two of four T effectors samples between the addition of CM obtained from HNSCC cell line and primary-derived fibroblast CM cultured under normoxic conditions (figure 6.6a). In one sample the normoxic fibroblast CM caused a significant increase in IL-10 production compared to normoxic cell line CM, however in T effector cells from healthy PBMC 4 the opposite was true, with hypoxic cell line CM causing a significant increase in IL-10 production compared to hypoxic primary-derived fibroblast CM.

There was a significant difference in IL-10 release from one of four T effector cells between the addition of CM obtained from HNSCC cell line and primary-derived fibroblast CM cultured under hypoxic conditions (figure 6.6b). With cell line CM causing the greatest release of IL-10 when compared to hypoxic fibroblast CM.

*Significant when compared to medium only; paired student t test.
In all other cases, there was no significant difference in IL-10 production from T effectors when comparing the effects of the addition of HNSCC cell line and primary-derived fibroblast CM.

**Figure 6-6** – Bar chart showing the effect of the addition of CM from a) HNSCC cell lines and primary-derived fibroblasts cultured under normoxic conditions and b) HNSCC cell lines and primary-derived fibroblasts cultured under hypoxic conditions; on the production of IL-10 by T effector cells from healthy PBMC (mean ±SEM).

* Significant difference; paired student t test
6.4 - The effect of CM from overnight dispersed tumour on the release of Th1/Th2 cytokines by T effector cells from healthy PBMC

CM from overnight dispersed tumour of laryngeal (n=5) and oropharyngeal (n=5) origin was added to cultures of T effector cells before the CM was harvested. IFN-γ, IL-2, IL-4 and IL-10 ELISAs were carried out on the resultant CM.

6.4.1 – Release of the Th1-like cytokine IFN-γ

In one of four T effector cell populations, the addition of CM from overnight dispersed tumour of laryngeal origin caused a significant increased in IFN-γ production compared to the addition of medium alone. In two out of four samples the addition of CM from overnight dispersed tumour of oropharyngeal origin caused a significant difference in IFN-γ production by T effector cells compared to the addition of medium alone. In one sample the level of IFN-γ decreased upon the addition of oropharyngeal tumour CM compared to medium alone and in the second sample the IFN-γ levels significantly increased upon the addition of oropharyngeal tumour CM (figure 6.7).

The difference in IFN-γ production by T effector cells upon the addition of overnight dispersed tumour CM of different origins (laryngeal and oropharyngeal) was also compared using student t tests. In three out of four T effector cell populations there was a significant difference in IFN-γ production upon the addition of CM from overnight dispersed tumour of laryngeal vs oropharyngeal origin. In two cases the addition of oropharyngeal tumour CM caused reduced production of IFN-γ compared to the addition of laryngeal tumour CM. In one case the opposite happened, with oropharyngeal tumour CM causing a higher level of IFN-γ production by T effector cells compared to laryngeal tumour (figure 6.7).
The addition of laryngeal tumour CM caused a significant difference in IL-2 production by T effector cells in all four samples compared to the addition of medium alone. In one sample the addition of laryngeal tumour CM caused a significant increase in IL-2 compared to the addition of medium alone. In the other three samples the opposite happened with the addition of laryngeal tumour CM causing approximately, a twofold reduction in IL-2 production compared to the addition of medium alone (figure 6.8).

Upon the addition of oropharyngeal tumour CM there was a significant large decrease in IL-2 production by T effector cells in three out of four samples when compared to the addition of medium alone (figure 6.8).

The difference in IL-2 production by T effector cells upon the addition of overnight dispersed tumour CM of different origins (laryngeal and oropharyngeal) was also compared. In three out of four T effector cell populations there was a significant
difference in IL-2 production upon the addition of tumour CM of differing origins. In all three cases the addition of oropharyngeal tumour CM caused the lowest levels of IL-2 production compared to the addition of laryngeal tumour CM (figure 6.8).

![Figure 6-8](image-url) – Bar chart showing the effect of the addition of CM from overnight dispersed tumour of laryngeal (n=5) and oropharyngeal (n=5) origin, on the production of IL-2 by T effector cells from healthy PBMC (mean ±SEM).

* Significant difference compared to medium only; paired student t test

^Significant difference between CM origin

6.3.5.3 - Release of the Th2-like cytokine IL-4

IL-4 levels were below the detectable limit of the ELISA in all T effector cell CM with the addition of HNSCC derived CM making no difference.
6.3.5.4 – Release of the Th2-like cytokine IL-10

In three out of four T effector cell populations, there was no IL-10 production on the addition of medium alone. In one samples there was a low level of IL-10 production. The addition of HNSCC tumour derived CM caused the release of very low level of IL-10, however this was not significantly different from the addition of medium alone. In the T effector cell samples that released IL-10 upon the addition of medium, there was a significant reduction in production upon the addition of both laryngeal and oropharyngeal tumour CM (figure 6.9).

Figure 6-9 – Bar chart showing the effect of the addition of CM from overnight dispersed tumour of laryngeal (n=5) and oropharyngeal (n=5) origin, on the production of IL-10 by T effector cells from healthy PBMC (mean ±SEM).

* Significant difference compared to medium only; paired student t test

^Significant difference between CM origin
6.4 - Discussion

6.4.1 - Th1/Th2 cytokines released by T effector cells obtained from healthy PBMC

As a control, T effector cells obtained from healthy PBMC were cultured with medium alone in order to remove any effect the medium had on the secretion of cytokines from effector T cells, leaving any changes in cytokine levels (IFN-γ, IL-2, IL-4 and IL-10) down to the CM derived from HNSCC cell lines, primary-derived fibroblast and overnight dispersed tumour. This gave the opportunity to look at the Th1/Th2 balance of healthy T effector cells in culture with only the IL-2 and CD3/CD28 bead stimulation.

Results showed that as expected T effector cells from healthy PBMC had a predominantly Th1 cytokine profile. After 48 hours of incubation with medium alone, all four T effector cell samples produced both IFN-γ and IL-2, both of which are the main cytokines known to be released by Th1 cells (Luckheeram et al., 2012). In contrast, only one out of four T effector cell samples released the Th2-like cytokines IL-4 and IL-10 (Luckheeram et al., 2012; Nakayamada et al., 2012; Zhu et al., 2010). Healthy PBMC have not been influenced by cancer derived cytokines and therefore it was thought that the Th1/Th2 balance would lean more towards Th1 in comparison to cancer patients.

Other studies have looked at the Th1/Th2 balance in healthy individuals, though many use whole PBMC as opposed to sorted T effector cells as done in this study. Using whole PBMC has its advantages, as it keep all the cell to cell interaction in place, however, many of the Th1/Th2 cytokines can be released by other cells types making results confusing without further experimental clarification. Agarwal et al (2003) harvested PBMC from 21 normal subjects to look at their expression of IFN-γ, IL-2, IL-4 and IL-10 with the aim of comparing the cytokine production to PBMC obtained from 35 oral squamous cell carcinoma patients. They extracted the mRNA from PBMC and used reverse transcriptase-polymerase chain reaction (RT-PCR) to identify the cytokine expression. They found that 66.6% of healthy PBMC released all cytokines (IFN-γ, IL-2, IL-4 and IL-10) suggesting there was no skew in the Th1/Th2 balance. The majority of the remaining healthy PBMC had a cytokine profile which was skewed towards the
anti-tumour Th1 cytokine production. The production of Th1 cytokines is in agreement with the data expressed in this chapter, however only one of four healthy samples expressed Th2 cytokines, unlike in Ararwal et al (2003) where a large proportion of samples expressed both IL-4 and IL-10. This difference may be due to the different method of detection used as PCR only detects expression but not synthesis and secretion or the fact that it was whole PBMC and not isolated CD4+ T effector cells.

Another study by Goto et al (1999) assessed the cytokine profile of PBMC from healthy subjects and again compared them against PBMC from cancer patients using ELISA on collected supernatant. They found a higher level of secretion of the Th1-like cytokines IL-2 and IFN-γ and the Th2-like cytokine IL-10 in healthy PBMC, but lower levels of the Th2-like cytokine IL-4, compared to PBMC obtained from cancer patients. The presence of IFN-γ, IL-2 and the lower levels of IL-4 agree with the data obtained in this chapter, although the presence of IL-10 is contradictory. This difference may be attributable to incubation length as Goto et al (1999) opted for a shorter incubation time and also used different stimulation.

These data show that healthy T effector cells produce predominantly Th1 cytokines, suggesting they have good capacity for combating tumour growth before they encounter the cytokines in the tumour microenvironment.

6.4.2 – The effect of tumour-derived soluble factors on the release of Th1/Th2 cytokines from healthy T effector cells

Upon the addition of CM from HNSCC cell lines cultured under both normoxic and hypoxic conditions there was a significant increase in the levels of IFN-γ released by T effector cells in three of four samples with two samples showing more than a two-fold increase. Secondly, there was a decrease in IL-2 levels in three of four samples when compared to the addition of medium alone. Both IFN-γ and IL-2 are Th1 cytokines released by Th1 cells and are thought to be anti-tumour (Ruffell et al., 2010; Schreck et al., 2009; Johansson et al., 2008; Sparano et al., 2015). In contrast to what was expected the CM from HNSCC cell lines cultured under both normoxic and hypoxic conditions, only stimulated T effector cells to release IL-4 and IL-10 in one of four samples. It was hypothesised that the tumour-derived CM would tip the Th1/Th2 balance towards the
Th2 response, especially under hypoxic conditions, but these data suggest that the response seen might be sample dependant with effector cells from different individuals responding in an individual manner.

CM from HNSCC cell lines contained various cytokines, including IFN-γ which can cause the differentiation of CD4+ T cells into Th1 cells (Luckheeram et al., 2012), which may explain the increase in the levels of IFN-γ release by the T effector populations upon co-culture. IL-2 and IL-4 are also present in cell line CM which are both known to be involved in the differentiation of CD4+ T cells into Th2 type cells (Zhu et al., 2010), however it appears that in this case the levels of IL-2 and IL-4 were insufficient to push the balance of Th1/Th2 towards a Th2 type pro-tumour environment. The results obtained from the current study suggest that in some cases cytokines alone may not be enough to tip the Th1/Th2 balance towards Th2 and that cell to cell interactions within the tumour microenvironment might need to be present.

The topic of cell to cell interaction contributing to the Th1/Th2 balance has been well reviewed by Burkholder et al (2014). Th1 and Th2 cell are able to self propagate themselves, producing cytokines that can further increase numbers of their parent cell by initiating differentiation of naïve T cells. For example, IL-12 and IFN-γ are released by Th1 cells but can also induce the differentiation of naïve T cells into Th1 cells. Other cells within the TME such as tumour associated macrophages (TAMs) and MDSCs are also able to directly promote Th2 cells and suppressive Th1 cells (Burkholder et al., 2014). Tregs are also able to suppress the differentiation of Th1 effector cells and promote Th2 through the release of TGF-β (Burkholder et al., 2014). This suggests that the Th2 balance may require the presence of other cell types, and that soluble factors alone are not able to drive the increase in pro-tumour Th2 type cells.

In contrast to the cell lines, CM obtained from primary-derived fibroblasts cultured under both normoxic and hypoxic conditions induced no significant change in the levels of IFN-γ but caused a decrease in IL-2 secretion when added to two out of four healthy T effector cell samples. Only one out of four T effector cell populations released IL-4 and two out of four released IL-10 in response to CM from primary-derived fibroblasts. The results suggest that soluble factors released by primary-derived fibroblasts do not
encourage a more anti-tumour microenvironment but nor do they encourage a pro-tumour microenvironment either. Again it suggests that cell to cell interactions are important for a change to the Th1/Th2 balance.

CM from primary-derived fibroblasts cultured under both hypoxic and normoxic conditions contained all the cytokines important in stimulating the differentiation of CD4+ T cells into both Th1 and Th2 subtypes (IFN-γ and IL-2 for Th1 and IL-2 and IL-4 for Th2; chapter 3). Even though the necessary cytokines were available in the CM it appears that no specific trend in Th1/Th2 ratio can be induced by the addition of fibroblast CM. Again, this may be due to the need for cell to cell interactions, or it may be due to the presence of other factors within the CM dampening down the effect.

Again this disagrees with the original hypothesis which suggested that HNSCC-derived CM would tip the Th1/Th2 balance towards a more Th2-like response.

In this thesis, the role of soluble factors in isolation on the function of immune cells was assessed. However, again it appears that CM from primary-derived fibroblasts was unable to produce the Th2 skew reported in many other research journals. Others have found that CAFs are able to influence the Th1/Th2 balance, but require the presence of other cell types. For example, CAFs have been found to produce thymic stromal lymphopoietin (TSLP) which conditions DCs to induce the differentiation of naïve T cells into Th2-type cells (Protti and Monte, 2012; Monte et al., 2011).

Culture medium obtained from overnight dispersed tumour was also cultured alongside T effector cells to determine if soluble factors from the whole tumour could affect the Th1/Th2 balance. The tumour cultures were a mixture of all the cells present within the tumour microenvironment, including cancerous epithelial cells, cancer associated fibroblasts and various immune cells including Tregs, CD4+ T effector cells and CD8+ cytotoxic T cells. Therefore, any soluble factors released were a product of the entire tumour microenvironment. In addition, using the whole dispersed tumour maintains cell to cell communications between different cell types to a certain extent, potentially leading to the secretion of additional factors.
The addition of overnight dispersed tumour CM to healthy T effector cells had no consistent effect on IFN-γ production but caused a significant decrease in IL-2 production in three out of four T effector cells populations. The results for IFN-γ production are in contrast to the results obtained for the addition of HNSCC cell line CM which tended to increase IFN-γ secretion. However, results obtained for IL-2 are similar upon the addition of all HNSCC-derived CM. It is interesting that HNSCC cell line CM can cause an increase in IFN-γ production by T effector cells but whole tumour CM in most instances cannot when the tumour sample also contains cancerous epithelial cells. It may be that epithelial cells in isolation produce a different array of cytokines when not under the influence of cancer associated fibroblasts and immune cells. Some of the differences observed may be down to differences in cytokine content between the CM. As determined in chapter 3 overnight dispersed tumour CM lacked the presence of both IL-4 and IL-17 whereas HNSCC cell line CM contained both. The only cytokine that was present in overnight dispersed tumour CM that was absent in HNSCC cell line CM was IL-22. Both IL-22 and IL-17 are Th17 related cytokines. IL-22 is released by Th17 cells and is involved in inducing a pro-inflammatory response (Ouyang et al., 2008). IL-4 is a cytokine involved in the induction of Th2 cells and inhibition of Th1 cells (Sokol et al., 2008), suggesting that its presence may reduce IFN-γ levels. However, in this case the presence in IL-4 in HNSCC cell line CM did not prevent IFN-γ production. This suggests that other mechanisms are at work in creating a Th2 biased immunosuppressive microenvironment. The effect of immune cells CM on T effector cell function may also contribute to the function of T effector cells.

Overnight dispersed tumour CM caused the greatest reduction overall in Th1 cytokines, which causes a more pro-tumour environment. This suggests a possible role for immune cells which will have been present in the whole tumour.

It was hypothesised that CM from tumour derived cells would result in a skew in the Th1/Th2 balance of CD4+ T effector cells towards a Th2 phenotype from which Th2-like cytokines such as IL-4 and IL-10 are produced. However, this was not found to be the case. No IL-4 and very little IL-10 was detected following the addition of any tumour derived CM to the T effector cells.
The current study is unique in the way it has isolated CD4\(^+\) T effector cells to look at the effect of HNSCC-derived soluble factors on their function. Previous research often uses PBMC or serum from cancer patients to assess whether the immune system of a patient is skewed towards Th2. For example, Bleotu et al (2013) took the serum from 50 laryngo-pharyngeal patients and assessed the levels of IFN-\(\gamma\), IL2, IL-4 and IL-10 using ELISA, therefore was looking at the cytokine profile of all cells present within patient blood. In agreement with some of the results from the current study, they found decreased IL-2 levels and increased INF-\(\gamma\), with the levels of IFN-\(\gamma\) decreasing especially towards the later stage of cancer. However, in contrast to the current results Bleotu et al (2013) observed increases in IL-4 and IL-10 levels in cancer patients. Lathers and Young (2004) looked at plasma levels of Th1 and Th2 associated cytokines in 101 HNSCC patients and 40 age matched controls and found that patient plasma had significantly higher levels of Th2-like cytokines IL-4 and IL-10 and increased level of Th1-like cytokine IL-2 compared to controls. They also found significantly reduced levels of IFN-\(\gamma\) in patients when compared to controls. This suggested a partial Th2 bias, which agrees with the data obtained in this chapter.

In another paper by Sheu et al (2001), co-cultured PBMC from cervical cancer patients with autologous cancer cells to determine how the cancer cells effect the Th1/Th2 balance of cytokines. This is similar to the research in this chapter, however in our case we chose to add just the soluble factors from cancer cells in order to determine their role without the influence of cell to cell interaction. It took 5 days of co-culture before Sheu et al (2001) observed any significant drop in IFN-\(\gamma\) production and 3 to 5 days to see an increase in IL-4. This suggests that 48 hours of incubation of the CD4\(^+\)effector T cells with the CM in this chapter may not have been sufficient time to induce the skew towards Th2.

Another theory as to why a Th2 skew was not observed was the lack of IL-10 secretion by T effector cells. IL-10 is able to inhibit Th1 cytokines. Though there was relatively low levels of IL-10 in the HNSCC-derived CM (chapter 3), very low levels of IL-10 was produced by the T effector cells. If whole PBMC had been cultured instead of just the T effector cells, it might be that the T regulatory cells presence would have been able to kick start the skew towards Th2 by producing IL-10.
Other studies have investigated the release of Th1/Th2 cytokines by whole PBMC from cancer patients and compared it to healthy subjects. For example Agarwal et al (2003) collected mRNA from PBMC harvested from 35 oral squamous cell carcinoma patients and 21 healthy controls and using RT-PCR showed that 63% of patients had a skew in the Th1/Th2 balance with 34% expressing only Th1 cytokines (IFN-γ and IL-2) and 28.5% expressing just Th2 cytokines (IL-4 an IL-10). Though the results are not fully comparable due to the use of RT-PCR and whole PBMC, the fact that some of the oral SCC patients still retain their Th1 cells is in agreement with the data obtained in this chapter where in several cases levels of IFN-γ were raised upon the exposure of T effector cells to HNSCC derived cytokines. As stated previously, the cells may be expressing Th2 cytokines but not releasing them which may be why the ELISA could not detect the Th2 cytokines but the RT-PCR could. In contrast to the current study Goto et al (1999) used ELISA to detect the release of cytokines by PBMC obtained from 38 cancer patients with advanced disease of different origins compared to those of 20 healthy controls and found that patients had lower levels of Th1-like cytokines including IFN-γ, however they did find lower levels of IL-2 similar to the current data.

6.4.3 – The effect of hypoxia-derived CM on the release of Th1/Th2 cytokines by T effector cells

Tumours are known to have hypoxic areas due to the chaotic vasculature (Kumar and Gabrilovich, 2014), therefore HNSCC cell line and primary-derived fibroblasts were cultured in both normoxic and hypoxic conditions before CM was collected to determine whether reduced oxygen levels altered the cytokine profile of the cells (chapter 3) and thus the ability to alter CD4+ T effector cell function. The oxygen levels present when culturing and collecting CM from HNSCC cell lines and primary-derived fibroblasts made no significant difference to the T effector cells ability to produce IFN-γ and IL-10.

As stated previously, in one of four effector T cell populations, the addition of CM from HNSCC cell lines cultured under both normoxic and hypoxic conditions cause a reduction in IL-2 production when compared to the addition of medium alone, and one of four saw a reduction on the addition of primary-fibroblast derived CM. However in one case the addition of CM obtained from cell lines cultured under normoxic
conditions caused a significant difference in the T effector cells ability to release IL-2 when compared to the addition of hypoxic cell line CM with CM collected under normoxia causing a smaller reduction in IL-2. In the second T effector cell population, the addition of CM from primary-derived fibroblasts cultured under normoxic conditions cause a smaller reduction in IL-2 compared to hypoxic conditions. So in both cases the CM collected under hypoxic conditions caused the greatest reduction in IL-2 compared to medium alone. IL-2 is a Th1 cytokine and therefore it could be remarked on that the hypoxic environment has altered the cell line and fibroblasts cytokine profile enough to cause greater reduction in Th1 cytokines to be released by T effector cells, therefore causing a more pro-tumour environment. However, this pattern was not seen in the majority of cases, so further work needs to be carried out to determine how hypoxia can cause the release of cytokines that alter the secretion of IL-2 by T effectors into the TME. Finally, in the majority of cases adding CM obtained under normoxic or hypoxic conditions made no difference to IL-4 production by T effector cells. However, there was one case where the addition of CM from cell lines cultured under normoxia caused the greater release of IL-4 when compared to CM collected under hypoxic.

There was no difference to IFN-γ and IL-10 production when comparing the addition of hypoxia-derived CM and normoxia-derived CM.

The original hypothesis was that CM collected under hypoxia conditions would cause a greater Th2 bias when compared to the addition of CM collected under normoxia. The data in this chapter leads to the rejection of the initial hypothesis as no bias was found and any differences found were sporadic.

Though there was little evidence that soluble factor derived in hypoxia from cell lines and primary-derived fibroblasts were able to create a Th2 cytokines skew, it may be that the T effectors themselves must be exposed to hypoxia and that other cell types must be present. The co-culture of T effector cells and HNSCC-derived CM occurred at normal atmospheric levels of oxygen, however the environment experienced by the T effector cells would have been oxygen depleted. T effectors cells grown in hypoxia have lower proliferation rates and do not expand to the same extent upon TCR stimulation as they would in higher oxygen levels (McNamee et al., 2013). Hypoxia can also directly
reduced the production of Th1 cytokines IL-2 and IFN-γ (McNamee et al., 2013). This suggests that T effector cell function is diminished in hypoxia.

Other cells that are present in the tumour microenvironment such as DCs in the presence of HIF-1α are able to stimulate the production of Th2 cells (Yang et al., 2015; Yang et al., 2009). HIF-1α through a series of events is able to prevent the shedding of the surface molecule CD44 which then interacts with naïve T cells causing them to differentiate into Th2-type cells (Yang et al., 2015). This suggests that soluble factors may be involved in creating the Th2 skew, but require others cells to be present at the time. The HNSCC cell lines and primary-derived fibroblasts cultured in hypoxia may have released HIF-1α which in the right conditions may have caused the Th2 shift to occur.

In conclusion, it is clear that hypoxia plays a role in the Th2 skew, but the cytokines derived from epithelial cell lines and primary-derived fibroblasts cultured in hypoxia were not sufficient to induce a Th2 skew in healthy T effector cells. Further work must be carried out to determine how the soluble factors are involved.

6.4.4 – The effect of tumour subsite on the Th1/Th2 balance

The CM obtained from dispersed tumours of oropharyngeal origin appeared to have a more suppressive effect on the CD4+ T cells than that of laryngeal origin in terms of IFN-γ release with two out of four samples showing a lower level of IFN-γ upon the addition of oropharyngeal CM compared to laryngeal CM. However, the only difference in cytokine production observed between laryngeal and oropharyngeal tumour was the absence of IL-1β in oropharyngeal overnight dispersed tumour CM (chapter 3). IL-1β is a pro-inflammatory molecule predominantly released by monocytes and is thought to be partly involved in the production of IFN-γ (Cooper et al., 2001). The lack of IL-1β may therefore be one reason why IFN-γ production from the CD4+ T effector cells is reduced upon the addition of oropharyngeal tumour CM when compared to laryngeal tumour CM which contained IL-1β. The addition of CM from overnight dispersed tumour of both laryngeal and oropharyngeal origin caused a decrease in the level of IL-2 produced by T effector cells in three out of four samples compared to the addition of medium alone. However, in all three cases, the addition of CM from oropharyngeal origins
caused the greatest reduction in IL-2 production suggesting that soluble factors within this CM have greater suppressive abilities in terms of IL-2 production by T effector cells. IL-4 and IL-10 production by T effector cells was not influenced by overnight dispersed tumour CM. This suggests that the soluble factors produced by HNSCC-derived cells alone are not capable of increasing Th2-like cytokine release. This suggests that other mechanisms are required for the Th1/Th2 balance to shift towards Th2.

It was hard to find studies to directly compare this data with, though one paper looked at the serum levels of Th1 and Th2 cytokines in HNSCC patients. Green et al (2012) used a Quantibody cytokine array to assess levels of IL-2, IL-4, IL-10 and IFN-γ in serum obtained from HNSCC of various origins, including oropharyngeal (n=27) and laryngeal carcinomas (n=57). They found no differences in cytokine levels between the different subsets. This suggests that without the influence of tumour derived cytokines there is no difference between the subsites in terms of Th1 and Th2 cytokines. This partially agrees with the data in this chapter as there was no difference observed in IL-4 and IL-10 levels. However it disagrees with the levels of IFN-γ and IL-2 levels, whereas the data in this chapter found differences. This difference is likely due to the fact that serum was tested in Green et al (2012) which will include cytokines released by all cells types within the blood as opposed to just T effector cells in this chapter.

Overall it appears that soluble factors obtained from oropharyngeal tumour were able to prevent excessive production of Th1-like cytokine IFN-γ and suppress the production of Th1-like cytokine IL-2 to a greater extent than laryngeal-derived CM. Though little difference was detected in the soluble factors in laryngeal and oropharyngeal CM, other soluble factors not included in the assay may be expressed differentially between tumour of different origins.

6.5 – Conclusion

Other research has found that there is a skew towards the Th2 profile in HNSCC patients, particularly patients with late stage disease (Bleotu et al., 2013; Lathers and Young, 2004). In this case there was a slight increase in Th1 type cytokine IFN-γ but a decrease in Th1 cytokine IL-2 but little change in the release of Th2 cytokines (IL-4 and
IL-10) by healthy T effector cells. This data does not show a Th2 skew. Further work must be carried out to determine whether different experimental conditions can bring about the skew toward Th2 seen in other research.
Chapter 7

The effect of HNSCC-derived CM on the function of CD8+ T cells
CD8\(^+\) T cells play an important role in the immune response against foreign antigens, including tumour-associated antigens (Rad et al., 2015; Prado-Garica et al., 2012; Pretsch, et al., 2009). In a healthy individual, a foreign antigen is presented on MHC class I molecules and the CD8\(^+\) T cells become activated causing them to proliferate and differentiate killing target cells by releasing cytokines (TNF\(\alpha\) and IFN-\(\gamma\)), cytotoxic granules (perforin and granzyme) and also by binding of the FasL on the CD8\(^+\) T cell to Fas on the target cell. Once the target has been neutralised, the cells are destroyed via apoptosis (Prado-Garcia et al., 2012).

CD8\(^+\) T cells can be recruited into the tumour mass upon activation (Conlon et al., 2014). In fact it has been shown that CD8\(^+\) T cells accumulate within tumour masses of many types of cancer including breast (Rad et al., 2015), lung (Prado-Garica et al., 2012) and HNSCC (Schoenfeld, 2015) though levels in the periphery remain unchanged (Rad et al., 2015). The presence of CD8\(^+\) T cells within the tumour mass has also been associated with an improved prognosis (Nordfors et al., 2013; Prado-Garcia et al., 2012; Trojan et al., 2004) and many trial therapies have involved increasing tumour-antigen specific CD8\(^+\) T cell levels. However, this anti-tumour response is all too often, ineffective in eradicating the tumour and the lack of success is thought to be due to the tumour suppressing the CD8\(^+\) T cell activity by inhibiting their proliferation, their ability to release Th1 cytokines as well as perforin and granzyme and therefore their cytotoxic potential (Prado-Garcia et al., 2012; Trojan et al., 2004). The exact mechanisms for this suppression of CD8\(^+\) activity by the tumour remains to be fully elucidated but could be due to cell to cell contact or via the secretion of cytokines into the tumour microenvironment. For example, it is thought that tumour cells can directly affect the CD8\(^+\) T cell function and cause their death via apoptosis (Duray et al., 2011), however cancer associated fibroblasts may also have a role to play in the control of anti-tumour immunity.

The current chapter focuses on the ability of the soluble factors released into the tumour microenvironment to alter the function of CD8\(^+\) T cells and aims to determine which cells may be responsible for immunosuppression. Immunosuppressive factors including IL-10 and TGF-\(\beta\) have been identified in the CM obtained from HNSCC-derived cells.
and are thought to be involved in affecting the function of CD8\(^+\) T cells (Prado-Garcia et al., 2012), decreasing both their function and number in the tumour mass (Duray et al., 2010).

In a healthy immune response CD8\(^+\) T cells can be co-stimulated by CD4\(^+\) T effector cells via the release of cytokines, however, in the TME the CD4\(^+\) T effector cells produce less of these cytokines (chapter 3), which leads to a concurrent reduction in CD8\(^+\) function. In the TME there could also be direct suppression from Treg cells which have been found to be present in greater numbers in the TME compared to normal tissue (Prado-Garcia et al., 2012; Duray et al., 2010).

Various methods are available to measure the function of CD8\(^+\) T cells including the radioactive chromium release assay which involves the internalisation of the radioactive isotope by target cells and measurement of its release when lysed by CD8\(^+\) T cells (Zaritskaya et al., 2010). This method has low sensitivity and is subject to false positives due to tumour cells spontaneously releasing the chromium (Zaritskaya et al., 2010). The lack of facilities to handle the radioactive isotope meant an alternative was required for the current study. The intention was to use autologous dendritic cells pulsed with a target protein to activate the CD8\(^+\) T cells and then to use an IFN-\(\gamma\) ELISpot assay to identify cells secreting IFN-\(\gamma\). ELISpot does not directly measure cytotoxic activity so it would have been essential to look for multiple markers such as granzyme B (Zaritskaya et al., 2010). However, despite repeated attempts to culture dendritic cells optimisation was not possible and so the ability of the CD8\(^+\) cells to produce IFN-\(\gamma\) in response to CM from different cell types within the TME was measured using flow cytometry.

7.1.1 – Aims and hypothesis

The aim of this chapter was to determine whether HNSCC derived CM effected the function of CD8\(^+\) cytotoxic T cells from healthy donors and HNSCC patients when compared to the addition of medium alone using IFN-\(\gamma\) production as a marker of function. The role of each cell subset was investigated separately as was the effect of hypoxia. It was hypothesised that HNSCC-derived CM would reduce the ability of CD8\(^+\) T cells to produce IFN-\(\gamma\) therefore acting in an immunosuppressive manner. It
was also hypothesised that the CD8$^+$ T cells from patient PBMC would react to the HNSCC-derived CM to a lesser extent than healthy CD8$^+$ T cells due the possibility of them being pre-primed by the presence of tumour. Finally it was hypothesised that CM collected from cell lines and primary-derived fibroblasts cultured in hypoxia would cause a greater reduction in IFN-$\gamma$ production by CD8$^+$ cytotoxic T cells when compared to the addition of CM obtained under normoxic conditions.

7.2 – Methods

7.2.1 - Assessing the Function of CD8$^+$ T cell using IFN-$\gamma$ production

In order to assess the function of CD8$^+$ T cells, their capacity to make IFN-$\gamma$ was assessed after the addition of HNSCC derived CM.

CD8$^+$ T cells were isolated from normal (n=4) and healthy (n=4) PBMC samples and cells counted using trypan blue exclusion (section 2.6; section 2.1.1.3; table 7.1). Cell concentration was adjusted to $5\times10^4$/66μl of medium before being plated out at 66μl/well in a round bottomed 96 well plate. Cells were incubated for 24 hours at 37 °C before CM from HNSCC cell lines, primary-derived fibroblast and overnight dispersed tumour cultured under both normoxia and hypoxia was added to the appropriate wells to pre-condition the CD8$^+$ T cells (table 3.3 and 7.2). Medium alone was added to some wells as a control and additional wells were included for an isotype control in preparation for flow cytometry. After a further 48 hours of incubation at 37 °C the CD8$^+$ cells were stimulated with PMA (20ng/ml; Sigma) and ionomycin (2μg/ml; Sigma). Brefeldin A (10μg/ml; Sigma) was also added to prevent the transport of IFN-$\gamma$ out of the cells. Media only was added to one well and just the PMA and ionomycin to the others as controls.
Table 7-1 - Patients from which PBMC were harvested to isolate CD8⁺ T cells for culture

<table>
<thead>
<tr>
<th>PBMC samples</th>
<th>Gender</th>
<th>Age</th>
<th>TNM</th>
<th>Specimen site</th>
<th>Node or Tumour (N/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR18</td>
<td>Male</td>
<td>53</td>
<td>T4</td>
<td>Oropharyngeal</td>
<td>T</td>
</tr>
<tr>
<td>TR118</td>
<td>Female</td>
<td>79</td>
<td>T4N0</td>
<td>Oropharyngeal</td>
<td>T</td>
</tr>
<tr>
<td>TR129</td>
<td>Male</td>
<td>60</td>
<td>T4N0</td>
<td>Laryngeal</td>
<td>T</td>
</tr>
<tr>
<td>TR90</td>
<td>Male</td>
<td>56</td>
<td>T1/T2</td>
<td>Laryngeal</td>
<td>T</td>
</tr>
</tbody>
</table>

Table 7-2 - List of overnight dispersed tumour sample from which CM was harvested

<table>
<thead>
<tr>
<th>ON dispersed CM</th>
<th>Gender</th>
<th>Age</th>
<th>TNM</th>
<th>Specimen site</th>
<th>Node or Tumour (N/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laryngeal ON CM 1</td>
<td>Female</td>
<td>65</td>
<td>?</td>
<td>Larynx</td>
<td>N</td>
</tr>
<tr>
<td>Laryngeal ON CM 2</td>
<td>Male</td>
<td>48</td>
<td>T4N2</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Laryngeal ON CM 3</td>
<td>Male</td>
<td>70</td>
<td>T4N0</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Laryngeal ON CM 4</td>
<td>Male</td>
<td>73</td>
<td>T1aN0</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Laryngeal ON CM 5</td>
<td>Male</td>
<td>78</td>
<td>T4N3</td>
<td>Larynx</td>
<td>T</td>
</tr>
<tr>
<td>Laryngeal ON CM 6</td>
<td>Female</td>
<td>72</td>
<td>T4N2b</td>
<td>Larynx</td>
<td>N</td>
</tr>
<tr>
<td>Oropharyngeal ON CM 1</td>
<td>Male</td>
<td>56</td>
<td>?</td>
<td>Oropharynx</td>
<td>N</td>
</tr>
<tr>
<td>Oropharyngeal ON CM 2</td>
<td>Female</td>
<td>70</td>
<td>T3N1</td>
<td>Oropharynx</td>
<td>T</td>
</tr>
<tr>
<td>Oropharyngeal ON CM 3</td>
<td>Male</td>
<td>54</td>
<td>T4N2b</td>
<td>Oropharynx</td>
<td>N</td>
</tr>
<tr>
<td>Oropharyngeal ON CM 4</td>
<td>Male</td>
<td>61</td>
<td>T1N2a</td>
<td>Oropharynx</td>
<td>N</td>
</tr>
<tr>
<td>Oropharyngeal ON CM 5</td>
<td>Male</td>
<td>63</td>
<td>T1N2b</td>
<td>Oropharynx</td>
<td>N</td>
</tr>
</tbody>
</table>
Cells were left for 5 hours at 37 °C to allow full stimulation before the cells were fixed and permeabilised with Leucoperm (Bio-rad). Cells were then stained using a PerCP-Cy5.5 mouse anti-human IFN-γ antibody except for one well which was stained with PerCP-Cy5.5 isotype control.

The FACS Aria™ II was set up for flow cytometry as described in section 2.5.1. A dot plot and a histogram with PerCP-cy5.5 on the x axis were drawn. The first tube (cells only) was run through the analyser to gate the CD8⁺ T cell population, followed by the isotype control which was used to set the P3 gate in order to exclude background staining (figure 7.1a & b).

Figure 7-1 - (a) Dot plot showing the events acquired after CD8⁺ cells were incubated with head and neck derived CM. A gate was drawn around the population that had positive for IFN-γ in later tubes. A high proportion of events were seen up the Y axis (side scatter) due to a high proportion of dead cells from treatment. (b) Histogram of the isotype control tube showing the P3 gate drawn to exclude background staining. The gate was placed to ensure that 99% of events were to the left.

After gates were set, all other tubes were analysed in the same way, ensuring that the P1 gate remained around the desired population.

The mean fluorescence of PerCP-cy5.5 was recorded for each tube along with the percentage of events that were positive for IFN-γ.
7.3 – Results

The first aim was to determine the effect of CM obtained from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under both normoxic and hypoxic conditions on the ability of CD8\(^+\) T cells isolated from healthy and HNSCC patient PBMC to produce IFN-\(\gamma\).

7.3.1 - The effect of cell line and fibroblast derived CM on IFN-\(\gamma\) production by CD8\(^+\) T cells obtained from healthy PBMC

The results from each PBMC sample are presented separately to show the variation in response between samples. Results for each sample were all obtained in the same run and each condition was done only once due to the cell numbers. Results were grouped into the addition of CM from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under both normoxic and hypoxic conditions (table 7.3).

Table 7.3 - Showing the grouping of results for statistical purposes

<table>
<thead>
<tr>
<th>Group</th>
<th>Oxygen status</th>
<th>Sample number (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNSCC cell lines (UMSCC 11b, 81b, 4a, 47b)</td>
<td>Normoxia</td>
<td>4</td>
</tr>
<tr>
<td>HNSCC cell lines (UMSCC 11b, 81b, 4a, 47b)</td>
<td>Hypoxia</td>
<td>4</td>
</tr>
<tr>
<td>Primary-derived fibroblasts</td>
<td>Normoxia</td>
<td>4</td>
</tr>
<tr>
<td>Primary-derived fibroblasts</td>
<td>Hypoxia</td>
<td>4</td>
</tr>
</tbody>
</table>

There was a significant difference in IFN-\(\gamma\) production between the CD8\(^+\) T cell populations obtained from different healthy PBMC controls. The CD8\(^+\) T cells from PBMC sample 1 had approximately four times the base level of IFN-\(\gamma\) production (mean fluorescence) compared with the other three samples (figure 7.2).

CM obtained from HNSCC cell lines cultured under hypoxic conditions caused a significant decrease in IFN-\(\gamma\) production in one out of four CD8\(^+\) T cell populations (sample 1) when compared to addition of medium alone (figure 7.2a). Mean fluorescence dropped from an average of 4040 to 3105. In no other cases did HNSCC
cell line CM cause a significant difference in IFN-γ production when compared to the addition of medium alone.

CM obtained from primary-derived fibroblasts cultured under normoxic and hypoxic conditions caused a significant decrease in IFN-γ production (mean fluorescence) in two out of four CD8⁺ T cell populations (samples 1 and 4) when compared to the addition of medium alone (figure 7.2a & d).

In only one out of four samples did the oxygen levels during the culture of HNSCC cell lines cause a differential effect in IFN-γ production by healthy CD8⁺ T cells. IFN-γ production dropped from an average of 3914 upon the addition of CM from HNSCC cell lines cultured under normoxic conditions to 3105 with hypoxia-derived media (figure 7.2a).

No further significant differences or trends were seen when comparing the addition of CM obtained from cells grown under normoxic and hypoxic conditions. There was also no significant difference between the IFN-γ production by CD8⁺ T cells upon the addition of CM from HNSCC cell line or primary-derived fibroblast origin.

An example of the dot plots obtained show a visual difference in IFN-γ production between the addition of medium compared to CM from cell lines cultured under hypoxic conditions (figure 7.3).
Figure 7-2 – Bar chart showing the effect of CM from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under normoxic and hypoxic conditions on the production of IFN-γ by CD8+ T cells from four healthy PBMC samples (represented by mean fluorescence; a, b, c & d). Each condition for PBMC sample was assayed in duplicate then grouped by CM origin and averaged (mean; ±SEM).

*significant difference when comparing the addition of HNSCC CM to medium alone

^ Significant difference between data sets (p<0.05); student t test.
7.3.2 - The effect of HNSCC cell line and primary-derived fibroblast CM on IFN-γ production from CD8⁺ T cells obtained from HNSCC patient PBMC

The above was repeated on four HNSCC patient PBMC samples.

The base levels (addition of medium alone) of IFN-γ by CD8⁺ T cells varied between the four patient PBMC samples, with the lowest averaging at 384 and the highest at 2080 (figure 7.4).

CM obtained from HNSCC cell lines cultured under normoxic and hypoxic conditions caused a significant decrease in IFN-γ production in two out of four patient PBMC (samples 1 and 3) samples when compared to addition of medium alone (figure 7.4a & c). In patient sample 1 the mean fluorescence went from an average of 1035 upon the addition of medium alone to 765 upon the addition of CM from HNSCC cell lines cultured under normoxic conditions and 728 upon the addition of CM from HNSCC cell lines cultured under hypoxia. There was no significant change in IFN-γ production by CD8⁺ T cells upon the addition of HNSCC cell line CM in the other two patient PBMC samples.
CM obtained from primary-derived fibroblasts cultured under both normoxic and hypoxic conditions caused a significant decrease in IFN-γ production in two out of four CD8⁺ T cell populations and in one case CM from primary-derived fibroblasts cultured under normoxia caused a significant increase in IFN-γ when compared to the addition of medium alone (figure 7.4a, c & d). In PBMC sample 1 the mean fluorescence dropped from an average of 1035 upon the addition of medium alone to an average of 755 upon the addition of CM from primary-derived fibroblasts and in PBMC sample 3 went from 2081 upon the addition of medium to an average 1325 upon the addition of fibroblast CM (figure 7.4a & c). For patient PBMC 4 the mean fluorescence increased from an average of 384 upon the addition of medium to 498 upon the addition of CM from primary-derived fibroblasts cultured under normoxia (figure 7.4d).

A significant difference was seen in the IFN-γ production by CD8⁺ T cells in one out of four patient PBMC samples when comparing the effect of adding CM from HNSCC cell lines and primary-derived fibroblasts cultured under normoxic conditions (figure 7.4c & figure 7.4). The mean fluorescence was 1553 upon the addition of CM from HNSCC cell lines culture under normoxia and dropped to 1325 upon the addition of CM from primary-derived fibroblasts cultured under normoxic conditions. No other significant differences were seen when comparing the addition of CM from HNSCC cell lines and primary-derived fibroblast origin.

There was no significant difference in IFN-γ production from CD8⁺ T cells from patient PBMC when comparing the addition of HNSCC derived CM from normoxic vs hypoxic culture conditions.

An example of the data obtained from the FACS Diva software is shown (figure 7.5). It shows the difference in IFN-γ production between the addition CM from HNSCC cell lines and primary-derived fibroblasts cultured under normoxic conditions to CD8⁺ T cells obtained from HNSCC patient PBMC 3 (figure 7.4c).
Figure 7-4 – Bar chart showing the effect of CM from HNSCC cell lines (n=4) and primary-derived fibroblasts (n=4) cultured under normoxic and hypoxic conditions on the production of IFN-γ by CD8+ T cells from four HNSCC patient PBMC samples (represented by mean fluorescence; a, b, c & d). Each condition for PBMC sample was assayed in singlicate then grouped by CM origin and averaged (mean; ±SEM).

*significant difference when comparing the addition of HNSCC CM to medium alone.

^ Significant difference between data sets (p<0.05).
Figure 7-5 – Example histogram showing the difference in PerCP-Cy5.5 fluorescence produced by lysed CD8$^+$ T cells after culture with (a) CM from HNSCC cell lines (mean fluorescence = 1530) and (b) CM from a primary-derived fibroblast, both cultured under normoxic conditions (mean fluorescence = 1340). PerCP-Cy5.5 fluorescence represents the production of IFN-γ by CD8$^+$ T cells obtained from HNSCC patient PBMC 3 (figure 7.4c).
7.3.3 - The effect of HNSCC-derived overnight dispersed tumour CM on CD8+ T cell IFN-γ production obtained from healthy PBMC

As above, CD8+ T cells were sorted from healthy PBMC (n=4) samples and cultured alongside CM from HNSCC-derived overnight dispersed tumour of laryngeal (n=6) and oropharyngeal (n=5) origin, before IFN-γ production was assessed by FACS. Results obtained were not directly comparable to those obtained from the addition of HNSCC cell lines and primary-derived fibroblast CM due to the inability to enumerate the cell number in the tumour samples, however similar trends could be studied.

Following the addition of CM from HNSCC-derived overnight cultured dispersed tumour of both laryngeal (n=6) and oropharyngeal (n=5) tumours there was a significant decrease in IFN-γ production by healthy CD8+ T cells in two out of four normal PBMC samples (figure 7.6). In healthy PBMC sample 1, mean fluorescence (IFN-γ levels) went from an average of 4040 upon the addition of medium alone to 3102 upon the addition of CM from overnight dispersed tumour of laryngeal origin and 2846 upon the addition CM from overnight dispersed tumour of oropharyngeal origin (figure 7.6a and 7.7). In healthy PBMC sample 4, mean fluorescence went from an average of 1125 upon the addition of medium alone to 932 upon the addition of CM from overnight dispersed tumour of laryngeal origin and 802 upon the addition of CM from overnight dispersed tumour of oropharyngeal origin (figure 7.6d).

There was no significant difference observed in the IFN-γ production between those CD8+ T cells incubated with laryngeal derived CM and those incubated with oropharyngeal derived CM.
Figure 7-6 – Bar chart showing the effect of CM from HNSCC-derived overnight dispersed tumour tissue of laryngeal (n=6) and oropharyngeal (n=5) origin on the production of IFN-γ by CD8⁺ T cells from healthy PBMC samples (n=4) represented by mean fluorescence; a, b, c & d). Each condition for PBMC sample was assayed once then grouped by CM origin and averaged (mean; ±SEM).

*significant difference when comparing the addition of HNSCC CM to medium alone.
7.3.4 - The effect of HNSCC-derived overnight dispersed tumour CM on CD8$^+$ T cell IFN-$\gamma$ production obtained from HNSCC patient PBMC

As above, CD8$^+$ T cells were sorted from HNSCC patient PBMC samples (n=4) and cultured alongside HNSCC-derived overnight dispersed tumour CM of laryngeal (n=5) and oropharyngeal (n=4) origin, before IFN-$\gamma$ production was assessed.

A significant decrease in IFN-$\gamma$ production from CD8$^+$ T cells was seen in three out of four patient PBMC samples upon the addition of CM from HNSCC-derived overnight dispersed tumour of laryngeal origin when compared to medium alone (figure 7.8). In Patient PBMC sample 1 the IFN-$\gamma$ production went from an average mean fluorescence of 1035 upon the addition of medium to 767 upon the addition of overnight CM of laryngeal origin. Sample 2 went from 1805 to 1636 and sample 3 went from 2081 to

**Figure 7.7** - Example histogram showing the difference in PerCP-Cy5.5 fluorescence produced by lysed CD8$^+$ T cells after culture with (a) medium (mean fluorescence = 536), (b) CM from HNSCC overnight dispersed tumour of laryngeal origin (mean fluorescence = 805) and c) HNSCC overnight dispersed tumour of oropharyngeal origin (mean fluorescence = 702). PerCP-Cy5.5 fluorescence represents the production of IFN-$\gamma$ by CD8$^+$ T cells obtained from healthy PBMC 1 (figure 7.6a).
CM from HNSCC-derived overnight tumour of oropharyngeal origin caused a significant decrease in IFN-γ in two out of four patient PBMC samples. Patient PBMC sample 1 went from an average mean fluorescence of 1035 upon the addition of medium to 715 upon the addition of overnight dispersed tumour of oropharyngeal origin and patient PBMC sample 3 went from 2081 to 1544 (figure 7.8a & c).

No significant difference was seen in CD8+ T cell IFN-γ production upon the addition of CM from overnight dispersed tumour from different subsites (laryngeal and oropharyngeal; figure 7.8).

7.3.5 – Differences in IFN-γ production between CD8+ T cells obtained from healthy and HNSCC patient PBMC

No significant difference in the presence of IFN-γ positivity in CD8+ cells was seen between PBMC obtained from HNSCC patients (n=4) and healthy donors (n=4) upon the addition of medium only and HNSCC derived CM (figure 7.6 and 7.8).
Figure 7-8 – Bar chart showing the effect of CM from dispersed tumour tissue of laryngeal (n=6) and oropharyngeal (n=5) origin on the production of IFN-γ by CD8⁺ T cells from four patient PBMC samples (represented by mean fluorescence; a, b, c & d). Each condition for PBMC sample was assayed in singlicate then grouped by CM origin and averaged (mean; ±SEM).

*significant difference when comparing the addition of HNSCC CM to medium alone
7.4 - Discussion

CD8+ T cells have been found to infiltrate many types of tumour including colorectal carcinoma (Chaput et al., 2009) and HNSCC and have reduced proliferation and suppressed cytotoxic function once in the TME (Prado-Garcia et al., 2012; Pittet, 2009; Zippelius et al., 2004). The CD8+ T cells in the periphery of cancer patients have full functionality (Pittet, 2009; Zippelius et al., 2004) suggesting that either soluble factors such as cytokines and/or the different cell types present within the TME are responsible for altering CD8+ T cell function. In this chapter, the role of soluble factors, released from specific cell types within the TME, in altering the function of CD8+ T cells was assessed. CD8+ T cells from the peripheral blood of both healthy donors and HNSCC patients were used to determine whether HNSCC patient derived peripheral CD8+ cells were pre-conditioned by the presence of tumour by comparing the results with those from healthy donor PBMC. The CD8+ cells were cultured alongside HNSCC derived CM in the hope of emulating the conditions experienced by the immune cells within the TME in terms of soluble factors.

7.4.1 – The effect of HNSCC derived CM on the function of CD8+ T cells from healthy and HNSCC patient PBMC

In two out of four healthy and HNSCC patient CD8+ T cells, soluble factors present within the TME were able to suppress the function of CD8+ T cells by reducing their ability to produce IFN-γ. CM from primary-derived fibroblasts cultured in both normoxia and hypoxia and overnight dispersed tumour of laryngeal and oropharyngeal origin were able to suppress the production of IFN-γ in both normal and patient-derived CD8+ T cells and CM from cell lines were able to suppress IFN-γ production of CD8+ T cells from patient PBMC. The results were very consistent, with the same two healthy CD8+ T cell populations seeing a decrease in IFN-γ upon the addition of CM from primary-derived fibroblasts and overnight dispersed tumour. Also the same two HNSCC patient populations saw a decrease in IFN-γ production upon the addition of all HNSCC-derived CM. Both patient and healthy derived CD8+ T cells reacted to some CM but not to others suggesting that the response seen is specific to the individual and that the cancer has not pre-primed the CD8+ T cells in a way that makes them more or less susceptible to influence from tumour derived factors.
In the CD8\(^+\) T cell populations from both healthy and patient PBMC that reacted to the HNSCC CM, there seemed to be the same level of reduction of IFN-\(\gamma\) production following the addition of HNSCC cell line and primary-derived fibroblast CM as well as overnight dispersed tumour CM. This suggests that both the cancerous epithelial cells and cancer associated fibroblasts have the ability to release cytokines that reduce the cytotoxic function of CD8\(^+\) T cells. Although the effect may not be additive since there was no further reduction in IFN-\(\gamma\) production following the addition of overnight dispersed tumour CM which is from cultures which contain both these cell types and also immune cells. However, it is important to remember that it was not possible to obtain a cell count for overnight dispersed tumour cultures so results are not directly comparable.

As well as aiding in cytotoxicity by CD8\(^+\) T cells, IFN-\(\gamma\) is also involved in stimulating the differentiation of CD4\(^+\) Th1 T cells which have anti-tumour activity (Luckheeram et al., 2012; Pepper and Jenkins, 2011). Therefore a reduction in IFN-\(\gamma\) may also reduce the ability of the immune system to prevent tumour growth.

Although these exact experiments have not been carried out by others, similar research has found comparable results to those highlighted here. In most cases tumour infiltrating lymphocytes were harvested from tumours and cultured alongside autologous tumour cells to see if a cytotoxic response was initiated. Yoshino et al (1992) took this approach and found that 100% of TILs obtained from lung cancer patients failed to react to the presence of autologous tumour cells, even in the presence of IL-2. They concluded that this may be down to the presence of immune-suppressive factors within the TME having pre-conditioned the immune cells to become immunosuppressive or inert. The same outcome from lung cancer was also obtained by Prado-Garcia et al (2012) who in addition found that CD8\(^+\) T cell function could be restored post TME exposure by treatment with IL-2.

Other cytotoxic effector molecules not assayed in this chapter, such as perforin and granzymes were also found to be reduced in TILs from lung cancer patients, showing an overall reduction in function (Prado-Garcia et al., 2012). Zippelius et al (2004) also found that TILs obtained from melanoma tumours had reduced levels of IFN-\(\gamma\), granzymes B and perforin compared to those in the periphery suggesting a dampening
of their function in the TME. Overall the same reduction in function of CD8+ T cells induced by factors within the TME appears to be a similar theme in various types of cancer.

Another possible reason for the reduction of IFN-γ production by the CD8+ T cell cultures is programmed cell death (Prado-Garcia et al., 2012; Ahmadzadeh et al., 2009). It has been found that CD8+ T cells within the TME are positive for the programmed death-1 receptor (PD-1) in contrast to CD8+ T cells from normal tissue where PD-1 is negative causing CD8+ cells from the tumour to be more susceptible to death (Ahmadzadeh et al., 2009). It is possible that soluble factors within the TME up-regulate PD-1 on CD8+ T cells causing increased apoptosis of these cytotoxic cells as a means of immune evasion. In addition Ahmadzadeh et al (2009) found that PD-1 positive CD8+ T cells had lower levels of proliferation and release lower levels of effector cytokines (such as IFN-γ). They used immune-histochemical techniques to stain for IFN-γ in TILs that were PD-1 positive and PD-1 negative and found that PD-1 positive cells produced significantly less IFN-γ. In terms of this experiment, it could mean more cell death upon co-culture with HNSCC derived CM, leading to lower levels of IFN-γ being produced.

Soluble factors present within the tumour microenvironment which may be responsible for the results observed include the immunosuppressive factors IL-10 and TGF-β which are both able to suppress the function of CD8+ T cells (Prado-Garcia et al., 2012; Thomas and Massague, 2005). Thomas and Massague (2005) purified CD8+ T cells from mouse spleens in order to culture them in the presence and absence of TGF-β and found that the presence of TGF-β caused a reduction in the expression of perforin, granzymes and IFN-γ by CD8+ T cells. The HNSCC derived CM from all sources in the current study, co-cultured with CD8+ T cells contained both IL-10 and TGF-β (shown using ELISA; chapter 3) making this a viable theory regarding the reduction of IFN-γ production in 50% of CD8+ T cell populations.
7.4.2 – The differential effect of HNSCC derived CM on CD8+ T cells IFN-γ production from healthy and HNSCC patient PBMC

The hypothesis was that CD8+ T cells obtained from peripheral blood of HNSCC patients had been pre-primed by the presence of the tumour and would therefore already have reduced cytotoxicity and therefore would not react to the addition of CM to the same extent of un-primed healthy CD8+ T cells. This knowledge would give the opportunity to see if peripheral CD8+ T cells are primed by the TME, so allowing an assessment to whether immunotherapies should be targeted at the immune system as a whole or just at the tumour level.

In the current study the hypothesis was disproved suggesting that pre-priming of peripheral CD8+ cells does not occur, and that proximity to the tumour is required for suppressive effects to be induced. The work by Trojan et al (2004) further validates this theory. They took TILS from 31 human non-small cell lung cancer tumours and compared the IFN-γ expression in these cells with that in lymphocytes from peritumoral areas using qPCR. IFN-γ expression was found to be 2.5x higher in the lymphocytes isolated from the tumours compared to those from the peritumoral area indicating that the CD8+ T cells must be within the confines of the tumour itself to be suppressed.

7.4.3 – The effect of hypoxia-derived CM on the IFN-γ production of CD8+ T cells

The data from the current study found that in the majority of cases CM obtained from cell lines and primary-derived fibroblasts cultured in hypoxia had no differential effect on CD8+ T cells ability to produce IFN-γ when compared to CM from normoxia. It was hypothesised that the CM obtained from cell lines and fibroblasts cultured in hypoxia would cause a greater decrease in IFN-γ production by CD8+ T cells. The data in this chapter disproves this hypothesis.

The data obtained in chapter 3 found that cell lines cultured in hypoxia produced significantly greater levels of IL-2, TGF-β and TNF-β compared to cell lines cultured in normoxia. There was no significant difference in cytokine levels between primary-derived fibroblasts cultured in hypoxia and normoxia. As CM from cell lines cultured in hypoxia caused no difference in IFN-γ the data suggests that the soluble factors produced in these culture conditions are unable to affect CD8+ T cell function in this
way. It may be that the CD8$^+$ T cells themselves need to be exposed to the hypoxic environment for them to be suppressed or that they require the presence of other cell types such as immune cells. As there was also little differential effect between CM collected from cell lines and primary-derived fibroblasts, it may be that other cells such immune cells present within the TME, such as Tregs are more involved in suppression of CD8$^+$ T cells than soluble factors or that they work in tandem. Yang et al (2011) found that CD4$^+$ T cells treated with IL-2 are able to suppress the proliferation of CD8$^+$ T cells and production of perforin and granzyme B compared to CD4$^+$ T cells not treated with IL-2. Hilchey et al (2007) also found that Treg can suppress the proliferation for CD8$^+$ T cells and also the ability of CD8$^+$ T cells to release IFN-γ. These data suggest that soluble factors are important in the TME and are involved in the suppression of CD8$^+$ T cells but require the presence of other immune cells. It has also been found that direct contact of CD8$^+$ T cells with tumour cells causes their suppression via the binding of PD-1 which will lead to anergy or apoptosis (Barsoum et al., 2014). Again this points to a more cell to cell contact independent suppression.

7.5 - Conclusion

Due to the ability to restore CD8$^+$ T cell function (Prado-Garcia et al., 2012) tumour infiltrating CD8$^+$ T cells make a very promising target for immunotherapy. Therefore it is of upmost importance to gain a better understanding of the methods by which the tumour influences them in the hope of producing a more effective therapy.

In the current chapter it appears that the soluble factors within the TME may play a role in suppressing the function of infiltrating CD8$^+$ T cells, but further work into the exact mechanisms involved in the suppression is necessary. If the soluble factors involved in this suppression could be identified, targeted immunotherapy towards these may be possible to restore cytotoxic function therefore increasing the chance of tumour eradication.
Chapter 8

Final discussion
The overall aim of this thesis was to assess the ability of HNSCC-derived soluble factors to alter the proliferation and function of immune cells. It has been well documented that various immune cells infiltrate the tumour micro-environment in many cancer types and that once inside, are subject to immune-suppressive activities. These infiltrating immune cells include Tregs, CD4\(^+\) T effector cells, CD8\(^+\) T cells, DCs and macrophages, all of which release their own array of cytokines (Fridman et al., 2012). Immune-suppression comes in many forms, including both cell to cell interactions with cancer cells and associated stroma and the presence of soluble factors (Whiteside, 2008). Though cell to cell interactions clearly play an important role, this work focused on the ability of soluble factors to induce a more immune-suppressive tumour microenvironment. By studying the role of soluble factors within the TME, it may lead to a better understanding of the suppressive mechanisms at work, possibly leading to new approaches in immune-therapies. This could lead to better prognostic markers, tailored therapies and improved long-term survival for HNSCC patients.

The current study has shown that soluble factors derived from HNSCC-cells are capable of affecting the proliferation and function of Treg, T effectors cells and CD8\(^+\) T cells in the absence of cell to cell contact. CM collected from overnight dispersed tumour was able to reduce the percentage of CD8\(^+\) T cells whilst increasing the number of Tregs within all healthy PBMC samples investigated. The CM from overnight dispersed tumour was also able to significantly increase the suppressive activity of the Tregs in 20\% (2/5) of healthy PBMC samples and decrease the cytotoxic capabilities of healthy CD8\(^+\) T cells in 50\% (2/4) compared with the addition of medium alone. Suppressive factors such as IL-10 and TGF-\(\beta\) were found within the majority of this overnight dispersed tumour CM which could have contributed towards the effects.

To date, the main form of immunotherapy for HNSCC is the use of Cetuximab used in conjunction with radiotherapy (Lalami and Awada, 2016). However, long term survival rates remain low with only around 50\% of HNSCC patients surviving 5 years (Teymoortash et al., 2014). There is a need for new and improved targeted therapies. One branch of research has looked to immunotherapies as the answer.
One promising form of treatment is T cell therapy where autologous T cells from the patient are genetically engineered to be able to target tumour-associated antigens (Sharpe and Mount, 2015). The aim is to harness the dormant potential of the immune system within cancer patients, which has been shown to be suppressed but not beyond repair. This form of treatment requires initial lymphocyte-depletion to reduce the level of Tregs in the patient to prevent these cells from suppressing the new set of T cells being infused (Sharpe and Mount, 2015). However, during trials varying responses to this form of therapy was found, suggesting other factors may prevent the potential tumour killing power of the adopted T cells. It may be that in some patients the soluble factors within the tumour-microenvironment, such as the immune-suppressing factors IL-10 and TGF-β, or one of the many other factors identified using the cytokines array, are preventing the full potential of these transferred T cells. The importance of these soluble factors within the TME is becoming more known among those trying to design and improve immune-therapies leading to new ideas and potential improvements to existing therapies.

Headway is being made in the development of a cancer therapy which uses a TGF-β antagonist to inhibit the immunosuppressive nature of this cytokine. Mouse models have shown some promising results, with some recipients showing a positive anti-tumour response (Yang et al., 2015). However, the same study also found that TGF-β therapy produced undesirable effects in others, such as stimulation of metastases (Yang et al., 2015). It is important to remember the dual role of TGF-β and the heterogeneity in human cancers. In some cancers as described previously, TGF-β can be pro-tumourigenic whereas in others it can be anti-tumourigenic (Pickup et al., 2013). It would be important therefore to identify the patients who would benefit from this form of therapy due to their unique tumour/ immune system interaction; otherwise blanket use could cause further progression of the cancer, and therefore a poorer prognosis.

Hypoxia is a well documented characteristic of solid tumours and has been found to play a role in the immunosuppressive tumour microenvironment by the production of certain soluble factors such as HIF-1α (Labiano et al., 2015; Barsoum et al., 2014). The current study assessed the effect of hypoxia-induced soluble factors on the proliferation and function of immune cells cultured in normoxia and found that the cell lines cultured
under hypoxia produced significantly greater levels of the immunosuppressive factor TGF-β compared to those grown in normoxia, but failed to cause any significant difference in proliferation or function of immune cells compared to soluble factors derived from cell lines cultured in normoxia. These data suggest that any additional immune-suppression induced by hypoxia may require the presence of reduced oxygen levels at the time of the experiment and/or cell to cell contact. This in contrast to other information suggesting that hypoxia has the ability to interfere with the anti-tumour immune response by reducing the proliferation and function of T cells within the TME (Barsoum *et al*., 2014). One of the main factors produced in response to hypoxia is HIF-1α and the accumulation of adenosine within the TME has been observed; both of which act as negative regulators of immune cells (Labiano *et al*., 2015; Thiel *et al*., 2007).

Hypoxia is known to cause resistance to cancer therapies, therefore reducing a patient’s chance of long term survival, making hypoxia-related targets a growing topic of discussion (Barsoum *et al*., 2014; Lee *et al*., 2010). Popular ideas include targeting of the adenosine-A2AR pathway and HIF-1α (Noman and Chouaib, 2014; Sitkovsky and Ohta, 2013; section 1.11). One idea suggested the culture of tumour-reactive T cells with a reduced number of A2ARs, whereby T cells from patients are cultured in the presence of an A2AR antagonist. Cells which are able to proliferate in these conditions would be selected for further growth, leading to a population of cells with lower levels of A2AR to be transfused back into the patient, meaning they will not be subject to immune-suppression via increased levels of adenosine in the TME (Sitkovsky and Ohta, 2013). Other ideas revolve around trying to relieve the oxygen tension within the TME by using thermal therapies (Lee *et al*., 2010).

Overall, it seems that the effects of hypoxia must be in some way neutralised if therapies are going to be successful. Thus new treatment modalities that can target hypoxia are likely to be useful adjuvants for other immune therapies already available.

The TME includes many different cells types, including cancer cells and associated stroma, all of which play a role in tumour development. Cancer-associated fibroblasts are known to play a role in tumour growth, invasiveness and angiogenesis (Zhang and Liu, 2013). It has recently become apparent that they also contribute to the immune-
suppressive TME via the release of soluble factors such as TGF-β and HIF-1α (Takahashi et al., 2015). The current study found that CM collected from HNSCC primary-derived fibroblasts were able to increase the proliferation of whole PBMC and decrease CD8\(^+\) T cells in 25\% (1/4) of PBMC samples and increase levels of Tregs and T effector cells in 100\% and 75\% (4/4 and 3/4) of samples respectively. In some cases, but not all, primary-derived fibroblast CM was able to increase Treg percentage to a greater extent than CM from HNSCC cell lines, suggesting a tumour-specific response. Fibroblast CM was also able to alter the function of CD8\(^+\) T cells by reducing the production of IFN-\(\gamma\) in 50\% (2/4) of samples. They also reduced the production of Th1 inducing cytokine IL-2 by T effector cells in 50\% (2/4) of samples. These data suggest that both cancer cells and primary-derived fibroblasts are able to induce a more immunosuppressive environment, particularly through the ability to increase Treg levels. Knowledge of the mechanisms employed by cancer-associated fibroblast may lead to a more effective form of therapy. Although this may be the case with some individuals, it may be that the ability to increase Treg levels in this way is sample specific. This is a recurrent theme throughout this thesis; some healthy PBMC reacted to the HNSCC-derived soluble factors where other did not. Different people’s immune cells may respond to soluble factors in different ways or to differing degrees, which may be an indication as to whether a person is more susceptible to the development of cancer.

TGF-β, found to be released by CAFs is a promising target for new therapies due to the ability to stimulate Treg. By using neutralising anti-TGF-β antibodies it may be possible to prevent TGF-β signalling, therefore suppressing the levels of Tregs within the TME, and allowing other T cells to destroy the tumour (Zhang and Liu, 2013; Mazzocca et al., 2009). Other novel targets for CAFs include fibroblasts activation protein (FAP) which is expressed in tumour stroma (Zhang and Liu, 2013; Santos et al., 2009). Santos et al (2009) used mouse models to determine that depletion of FAP leads to the inhibition of tumour growth, suggesting a promising target for therapies.

The difficulty with targeting CAFs is again the variation between cancer types and patients. This is a factor that will have to be overcome in developing new therapies, and
it may be that a multimodal approach will be needed to gain positive results for the patient.

8.2 – Conclusion

In conclusion, immunotherapies are making steady progress towards being able to successfully treat cancer patients including HNSCC. The focus seems to be on targeting the immune cells within the TME, however, it is likely that a combination of therapies, aiming at a range of targets, including soluble factors will be required for full success of these treatments. The current project has added further to the knowledge behind the role of soluble factors within the tumour microenvironment, in the hope that these data will help develop new ways of boosting current immunotherapies by targeting soluble factors. A major obstacle for successful treatment is being appreciative of the heterogeneity within the TME between cancer types and between patients with the same disease. It may be that immunotherapies will have to be engineered on an individual patient basis to provide the best chance of survival (Lalami and Awada, 2016). This means the expression of immune suppressive molecules within each tumour would need to be identified in order to select the most appropriate treatment for each patient.

8.3 – Future work

The current study has established that HNSCC-derived soluble factors can influence the proliferation and function of immune cells. However, there are many questions that remain to be answered.

One of the original hypotheses was that HNSCC patient PBMC would be pre-primed by the TME and would therefore react to a lesser extent than healthy PBMC to HNSCC-derived CM. It was found that healthy PBMC proliferated to a greater extent upon the addition of HNSCC-derived CM compared to patient PBMC but this only goes part of the way to answering the question. Due to lack of HNSCC patient samples of the desired HNSCC sub-site, it was not possible to compare the effect of HNSCC-derived CM on the function of patient PBMC. Given time, it would be possible to gather enough patient PBMC samples and enough tumour to collect overnight dispersed tumour CM to get a full set of data. This would allow a more thorough investigation as to whether tumour-priming of peripheral immune cells occurs. If pre-priming was found to occur,
then it could possibly have effects on immunotherapies that use peripheral T cells. Some immunotherapies rely on extracting patient immune cells in order to expand them in culture before transferring them back into the patient. If these cells had already been altered or pre-primed by the presence of soluble factors from the TME, then this may decrease the success of this form of treatment.

Hypoxia-induced therapeutic targets are currently a hot topic. However, in the current thesis the CM was collected under hypoxia, but the reactions between this CM and the immune cells were carried out in normoxia. In contrast to other research, it was found that in this context, hypoxia did not have a significant effect on the function of Tregs, CD4\(^+\) T effector cells and CD8\(^+\) T cells. It would be interesting to see if carrying out the whole experiment within the hypoxic chamber had any effect on the ability of the CM to alter the immune cells function. This information would further the knowledge regarding the influence of hypoxia-induced soluble factors from the TME on immune suppression.

HPV-associated HNSCCs are linked with better prognosis and improved long term survival (Duray et al., 2014). Understanding why patients with HPV-associated HNSCC have improved survival may lead to a better understanding of the tumour microenvironment which consequently may lead to new therapy ideas. During the time of the project, tumours from patients presenting at Castle Hill hospital in Hull were not routinely tested for HPV status and following the same treatment regimen as their HPV negative counter parts. It would be interesting to investigate whether there are HPV associated soluble factors released from these HPV positive tumours and whether they are involved in the observed improved prognosis of HPV patients.

This thesis found evidence that tumour-derived soluble factors were able to alter proliferation and function of immune cells. However, which factors were responsible for these activities was not fully determined. Other research has focused on the role of IL-10 and TGF-\(\beta\) within the TME, yet the data in this thesis revealed high levels of other interesting cytokines within the CM, such as IL-21 and IL-23 which are both involved in T cell function. IL-21 is involved in the function of CD8\(^+\) cytotoxic T cells and IL-23 is involved in their infiltration into the tumour (Ettinger et al., 2008). It would be interesting to systematically block each cytokine of interest using antibodies
within the CM and repeat the experiments. This would identify which cytokines are involved and with which immune cells. Then further work could be carried out to identify the mechanisms behind their activity. This information would be of use when developing immunotherapeutic strategies that involve the infusion of autologous T cells into cancer patients. Improving the knowledge of the environment that these T cells will face may improve the treatments available.
Bibliography


fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 303, 848–851.


compared with the periphery of head and neck cancer patients. Clinical & Cellular Immunology 5, 1-7.


leads to treg recruitment through CCl22 production by tumor cells. Cancer Research 71, 6143–6152.


stromal fibroblasts is closely associated with the nodal metastasis and outcome of patients with invasive ductal carcinoma who received neoadjuvant therapy. Human Pathology 41, 262–270.


ccl4 and ccl20, are strong reciprocal predictive markers for survival of patients with oesophageal squamous cell carcinoma. British Journal of Cancer 113, 747–755.


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Appendix 1
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Secreted by</th>
<th>Function</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>GM-CSF</td>
<td>Macrophages, T cells, mast cells, NK cells, endothelial cell and fibroblasts</td>
<td>A hematopoietic growth factor which is involved in the immune/inflammatory cascade and so crucial for fighting infection. It stimulates stem cells to produce granulocytes and monocytes which mature into macrophages and DCs. It is also important in the proliferation and differentiation of hematopoietic precursors and also enhances the function of mature effector cells.</td>
<td>(Spiekermann et al: 1997)</td>
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<td>(Baldwin et al: 1989)</td>
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<tr>
<td>IFNγ</td>
<td>NK cells, CD4⁺ Th1 cells (T helper cells) and CD8⁺ cytotoxic T cells</td>
<td>Has immunostimulatory and immunomodulatory effect and is essential for innate and adaptive immunity. Vital for immune response against viral and bacterial infection as well as against tumour cells. Activates macrophages and promotes NK cell activity.</td>
<td>(Schroder et al: 2004)</td>
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<td>(Ikedaa et al: 2002)</td>
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<tr>
<td>IL-1β (aka catabolin)</td>
<td>Activated macrophages</td>
<td>Released in an inactive form before being cleaved to activate. A key mediator of the inflammatory response. An increased production of IL-1β leads to auto-immune conditions.</td>
<td>(Dinarello: 2009)</td>
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<tr>
<td>IL-2</td>
<td>Activated T cells (after antigen bind TCR)</td>
<td>Involved in the growth, proliferation and differentiation of CD4⁺ and CD8⁺ T cells. Involved in the development and maturation of T regulatory cells which work to then work to prevent the secretion of further IL-2 to protect from immune</td>
<td>(Gaffen &amp; Liu: 2004)</td>
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<td>Cytokine</td>
<td>Sources</td>
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<tr>
<td><strong>IL-4</strong></td>
<td>Possibly by Basophils (not confirmed)</td>
<td>Induces the differentiation of helper T cells into Th2 cells which will cause a decrease in the Th1 cells and the increase in production of immunosuppressant factors IL10 and TGF-β1. Stimulates the proliferation of B and T cells and the differentiation of B cells into plasma cells. Up regulates the production of MHC class II. Also down regulates IFNγ. (Sokol et al: 2008)</td>
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<tr>
<td><strong>IL-5</strong></td>
<td>Th2 Cells and mast cells</td>
<td>Stimulates B cell differentiation and growth. Increases Ig secretion. A mediator of eosinophil activation, recruitment and survival. (Dubucquoi et al: 1994)</td>
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<tr>
<td><strong>IL-6</strong></td>
<td>T cells and macrophages</td>
<td>A pro-inflammatory cytokine with many different functions. It is involved in the induction of the acute phase response, inflammation and oncogenesis. It work to increase production of neutrophils and is involved in B cell growth. It is also an antagonist of T regulatory cells. (Simpson et al: 1997) (Kishimoto: 2010)</td>
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<tr>
<td>Cytokine</td>
<td>Source/Function</td>
<td>References</td>
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<tr>
<td>IL-10</td>
<td>Produced primarily by monocytes and by lymphocytes (Th2 cells and CD4⁺CD25⁺FoxP3⁺ T regs) to a lesser extent.</td>
<td>(Moore et al: 2001)</td>
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<td></td>
<td>An anti-inflammatory cytokine which inhibits the function of T cells, monocytes and macrophages.</td>
<td>(Fiorentino et al: 1991)</td>
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<td></td>
<td>Reduces the synthesis of certain pro-inflammatory and Th1 cytokines such as IFNγ, IL-2, TNFα and GM-CSF.</td>
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<td></td>
<td>Involved in the proliferation and survival of B cells.</td>
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<td></td>
<td>So it generally down regulates the immune response.</td>
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<tr>
<td>IL-12p70</td>
<td>DCs and macrophages in response to antigen stimulation.</td>
<td>(Trinchieri: 1995)</td>
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<td></td>
<td>A pro-inflammatory cytokine which promotes the differentiation of naive T cells into Th1 cells.</td>
<td>(Trinchieri: 1994)</td>
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<td></td>
<td>It stimulates the production of other pro-inflammatory cytokines such as IFN-γ and TNFα.</td>
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<td></td>
<td>Reduces IL-4s ability to suppress the secretion of IFNγ.</td>
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<td></td>
<td>Enhances the cytotoxicity of CD8⁺ T cells and NK cells.</td>
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<td></td>
<td>It is anti-angiogenic, so has the ability to prevent the formation of new blood vessels.</td>
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<tr>
<td>IL-13</td>
<td>Activated Th2 cells</td>
<td>(Wynn: 2003)</td>
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<td></td>
<td>An anti-inflammatory cytokine which has very similar function to IL-4.</td>
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<td></td>
<td>It differs from IL-4 in that it is involved in certain physiological changes induced by allergic inflammation.</td>
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<td></td>
<td>Induces IgE secretion from B cells and is involved in the differentiation and maturation of B cells.</td>
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<td></td>
<td>Down-regulates macrophages which in turn reduces the production of pro-</td>
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<td>Cytokine</td>
<td>Source Cells</td>
<td>Function and Regulation</td>
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<tr>
<td>IL-17A and IL-17F</td>
<td>T helper cells (Th17 cells)</td>
<td>IL17A and IL17F are both members of the IL-17 family all with a similar structure and function. A pro-inflammatory cytokine which increases the production of other pro-inflammatory cytokines such as IL-6, GM-CSF, IL-1β, TGF-β1 and TNFα. This attracts monocytes and neutrophils to the site of inflammation. Plays an essential role in the function of Th17 CD4+ T cells.</td>
<td>(Korn et al: 2009) (Hurst et al: 2002)</td>
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<td>IL-22</td>
<td>Activated DCs and T cells</td>
<td>Part of the IL-10 family of cytokines. A pro-inflammatory cytokine which targets non-hematopoietic cells such as epithelial cells. Mediates the innate immune response and is Immunoregulatory in cancer. Enhances the barrier function of epithelial cells. Has a protective role in the body however can contribute to disease.</td>
<td>(Sonnenberg et al: 2010) (Rubino et al: 2012)</td>
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<td>IL-23</td>
<td>DCs and</td>
<td>A pro-inflammatory heterodimeric cytokine which exerts its effect on</td>
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<td><strong>IL-28A</strong></td>
<td>APCs upon viral activation</td>
<td>memory CD4+ T cells. Stimulates angiogenesis. Decreases the infiltration of CD8+ T cells. Stimulates naive CD4+ T cells to differentiate into Th17 T cells, which increases production of IL-17 and other pro-inflammatory proteins. Stimulates the production of IFNγ another pro-inflammatory protein.</td>
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<td><strong>MIP-3α (aka CCL20)</strong></td>
<td>Epithelial cells and monocytes</td>
<td>1 of 2 IL-28 isoforms. Involved in the immune reaction against viral antigens. It induces an antiviral state and can also promote an anti-tumour state. A pro-inflammatory molecule which promotes IFNγ and increases the cytotoxicity of CD8+ T cells against infected cells. It preferentially induces the Th1 immune response.</td>
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<td><strong>TGF-β1</strong></td>
<td>Lymphocytes (Tregs), macrophages and</td>
<td>An anti-inflammatory, immunosuppressive member of the TGF-β superfamily of cytokines. Involved in the growth, proliferation and differentiation of various immune cells. Involved in apoptosis.</td>
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(Kankoford & Frucht: 2003)  
(Hunter: 2005)  
(Koltsida et al: 2011)  
(Sheppard et al: 2003)  
(Akahoshi et al: 2003)  
(Schutyser et al: 2003)  
(Zou & Restifo. 2011)  
(Letterio & Roberts: 1998)
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<th>DCs</th>
<th>Inhibits effector T cell proliferation and function and inhibits cytotoxic T cells. Inhibits the secretion of pro-inflammatory molecules such as IFNγ and TNFα. Supports the function of CD4^+CD25^+ T regulatory cells.</th>
<th>(Marie et al: 2005)</th>
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<td>TNFα</td>
<td>Mainly produced by activated macrophages, and also to a lesser extent by CD4^+ lymphocytes, NK cells, FBs and endothelial cells.</td>
<td>A pro-inflammatory cytokine which is involved in the acute phase reaction. It induces inflammation and will inhibit tumorigenesis. Its signalling pathways lead to tumour cell apoptosis. It plays a role in the body’s resistance against cancer.</td>
<td>(Idriss &amp; Naismith: 2000) (Pfeffer: 2003)</td>
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<tr>
<td>TNFβ (aka lymphotoxin alpha)</td>
<td>Lymphocytes</td>
<td>A cytokine with similar function to TNFα though has alternative effect on different target cells. A pro-inflammatory cytokine which is thought to cause a cytotoxic response against tumour cells. It is inhibited by the immunosuppressive cytokine IL-10</td>
<td>(Kircheis et al: 1992) (Oster et al: 1987)</td>
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