FUNCTIONAL CHARACTERISATION OF CANCER-ASSOCIATED FIBROBLASTS FROM NASOPHARYNGEAL CARCINOMA

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FUNCTIONAL CHARACTERISATION OF CANCER-ASSOCIATED FIBROBLASTS FROM NASOPHARYNGEAL CARCINOMA

ABSTRACT

Nasopharyngeal carcinoma (NPC) remains a major health issue in Southern China and Southeast Asia where the disease is endemic. Whilst the cure rate for early stage NPC is high, patients often present with late stage diseases and have a 5-year survival rate of less than 50%. NPC patients often suffer from severe treatment side effects because of the location of the tumours that is in proximity to various important organs in the head and neck region. Therefore, alternative therapeutic approaches are needed to improve the treatment outcomes and reduce patient morbidity. Targeted therapies are not in routine use to treat NPC and therapeutic advances will require a more detailed understanding of the molecular basis of the disease. The tumour microenvironment (TME), which consists of various non-malignant cell types and extracellular matrix proteins, is known to support tumour development and progression in a number of cancer types. Cancer-associated fibroblasts (CAFs) are often the pre-eminent cell type within the TME of solid tumours and a number of tumour promoting properties of CAFs have been described. The role of CAFs in the pathogenesis of NPC, however, has received little attention. The present study was designed to phenotypically characterise a panel of CAFs derived from NPC tumours (NPC-CAFs) by examining their expression of CAF markers (α-SMA, PDPN, FAPα, PDGFRα/β and CAV-1), degree of senescence as well as to investigate their functional roles in NPC pathogenesis. The present study showed that the CAF strains expressed heterogeneous levels of alpha-smooth muscle actin (α-SMA), and interestingly, the CAF strains with high levels α-SMA also contained a high proportion of senescent cells. Notably, NPC-CAFs exclusively expressed podoplanin (PDPN), as PDPN expression was absent in normal fibroblasts. Next, the ability of the CAFs to promote NPC cell proliferation and migration, and also inhibit EBV-specific CD8 T cell responses

was investigated. MTT cell proliferation assays showed that conditioned media (CM) from NPC-CAFs had no effect in NPC cell proliferation. However, in transwell migration and T cell activation assays, CM from NPC-CAFs significantly enhanced the migration of NPC cells and inhibited IFN-γ production from antigen-stimulated EBV-specific CD8 T cells. Significantly, NPC-CAFs also inhibited CD8 T cells through cell-cell contact and this effect was likely to be PD-1/PD-L1 independent, as PD-L1 mRNA expression was not consistently upregulated in these NPC-CAFs. To begin to investigate which secreted proteins in the CM might be responsible for the effects on NPC cell migration and T cell activity, cytokine arrays were used to compare the profiles of cytokines secreted by normal fibroblasts and NPC-CAFs. The analyses revealed that NPC-CAFs had higher secretion of RANTES, MCP-3 and VEGF A than NPC-CAFs normal fibroblasts. Collectively, the present study showed that NPC-CAFs are phenotypically distinct from normal fibroblasts and may contribute to NPC pathogenesis by promoting a more motile and possibly metastatic phenotype in NPC cells, as well as by contributing to an immunosuppressive microenvironment. Therefore, the development of strategies to target CAFs could provide novel therapeutic opportunities for patients with NPC.

PENCIRIAN FUNGSIAN FIBROBLAS BERKAITAN KANSER DARI KARSINOMA NASOFARING

ABSTRAK

Karsinoma Nasofaring (NPC) kekal sebagai satu ancaman kesihatan di China Selatan dan Asia Tenggara. Walaupun kadar penyembuhan bagi NPC peringkat awal adalah tinggi, pesakit sering didiagnos pada peringkat akhir dan mempunyai kadar kelangsungan hidup 5 tahun yang tidak melebihi 50%. Pesakit NPC sering mengalami kesan sampingan rawatan yang teruk disebablan lokasi tumor yang rapat dengan banyak organ penting di kepala dan leher. Oleh itu, terapeutik alternatif diperlukan untuk meningkatkan hasil rawatan NPC dan mengurangkan morbiditi pesakit. Terapi yang disasarkan tidak kerap digunakan untuk merawat NPC dan kemajuan terapeutik memerlukan pemahaman yang lebih terperinci mengenai asas molekul penyakit. Mikropersekitaran tumor (TME) terdiri daripada matriks ekstraselular dan pelbagai jenis sel-sel stroma dan ia diketahui berpotensi menyokong perkembangan tumor. Fibroblas berkaitan dengan kanser (CAFs) adalah salah satu komponen stromal utama dan sumbangan CAFs dalam proses pembentukan tumor telah diterangkan dalam beberapa jenis tumor pepejal. Namun begitu, peranan CAF dalam patogenesis NPC tidak pernah dijelaskan. Kajian ini mencirikan secara fenotipikal panel CAFs diperolehi daripada NPCs (NPC-CAFs) dan mengkaji fungsi-fungsi CAFs dalam patogenesis NPC. Hasil kajian ini menunjukkan bahawa NPC-CAF ekspres α-actin otot licin (α-SMA) dan NPC-CAFs yang mempunyai tahap α-SMA yang tinggi adalah CAFs yang senescent. Tambahan pula, NPC-CAFs ekspres podoplanin (PDPN) secara eksklusif, kerana ekspresi PDPN tidak wujud dalam fibroblas biasa. Seterusnya, keupayaan NPC-CAF dalam menggalakkan percambahan dan penghijrahan sel-sel kanser, dan juga merencatkan respons EBV khusus sel T positif-CD8 juga dikaji.

MTT menunjukkan bahawa media terkondisi (CM) dari NPC-CAFs tidak mempunyai kesan dalam percambahan sel NPC. Walau bagaimanapun, melalui ujian migrasi transwell dan ujian pengaktifan sel T, CM dari NPC-CAF menggalakan penghijrahan sel NPC dan menghalang pengeluaran IFN-γ daripada sel-sel T positif-CD8 yang diaktifkan dengan antigen EBV. Di samping itu, hasil kajian ini menunjukkan bahawa NPC-CAF juga menghalang sel-sel T positif-CD8 melalui molekul permukaan sel dan kesan ini tidak bergantung kepada laluan PD-1/PD-L1. Profil rembesan sitokin daripada NPC-CAFs seterusnya berbanding dengan fibroblas biasa berasal dari mukosa mulut. Keputusan menunjukkan bahawa rembesan RANTES, MCP-3 dan VEGFA oleh NPC-CAFs adalah lebih tinggi daripada fibroblas biasa dan mungkin bertanggungjawab terhadap kesan-kesan tersebut. Kesimpulannya, keputusan kajian ini menunjukkan bahawa NPF-CAFs secara fenotipikal berbeza daripada fibroblas biasa dan berpotensi menyumbang kepada patogenesi NPC. Oleh itu, strategi untuk mensasarkan CAFs dapat memberikan peluang terapeutik baru bagi pesakit NPC..

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α-SMA	: Alpha-smooth muscle actin
AKT	: Protein kinase B
BART	: BamHI-A rightward transcript
Bcl-2	: B-cell lymphoma 2
CAF	: Cancer-associated fibroblast
Cas9	: CRISPR associated protein 9
CAV-1	: Caveolin-1
CCL	: Chemokine (C-C motif) ligand
CCR3	: CC chemokine receptor 3
CDKN2A	: Cyclin dependent kinase inhibitor 2A
CDKN2B	: Cyclin dependent kinase inhibitor 2B
cDNA	: Complementary DNA
CM	: Conditioned media
c-MET	: Tyrosine-protein kinase Met/ hepatocyte growth factor receptor
CRISPR	: Clustered regularly interspaced short palindromic repeat
CTLA-4	: Cytotoxic T-lymphocyte-associated protein 4
CXCL	: Chemokine (C-X-C motif)
CYP2A6	: Cytochrome P450 2A6
CYP2E1	: Cytochrome P450 family 2 subfamily E member 1)
DC	: Dendritic cell

DNA : Deoxyribonucleic acid

EBER : Epstein-Barr virus-encoded RNA

EBNA : Epstein-Barr nuclear antigen

EBV : Epstein-Barr virus

ECM : Extracellular matrix

EGF : Epidermal growth factor

EMT : Epithelial to mesenchymal transition

ERK : Extracellular signal-regulated kinase

FAPα : Fibroblast activation protein-alpha

FGF : Fibroblast growth factor

Flt-3 : Fms-like tyrosine kinase 3

FoxP3 : Forkhead box P3

GWAS : Genome-wide association studies

HCC : Hepatocellular carcinoma

HGF : Hepatocyte growth factor

HLA : Human leucocyte antigen

IDO : Indoleamine-2,3-dioxygenase

IFG : Insulin-like growth factor

IFN-γ : Interferon-gamma

IGF : Insulin-like growth factor

IGFBP-3 : Insulin-like growth factor-binding protein 3

IL	: Interleukin
JAK	: Janus kinases
LCL	: Lymphoblastoid cell line
LMP	: Latent membrane protein
LTBR	: Lymphotoxin beta receptor
MAPK	: Mitogen-activated protein kinase
MCP	: Monocyte-chemotactic protein
MDSC	: Myeloid-derived suppressor cell
MHC	: Major histocompatibility complex
MMP	: Matrix metalloproteinase
mRNA	: Messenger RNA
NFκB	: Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	: Next generation sequencing
NHOF	: Normal human oral fibroblasts
NK cell	: Natural killer cell
NO	: Nitric oxide
NPC	: Nasopharyngeal carcinoma
NPC-CAF	: NPC-derived CAF
NTC	: Non-template control
OSCC	: Oral squamous cell carcinoma
PBMC	: Peripheral blood mononuclear cell

PBS : Phosphate buffered saline

PD-1 : Programmed cell death-1

PDGF : Platelet-derived growth factor

PDGFRα/ β : Platelet-derived growth factor receptor alpha/beta

PD-L1 : Programmed cell death ligand-1

PDPN : Podoplanin

PHA : Phytohemagglutinin

PI3K : Phosphoinositide 3-kinase

PIGR : Polymeric immunoglobulin receptor

QPCR : Quantitative polymerase chain reaction

RANTES : Regulated on activation, normal T cell expressed and secreted

RASSF1A : Ras association domain family 1 isoform A

RNA : Ribonucleic acid

rpm : Revolutions per minute

RPMI : Roswell Park Memorial Institute

SA β-gal : Senescent-associated β-galactosidase

SASP : Senescence-associated secretory phenotype

SDF-1 : Stromal cell-derived factor-1

siRNA : Small interfering RNA

STAT3 : Signal transducer and activator of transcription 3

TAM : Tumour-associated macrophage

TAN	: Tumour-associated neutrophil
TGFBR2	: Transforming growth factor, beta receptor II
TGF-β	: Transforming growth factor-beta
Th1	: T helper 1
TIL	: Tumour-infiltrating lymphocyte
TIM-3	: T cell immunoglobulin and mucin-domain containing-3
TME	: Tumour microenvironment
TNF	: Tumour necrosis factor
TNFRSF19	: TNF Receptor Superfamily member 19
TRAF	: Tumor necrosis factor receptor-associated factor
Treg	: Regulatory T cell
VEGF A	: Vascular endothelial growth factor A

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Nasopharyngeal carcinoma (NPC) is a distinctive type of head and neck cancer that arises from the epithelium of nasopharynx. It is a relatively uncommon malignancy worldwide that has a skewed geographical distribution, with the disease being endemic in Southern China and Southeast Asia (Bray et al., 2018; Chua et al., 2016). In endemic regions, NPC often presents as an non-keratinising carcinoma that is highly associated with EBV infection (Raab-Traub, 2002). NPC is usually treated with radiotherapy or concurrent chemoradiotherapy and, whilst the treatment outcome for stage I NPC is good with a 5-year survival rate of 90% (Lee et al., 2005), the prognosis for late stage disease is very poor and the 5-year survival rate drastically reduces to 38-50%, according to the Cancer Facts & Figures 2017 published by the American Cancer Society. Furthermore, patients often suffer from severe treatment side effects because the location of the tumours is in close proximity to many vital organs in the head and neck region. Therefore, new therapeutic approaches are urgently needed to improve cure rates and reduce patient morbidity.

The tumour microenvironment (TME), also known as the tumour stroma, is the local environment where tumours develop and grow (Whiteside, 2008) The TME consists of various types of non-malignant cells and extra cellular matrix proteins that generally support tumour development and progression (Whiteside, 2008). Indeed, it is now generally accepted that a permissive TME is essential for tumour development as cancer cell-autonomous defects *per se* are insufficient to drive tumourigenesis (Catalano et al., 2013). It seems likely, therefore, that the identification of therapeutic targets within the TME will have a clinical impact in the near future.

Amongst the cellular components in the TME, cancer-associated fibroblasts (CAFs) are perhaps the most frequently studied cell type possibly because they are often found

abundantly in the stroma of many solid tumours. Different from quiescent fibroblasts, CAFs have been shown to possess enhanced contractile, proliferative and secretory capabilities and are usually perpetually activated, expressing alpha-smooth muscle actin (α-SMA) (Kalluri, 2016). Indeed, α-SMA expression in CAFs has been reported to be an unfavourable prognostic indicator for many malignancies, including NPC (Chen et al., 2017a; Marsh et al., 2011; Surowiak et al., 2007; Takatsuna et al., 2016; Yu et al., 2018). It is important to note, however, that there is often considerable heterogeneity within CAF populations, because not all CAFs are α-SMA positive and some have been shown to be prematurely senescent (Hassona et al., 2013). Functionally, CAFs have been shown to play a number of tumour-enhancing roles (Valkenburg et al., 2018), such as promoting angiogenesis, cancer cell proliferation, metastasis, and invasion, as well as contributing to immune evasion (Casey et al., 2008; Kalluri, 2016; Takahashi et al., 2015; Cremasco et al., 2018).

The TME of NPC has been shown to be permissive to tumour growth (Huang et al., 2018). However, the most extensively investigated cellular components in the NPC TME are the tumour-infiltrating lymphocytes (TILs) (Huang et al., 2018), particularly because marked lymphocytic infiltration is common in these tumours. By contrast, the role of CAFs in the pathogenesis of NPC has received little attention. Whilst it has been shown that α-SMA positive CAFs were present in NPC tumours and α-SMA expression levels in CAFs were associated with metastasis (Chen et al., 2017a) and poor prognosis (Yu et al., 2018), there have been no reports describing a functional role for NPC-derived CAFs. This represents a significant gap in our understanding of the pathogenesis of NPC. Therefore, the present study examined the expression of CAF markers in a panel of NPC-derived CAFs and investigated a possible role for these cells in NPC pathogenesis.

1.2 General Aims

CAFs have been shown to be pro-tumourigenic in various cancers. In the context of NPC, α-SMA positive CAFs have been shown to be present in tumours and their α-SMA expression was associated with metastasis and poor prognosis. However, the function of CAFs in the pathogenesis of NPC has never been investigated. Hence, the present study aimed to explore the possible tumour-promoting properties of CAFs from NPC.

Firstly, CAFs derived from NPCs (NPC-CAFs) were phenotypically characterized with regards to their state of senescence and expression of CAF markers. This could be important because CAFs are a highly heterogeneous population of cells and CAFs with different phenotypes are likely to be functionally distinct. Next, functional experiments were performed to examine whether the CAF strains could promote NPC cell proliferation and migration, as well as inhibit EBV-specific CD8 T cell activity.

During the course of this work, NPC-CAFs were shown to promote cancer cell migration and inhibit EBV-specific CD8 T cells. Hence, the final part of this study investigated the cytokine secretory profiles of NPC-CAFs to identify those cytokine(s) that may have contribute to these phenotypic effects of CAFs.

1.3 Objectives

The objectives of the present study are:

1. To phenotypically characterise NPC-CAFs with respect to their degree of senescence and expression of CAF markers.
2. To investigate the tumour-promoting potential of NPC-CAFs with regards to their effect on cell proliferation, migration and EBV-specific CD8 T cell activity.
3. To compare the profiles of cytokines secreted by normal human oral fibroblasts (NHOFs) and NPC-CAFs.

CHAPTER 2: LITERATURE REVIEW

2.1 Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is an epithelial cancer that arises in the mucosal lining of the nasopharynx (a cavity that connects the nasal cavity to the throat) with the Fossa of Rosenmuller being the most common site of origin. NPC is a relatively rare malignancy with a unique geographical distribution. The worldwide incidence rate of NPC is not more than 1 per 100, 000 persons per annum. However, in regions that have a high prevalence of the disease such as Southern China and Southeast Asia, the incidence rate rises dramatically to 20 to 50 per 100, 000 persons per annum (Bray et al., 2018; Chua et al., 2016). In addition to its unique geographical distribution, NPC seems to preferentially affect certain ethnicities, for example Cantonese in Guangdong and Hong Kong, Bidayuh in Borneo, Nagas in northern India, and Inuits in the Artic (Chua et al., 2016). Men are two to three times more susceptible to NPC than women (Chua et al., 2016) but the reason(s) behind these differences are unknown. The onset age of NPC is variable but peaks at 50-59 years.

Malaysia is one of the countries in Southeast Asia that has a very high prevalence of NPC. According to the Malaysian National Cancer Registry Report (2012-2016) published in 2019, NPC is the fifth most common cancer amongst Malaysian males with an age-standardised incidence rate of 8.6 per 100,000 persons per year, respectively. With regards ethnicity, Malaysian Chinese have been shown to be more susceptible to NPC than Malays and Indians and, additionally, some minority groups in Sarawak such as Bidayuh and Iban also have very high incidence rates of the disease (Devi et al., 2004).

2.1.1 Histopathology of NPC

NPC can be classified into three histopathological types, namely keratinising squamous cell carcinoma, non-keratinising carcinoma and basaloid squamous cell

carcinoma according and to the World Health Organisation (Barnes et al., 2005). The histopathology of keratinising NPC shares similar features with other head and neck carcinomas which are distinguished by cancer cells which are well-differentiated as well as the presence of intercellular bridges and epithelial (keratin) pearls in the tumours. This subtype of NPC is more prevalent in non-endemic regions (Wei & Sham, 2005).The non-keratinising NPC can be further sub-categorised into differentiated and undifferentiated carcinomas (Wei & Sham, 2005). NPCs of this category lack keratinisation and exhibit syncytial growth patterns with the cancer cells observed with large vesicular nuclei and prominent nucleoli. In contrast to keratinising NPCs, non-keratinising NPCs are the most common forms of NPCs in the endemic areas. Remarkably, both differentiated and undifferentiated non-keratinising NPCs are distinct from other subtypes by their strong association with Epstein-Barr Virus (EBV) infection (Young & Dawson, 2014). Additionally, massive lymphocyte infiltration is another characteristic of undifferentiated NPCs, also referred as lymphoepithelioma of the nasopharynx in the past (Peterson & Nelson, 2013).

2.1.2 Aetiology

The remarkable racial and geographical distribution of NPC strongly suggests that the onset of the disease is associated with both genetic susceptibility and environmental factors. In addition, the ubiquitous presence of EBV infection, which is clonal in origin in the undifferentiated NPC is indicative of a causal role for EBV (Tsao et al., 2014). It seems likely, therefore, that the development of NPC results from the interplay of these three major risk factors, which are discussed in more detail below.

2.1.2.1 Genetic susceptibility

Due to the unique geographical and ethnic distribution of NPC, genetic predisposition has been proposed as a risk factor for developing the disease. More specifically, familial clustering, whereby the first-degree-relatives of NPC patients have an elevated risk of

NPC, has been observed, particularly amongst the Cantonese in the Guangdong province of Southern China (Jia et al., 2004; Liu et al., 2017b). In addition, migration studies showed that while Caucasians rarely develop NPC, emigrants from Southern China and their next generation relatives born in western countries retain a higher risk of developing NPC (Mousavi et al., 2010; Wu et al., 2018). To delineate the significance of genetic factors in NPC pathogenesis, several genetic studies including genome-wide association studies (GWAS) have been carried out and various gene loci have been identified as being associated with the development of NPC. One such example is the MHC locus on chromosome 6p21 harbouring the Human Leucocyte Antigen (HLA) class I genes, which encode for proteins that are involved in the foreign antigen presentation machinery (Bei et al., 2010; Chin et al., 2015; Li et al., 2007b; Zhao et al., 2012), suggesting that a less efficient EBV antigen presentation to host immune surveillance may lead to immune evasion of EBV-infected tumour cells. In addition to HLA class I, allelic variants of some other genes such as *CDKN2A/2B*, *TNFRSF19*, *CYP2E1*, *CYP2A6*, *PIGR* and *MST1R* have also been reported to contribute to an increased risk of NPC (Bei et al., 2010; Dai et al., 2016; Hirunsatit et al., 2003; Tiwawech et al., 2006; Yao et al., 2017). CYP2E1 and CYP2A6 are involved in nitrosamine metabolism and PIGR governs EBV entry into nasopharyngeal epithelium, whilst *CDKN2A* and *CDKN2B* encode for cyclin-dependent kinase inhibitors, p16 and p15 and loss of these genes have been found in many NPCs (Wang et al., 2019). *MST1R* encodes for macrophage-stimulating 1 receptor, a cell surface receptor that plays an important role in regulating macrophage homeostasis. Interestingly, germ-line deleterious variants of *MST1R* have been found in a whole-exome sequencing study to be associated with early-age onset of the disease (Dai et al., 2016).

2.1.2.2 Environmental factors

Various environmental agents have been reported to be associated with an increased risk of NPC and these include genotoxic volatile nitrosamines, which are found in salt-

cured foods, such as salted fish (Zou et al., 1994). Salted fish can be considered as a staple and weaning food for the Cantonese population in Guangdong. Many early studies suggested that regular consumption of salted fish, especially from a young age, has a moderate to strong association with NPC risk (Guo et al., 2009; Jia et al., 2010; Ward et al., 2000). This was supported by a number of *in vivo* experimental studies showing that rats fed with salted fish developed tumours in the nasal and nasopharyngeal cavities (Yu et al., 1989b; Zheng et al., 1993).. Apart from salted-fish, childhood intake of other preserved food like fermented bean curds, salted eggs, pickled vegetables and salted shrimp paste have also been reported to increase the risk of developing NPC (Armstrong et al., 1998; Yu et al., 1989a). Other environmental factors besides dietary habits reported to have association with NPC susceptibility include smoking (Long et al., 2017; Xie et al., 2015), excessive alcohol consumption (Chen et al., 2009; Du et al., 2019), and occupational exposures like wood dust (Beigzadeh et al., 2019; Ekpanyaskul et al., 2015; Hildesheim et al., 2001) and formaldehyde (Vaughan et al., 2000).

2.1.2.3 EBV infection

EBV was the first human tumour virus to be discovered (Epstein et al., 1964). The association of EBV in the pathogenesis of NPC was first proposed when the sera of NPC patients were found to have higher titres of EBV-specific antibodies than healthy controls (Gunven et al., 1970). Multiple serological analyses subsequently revealed that EBV antibody titres were positively correlated with tumour stage. In addition, The presence of EBV in the precursor lesion of NPC as well as the invariable presence of clonal EBV in the undifferentiated form of NPC and the infrequent detection of EBV in normal nasopharyngeal epithelium *in vivo* further confirmed the close association of EBV with NPC development (Pathmanathan et al., 1995; Wolf et al., 1975) In addition to NPC, EBV has also been associated with other types of malignancy, such as Burkitt lymphoma, Hodgkin's lymphoma and gastric carcinomas (Young et al., 2016). EBV is a common

virus that can be easily transmitted interpersonally through saliva. Approximately 95% of the world's population are infected with this virus (Young et al., 2016) but the infection is generally asymptomatic, suggesting that other factors such as gene variations and environmental exposures may trigger EBV oncogenesis (Young et al., 2016).

EBV enters human hosts through the epithelium of the oropharynx to primarily infect B cells. Having naïve B cells infected, the virus enters a latent cycle and becomes dormant, whilst the infected naïve B cells become resting memory B cells. A life-long infection is established as the EBV-infected memory B cells are hidden from immune surveillance. Viral replication occurs when the memory B cells differentiate into plasma cells, triggering EBV lytic cycle to produce new virions that infect other naïve B cells or epithelial cells (Tsao et al., 2014). B cells are more readily infected by EBV than epithelial cells due to the expression of complement receptor type 2 (CR2), which is absent in epithelial cells (Tsao et al., 2014). Unlike B cells that acquire a hyperproliferative capacity upon EBV infection, primary epithelial cells infected with EBV undergo growth arrest (Babcock et al., 1998; Tsao et al., 2014). It has been suggested that in order for persistent EBV infection in premalignant nasopharyngeal epithelium to eventually lead to NPC some pre-existing genetic aberrations, such as those involving cyclin D1 and p16, must be present(Tsang et al., 2012b)

EBV strains can be broadly classified into type 1 and type 2 based on differences in the Epstein-Barr nuclear antigen 2 and 3 (EBNA2 and EBNA3) sequences, with the type 1 EBV strain being more prevalent (Zimber et al., 1986). Although the contribution of EBV strain variation to NPC pathogenesis has been unclear, previous studies and more recent work involving whole genome sequencing of EBV isolates from NPC tumours using next generation sequencing (NGS) approaches have revealed numerous EBV strains with genomic variations compared to the prototype EBV strain B95.8. For

example, EBV isolates from Chinese NPCs have been frequently found to have LMP1 variants with a common 10-amino-acid deletion (residues 343 to 352) and the variants have been shown to be more oncogenic than those of B95.8 *in vitro* (Dawson et al., 2000). Also, M81, a clonal EBV strain isolated from an NPC from a Chinese patient has also been shown to have enhanced pathogenic potential compared to B95.8 as it was prone to spontaneous lytic reactivation and hence, more likely to infect epithelial cells (Tsai et al., 2013). In addition, whilst B95.8 is defective in the synthesis of BART miRNAs (microRNAs encoded from the BamHI-A region of the viral genes), BART miRNAs are highly expressed in NPC tumours, suggesting that EBV strain with BART miRNA expression are important for NPC pathogenesis (Tsang et al., 2019). Most recently, two EBV variants with single nucleotide polymorphisms (SNPs) within *BALF2*, a gene which plays a role in the lytic DNA replication machinery, were identified as high risk EBV strains contributing to NPC pathogenesis in endemic regions. These variants were only found in NPC (and not other EBV-associated malignancies) and had a unique geographical distribution which was confined only to Asia (Xu et al., 2019).

2.1.3 Clinical presentation, diagnosis and prognosis

Neck lumps, persistent nasal obstruction and bleeding, hearing loss, blurred vision and sometimes cranial nerve palsy and headache are frequent clinical presentations of NPC (Peterson & Nelson, 2013). Endoscopic examination is first carried out to look for lesions in the nasopharynx of patients suspected with NPC and, if lesions are found, biopsies are taken for histopathological examination and diagnosis. Computerised tomography (CT) scan, integrated positron emission tomography (PET/CT) scan and magnetic resonance imaging (MRI) are then performed to identify the stage of the cancer based on the extent of regional lymph node metastasis and the presence of distant metastases. The staging of NPC follows the American Joint Committee on Cancer (AJCC) TNM (tumour-node-metastasis) staging system. Whilst NPC treated at early stage has a good prognosis and is

often curable, the disease is often diagnosed at late stages due to its deep anatomical location and unspecific symptoms at the early stage. The delayed diagnosis of NPC results in poor survival outcomes, whereby the 5-year survival rate for stage II and III disease is about 50%, which drops to only 38% for stage IV disease(American Cancer Society, 2017). This highlights the need for the development of early detection strategies. NPC patients have been found to have elevated levels of tumour-derived EBV DNA in their blood, and plasma EBV DNA load has been correlated to tumour burden (Zhao et al., 2015). A recent large scale prospective study involving more than 20,000 participants showed that plasma EBV DNA analysis had high sensitivity and specificity in identifying NPC in asymptomatic individuals, suggesting that plasma EBV DNA population screening could be a useful strategy to detect NPC at early stage (Chan et al., 2017)

2.1.4 Primary treatment

Unlike other head and neck cancers, undifferentiated NPC is highly sensitive to ionising radiation and hence, radiotherapy remains the mainstay of treatment for NPC. Intensity-modulated radiotherapy (IMRT) is currently the most preferred approach as it provides greater locoregional control with reduced side effects. Although radiotherapy alone is curative for early stage NPC with five-year survival rates of more than 90% (Lee et al., 2005), patients with signs of locoregional spread of the cancer are given radiotherapy concurrently with chemotherapy in order to achieve better overall- and disease-free survival (Chen et al., 2013). In some situations, adjuvant or induction chemotherapy is recommended to improve the efficacy of chemoradiotherapy. Cisplatin and 5-fluorouracil are the conventional chemotherapeutics drugs used in the chemoradiotherapy regimens for NPC patients.

2.1.5 Molecular basis of NPC

2.1.5.1 Oncogenic EBV latent genes

NPC cells harbouring EBV only express a restricted set of EBV latent genes (latency II), which are latent membrane proteins (LMP1, and LMP2), Epstein-Barr nuclear antigen 1 (EBNA1), Epstein-Barr virus-encoded RNAs (EBERs) and BamH1-A fragment transcripts (BARTs). The role of these latent genes in the pathogenesis of NPC has been well studied and has been reviewed recently (Young et al., 2016). LMP1 is a well-recognised viral oncogene which regulates a number of important cellular processes by activating various cellular signalling pathways, such as nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways (Dawson et al., 2012b). Further, it has been shown that LMP1 can induce NPC cell proliferation and resistance to apoptosis by inducing the upregulation of oncoproteins and anti-apoptotic proteins such as EGFR, Cyclin D1, survivin, and Mcl-1, as well as inhibiting growth inhibitory effects of TGF-β in NPC cells (Lo et al., 2010; Morris et al., 2009; Tsao et al., 2002b). Similar to LMP1, LMP2A is also a potent activator of several oncogenic pathways such as PI3K/AKT, Ras homolog family member A (RhoA) and extracellular-signal-regulated kinase (ERK) pathways (Tsang et al., 2019). Activation of PI3K/AKT pathway promotes cell proliferation and survival, as well as inhibiting epithelial squamous cell differentiation (Dawson et al., 2012a). In addition, LMP2A, as well as LMP1, also plays a significant role inducing epithelial-to-mesenchymal transition (EMT) and stemness acquisition in NPC cells (Dawson et al., 2012a; Kong et al., 2010). EBNA1 is essentially expressed by all cancers associated with EBV due to its critical role in maintaining EBV genomes in the cells during latency (Frappier, 2015). With regards to oncogenesis, EBNA-1 has been shown to affect cancer cell proliferation, survival and DNA repair (Frappier, 2012; Sivachandran et al., 2008; Yin & Flemington, 2006). The upregulation of proteins involved in metastases and angiogenesis in NPC cell lines

following EBNA-1 overexpression further suggests that EBNA-1 is involved in the development and progression of NPC (O'Neil et al., 2008). EBERs are non-coding RNAs which are abundantly present in EBV-infected cells. By having unique double-stranded secondary structures, EBERs can interact with various cellular proteins to modulate cell behaviour. For example, EBERs can promote NPC cell growth through upregulation of insulin-like growth factor-1 (IGF-1) (Iwakiri et al., 2005). BART miRNAs, on the other hand, could also promote cancer growth, survival and metastasis, as well as be involved in immune evasion and the regulation of cancer metabolism (Wang et al., 2017a).

2.1.5.2 Alterations in cellular genes

Pre-existing genetic alterations in several tumour suppressor genes particularly on chromosomes 3 and 9 are thought to favour the establishment of EBV latency in precancerous nasopharyngeal tissues. For example, *RASSF1A* (3p21.3), *CDKN2A* (9p21.3) and *TGFBR2* (3p24) are tumour suppressor genes within these loci that are often inactivated in NPCs (Tsang et al., 2019). More specifically, *CDKN2A* inactivation by homozygous deletion and/or promoter hypermethylation is a common genetic alteration in NPC (Lo et al., 1996; Lo et al., 1995). This aberration overrides EBV-induced growth arrest and supports EBV persistent infection in premalignant nasopharyngeal epithelial cells (Tsang et al., 2012b). *RASSF1A* inactivation, on the other hand is thought to induce genomic instability and resistance to apoptosis in NPC.

Further epigenetic alterations and somatic mutations accumulate as the cancer progresses. Genome-wide DNA hypermethylation resulting in inactivation of several tumour suppressor genes is a well-recognised feature of NPC which is induced by EBV infection (Dai et al., 2015). It was shown that EBV-induced DNA methylation was more frequent at CpG dinucleotides, making CpG island methylator phenotype (CIMP) another common characteristic of NPC (Dai et al., 2015). In addition to tumour suppressor gene

inactivation, amplification of oncogenes such as *CCND1* (11q13) (Hui et al., 2005), *PIK3CA* (3q26.3) (Lo et al., 1996), and *LTBR* (12p13) (Or et al., 2010) has also been reported in NPC. Similar to *CDK2A* inactivation, overexpression of *CCND1* supports stable EBV infection by overcoming EBV-induced growth arrest in nasopharyngeal epithelial cells (Tsang et al., 2012a). PIK3CA is a key molecule within the PI3K/AKT signalling pathway, whilst *LTBR* encodes for a member of the tumour necrosis factor (TNF) family of receptor which can activate NF-κB signalling pathway (Or et al., 2010); amplification of *PIK3CA* and *LTBR* results in aberrant activation of PI3K/AKT and NF-κB signalling pathways, respectively.

Constitutive activation of NF-κB is another common feature of NPC (Chung et al., 2013; Tsang et al., 2019). In an EBV-positive NPC cell line, C666-1, activation of NF-κB was shown to upregulate oncogenes *MYB* and *BCL2* as well as the expression of various chemokines involved in lymphocyte recruitment such as CXCL9, CXCL10, CX3CL1, and CCL20 (Chung et al., 2013). LMP1, which functions as a constitutively active tumour necrosis factor receptor (TNFR), has been shown to be a potent inducer of NF-κB signalling, particularly during the early development of NPC (Li et al., 2017; Tsao et al., 2002a) while somatic alterations such as *LTBR* amplification and loss of function mutation of three negative regulators of NF-κB signalling, namely *TRAF*s (14d32), *NFKBIA* (14q13) and *CYLD* (16q12) have also been reported to contribute to aberrant NF-κB signalling (Li et al., 2017; Or et al., 2010; Zheng et al., 2016a). Importantly, it was reported that high levels of LMP1 and somatic aberration-induced NF-κB activation were mutually exclusive in NPCs, implying that LMP1 expression may be essential for NF-κB activation during the early pathogenesis NPC but as the disease progresses, somatic mutations of NF-κB regulators might take over the NF-κB activating role of LMP1 (Li et al., 2017)

BART mi-RNAs and EBNA-1 have been reported to downregulate and inactivate the expression and function of p53 tumour suppressor protein (Saridakis et al., 2005; Zheng et al., 2018) although the incidence of *TP53* mutation in NPC is relatively low compared to other head and neck cancers. However, it is interesting to note that, the frequency of *TP53* mutation in recurrent and metastatic tumours were shown to be two times higher than in primary tumours, suggesting the potential contribution of *TP53* mutation in treatment resistance and disease progression (Li et al., 2017).

Collectively, these data have led to a genetic progression model for NPC being proposed (Tsang et al., 2019) (Figure 2.1).

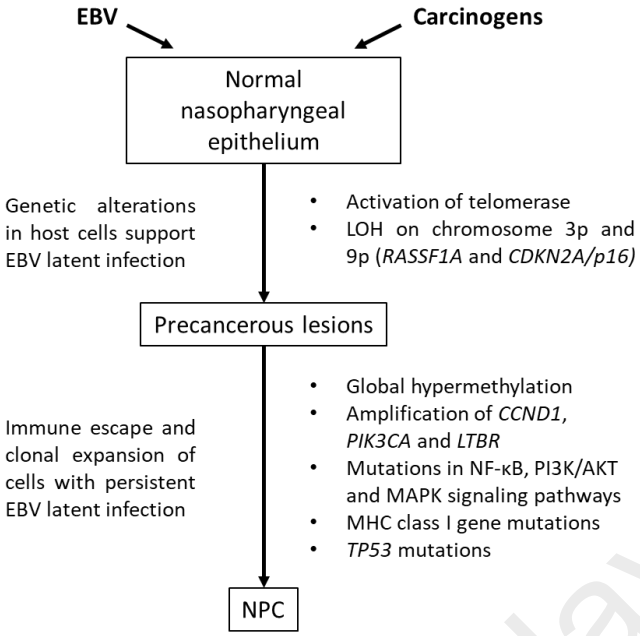

Figure 2.1: Genetic progression model of NPC. Normal nasopharyngeal cells constantly exposed to environmental risk factors may acquire various genetic alterations including *RASSF1A* and *CDKN2A/p16* LOH and telomerase activation. These genetic alterations result in cellular immortalisation and genome instability, thereby favouring EBV latent infection in the cells. The expression of oncogenic EBV latent genes allow the cells to escape immune surveillance and undergo clonal expansion. Further epigenetic alterations and somatic mutations accumulate as the cancer progresses and these include genome-wide hypermethylation, amplification of oncogenes (*CCND1*, *PIK3CA*, and *LTBR*), *TP53* suppressor gene mutations and downregulation of MHC class I genes. Mutations in NF-κB, PI3K/AKT and MAPK pathways distort a number of important cellular processes resulting in tumour progression (Tsang et al., 2019).

2.1.6 Immunotherapy in NPC

Although radiotherapy and chemoradiotherapy are the primary treatments, the development of more targeted therapies is likely to improve cure rates and reduce side effects. Whilst recent NGS studies (Chin et al., 2015; Li et al., 2017; Lin et al., 2014; Yu et al., 2019) have dramatically enhanced our understanding of the molecular alterations

that drive NPC, no molecular targeted therapies are yet in routine use. However, immunotherapy is a promising new strategy to manage NPC and a number of different approaches are currently being tested in clinical trials.

The EBV latent proteins EBNA1, LMP1 and LMP2 are expressed by most NPC tumours and, although having limited immunogenicity, they appear to be specific targets for immunotherapy. For example, infusion of autologous adoptive T cells specific to LMP1 and LMP2 resulted in near complete deletion of metastatic lesions in a NPC patient with pulmonary distant metastasis (Lutzky et al., 2014). Furthermore, first-line chemotherapy followed by adoptive EBV-specific T cell infusion was shown to prolong disease stabilization in a phase 2 clinical trial (Chia et al., 2014).

EBV-based vaccination is another approach to amplify host T cell responses against cancer cells. In the context of NPC, the first therapeutic vaccine developed and brought to clinical trial was a dendritic cell-based vaccine targeting LMP2 epitopes (Lin et al., 2002). This Phase 1 trial involved 16 patients who had residual NPC and low or undetectable levels of LMP2-specific T-cell responses. After 4 cycles of vaccination, LMP2-specific T cell responses were boosted in 9 patients. The effects were sustained for 3 months and 2 patients were coincidentally found to have partial tumour regression. Transduction of dendritic cells with an adenovirus expressing truncated LMP1 and full length LMP2 protein produced another type of dendritic cell vaccine that was able to boost LMP1/2-specific T cell responses regardless of the patients' HLA types. The vaccine was tested in a Phase 2 clinical trial involving 16 heavily pre-treated patients with advanced metastatic NPC and the study found that the vaccine could marginally improve clinical response and survival but did not increase the number of LMP1/2-specific T cells in the patients (Chia et al., 2012). Besides using dendritic cells as the vaccine vector, modified vaccinia virus Ankara (MVA) has also been used to generate vaccines to boost

EBV-specific T cell responses. A MVA-based vaccine against EBNA1 and LMP2 was found to be potently immunogenic and safe in a Phase 1 clinical trial (Hui et al., 2013).

Local immune tolerance can be a barrier to the success of immunotherapy regimens and this is very likely to be true in NPC. The undifferentiated tumours are often heavily infiltrated with lymphocytes whilst the tumours continue to grow and progress. This strongly suggests that potent immunosuppressive factors are present within the NPC tumour microenvironment and hence, strategies to negate the effects of these factors may help to augment patients' innate anti-tumour capacity, as well as to refine immunotherapy protocols. Programmed cell death ligand-1 (PD-L1) is a potent immune checkpoint inhibitor that can inhibit T cell function and survival following binding to its receptor, PD-1, expressed on T cells (Lipson et al., 2015). Notably, PD-L1 is frequently expressed by NPC cells and its expression may be induced by LMP1 as well as interferon-gamma (IFN-γ), a known inducer of PD-L1 (Fang et al., 2014). This makes NPC a good candidate for anti-PD-1/PD-L1 checkpoint blockade therapy. Indeed, monoclonal anti-PD-1 antibodies such as pembrolizumab (Hsu et al., 2017), nivolumab (Ma et al., 2018) and camrelizumab (Fang et al., 2018) have been reported in trials to have promising clinical activities.

2.2 The tumour microenvironment

The tumour microenvironment (TME), also known as the tumour stroma or reactive stroma, refers to the local environment in which tumours rely on to grow. It consists mainly of cancer-associated fibroblasts (CAFs), immune cells, pericytes, blood vessels and the extracellular matrix (ECM) (Whiteside, 2008) (Figure 2.1). CAFs are present in the TME of many solid tumours and will be discussed in more detail in section 2.3. In addition to CAFs, tumour-associated macrophages (TAMs), natural killer cells, effector T cells, regulatory T cells (Tregs), and B cells are the examples of immune cells

frequently present in the tumour stroma and often have cytotoxic and/or immunosuppressive functions (Laplagne et al., 2019). Various macromolecules such as collagens, hyaluronic acid, heparan sulfate, fibronectin, and laminins form an ECM network, which functions to provide structural integrity to the tumour mass (Liu et al., 2019) and also serves as a reservoir of stromal- and tumour-derived signalling factors (Hui & Chen, 2015).

2.2.1 Formation and function of the TME

In epithelial tissues, the reactive stroma is initially formed during the development of carcinoma *in situ*, as a result of host responses towards the developing malignant tissues (Bremnes et al., 2011). It begins with a phenomenon termed the desmoplastic response whereby fibrosis actively takes place in response to the chronic inflammatory cancer milieu. In addition to fibroblast accumulation, other phenomena such as angiogenesis, immune cell infiltration and enrichment of inflammatory cytokines, growth factors and ECM macromolecules also occur in a reactive tumour stroma. Interestingly, these features of reactive stroma can also be seen in the stroma during wound healing and hence, the formation of reactive stroma may be considered as a host's defence mechanism attempting to heal cancer (Bremnes et al., 2011). However, in contrast to the stroma at the site of wounds which has healing properties, the tumour stroma is permissive to tumour growth and is associated with tumour progression, metastasis, recurrence and chemoresistance (Bremnes et al., 2011). The tumour promoting TME is believed to result from tumour-stromal crosstalk whereby cancer cells can progressively reprogram an initially tumour-suppressive TME to one that is permissive for tumorigenesis (Bremnes et al., 2011). For example, through growth factor secretion or direct cell-cell contact, cancer cells can preferentially recruit tumour-promoting cell types (Argyle & Kitamura, 2018), modify the metabolism of neighbouring cells to support their growth (Avagliano et al., 2018) and alter the ECM to facilitate invasion and metastasis (Williams et al.,

2019). It is now generally accepted that the TME can influence cancer cell behaviour and cancer cell-autonomous defects *per se* cannot support cancer progression and metastasis (Catalano et al., 2013). Therefore, the TME is a major focus of contemporary cancer research and emerging studies on angiogenesis, hypoxia, interstitial pressure, ECM organisation, the function and metabolism of immune cells and CAF, as well as tumour-stromal crosstalk are likely to have an impact clinically in the near future. Among the cellular components in the TME, CAFs have been extensively studied, in part because they are often the most abundant cell type in the tumour stroma. Somewhat surprisingly, the function of CAFs in the pathogenesis of NPC has not been reported previously.

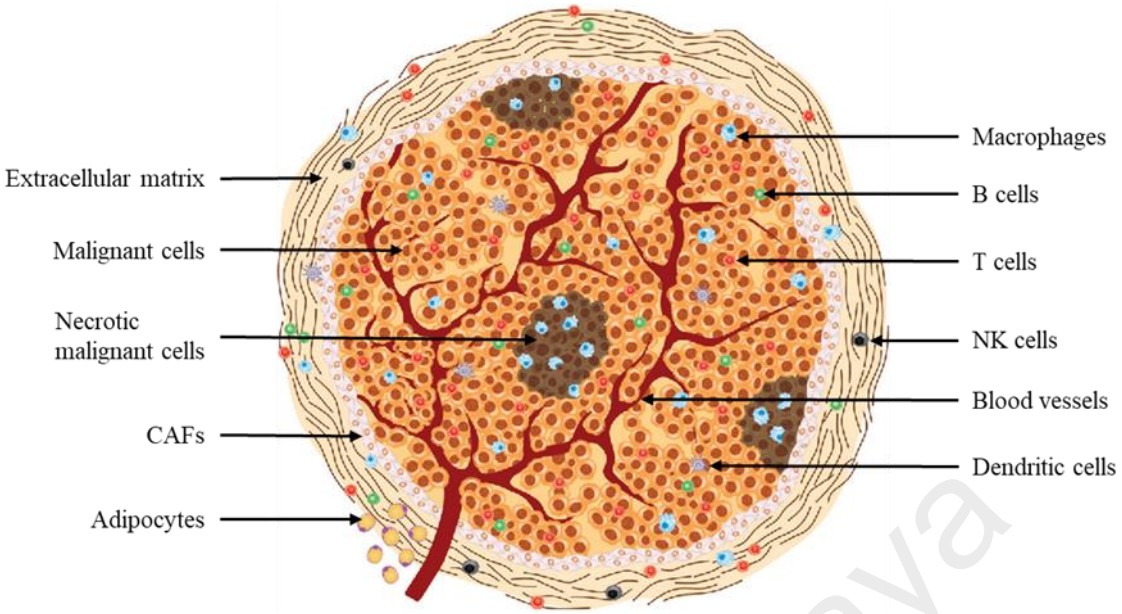

Figure 2.2: Schematic representation of the TME. The tumour mass consists not only of cancer cells but also numerous resident and recruited cells which are non-malignant. Surrounding the tumour cells are often cancer-associated fibroblasts (CAFs) and a dense extracellular matrix (ECM) which is rich in cytokines and macromolecules and which provides structural support to the tumour mass. Various immune cells such as lymphocytes, natural killer (NK) cells, macrophages and dendritic cells are also present in the TME. The figure was adapted from Asgharzadeh et al. (2017).

2.3 Cancer-Associated Fibroblasts

2.3.1 CAF heterogeneity and expression of CAF markers

CAFs are technically fibroblasts that reside in the vicinity of tumours and these cells are often the most prominent cell type in the TME and exhibit a high degree of heterogeneity. Compared to quiescent fibroblasts in the stroma from normal tissues, CAFs are generally activated fibroblasts that express alpha-smooth muscle actin (α-SMA) or fibroblast activation protein-alpha (FAPα) and which are actively proliferating with enhanced secretion of ECM proteins and cytokines. It is these acquired traits that provide activated CAFs with the ability to support tumour growth (Kalluri, 2016).

α-SMA-expressing CAFs can often be found in the TME and these cells resemble myofibroblasts that are usually found around wounds (Kalluri, 2016). The *de novo* expression of α-SMA in myofibroblasts allow the cells to participate in wound closure through the acquisition of contractile, proliferation and secretory capabilities and once the wounds are healed, myofibroblasts undergo apoptosis or may revert back to a quiescent state (Darby et al., 2014). However, in cancers, that have been referred to as "wounds that never heal" (Dvorak, 1986), CAFs remain perpetually activated in a chronic inflammatory environment that is constantly rich in growth factors and cytokines. Epigenetic regulation has also been thought to retain the activated state of CAFs (Kalluri, 2016). Also in contrast to myofibroblasts which possess wound healing properties, α-SMA expressing CAFs are pro-tumourigenic (Quante et al., 2011).

Although CAFs in the TME are mainly hyperproliferative, a sub-population of non-dividing CAFs which are prematurely senescent has been identified in some tumours, including those of the head and neck (Hassona et al., 2013). The onset of premature senescence in CAFs may be induced by cancer cells. For example, the co-culture of normal fibroblasts and oral squamous cell carcinoma (OSCC) induced senescence in the fibroblasts. During the co-culture, CXCL1 was upregulated in the normal fibroblasts and CXCL1 was shown to induce fibroblast senescence (Kim et al., 2018). In addition, a higher proportion of senescent cells have been found in CAFs from genetically unstable OSCCs compared to their counterparts from genetically stable tumours (Hassona et al., 2013). These genetically stable OSCC cells produced high levels of reactive oxygen species (ROS) and these induced fibroblasts to express TGF-β which subsequently promoted fibroblast activation and senescence (Hassona et al., 2013). Interestingly, senescent CAFs are mostly activated CAFs expressing α-SMA (Mellone et al., 2016) and this may be because myofibroblast transdifferentiation and senescence induction in fibroblasts are both dependent on the canonical TGF-β/Smad signalling (Mellone et al.,

2016). Senescent CAFs contribute to cancer progression by secreting a distinctive set of pro-inflammatory and ECM remodelling proteins known as the senescence-associated secretory phenotype (SASP) (Alspach et al., 2013; Hassona et al., 2014).

α-SMA is frequently expressed in CAFs of a wide variety of tumours. However, it is important to note that not all CAFs are activated and express this protein (Shiga et al., 2015). In fact, CAFs are highly heterogeneous and there is currently no single marker that can be used specifically to distinguish CAFs from normal fibroblasts. Some proteins other than α-SMA have been reported to be more highly expressed by some CAF populations. These include, fibroblast activation protein alpha (FAPα), podoplanin (PDPN), and platelet-derived growth factor receptor alpha/beta (PDGFRα/β) amongst others (Nurmik et al., 2019). In contrast, loss of caveolin-1 (CAV-1) expression has been observed in some CAF populations (Zhao et al., 2013). However, what is clear now is that none of these markers alone or in combination can define this highly heterogeneous population of cells. This heterogeneity may reflect the plasticity of CAFs within the dynamic cancer niche and the "intelligence" of cancer cells to recruit or induce fibroblasts of various phenotypes to support their growth. Presumably, CAFs with different expression profiles would have different tumour-promoting roles. Furthermore, the fact that CAFs can originate from various progenitors such as resident quiescent fibroblasts, mesenchymal stem cells, endothelial cells or tumour cells that have undergone epithelial to mesenchymal transition (EMT) may also contribute to the heterogeneity of CAFs (Ishii et al., 2016). In the following section, some relevant details on the various CAF markers will be highlighted, with more attention being given to α-SMA and podoplanin, as these markers were studies in more detail in the present study.

2.3.1.1 α-SMA

α-SMA, also known as smooth muscle aortic alpha-actin (ACTA2), is a cytoskeletal protein of the actin family which plays an important role in cellular contraction. The incorporation of the protein into the stress fibres of fibroblasts enhances the contractility of the cells and due to the acquisition of contractile phenotype resembling smooth muscle cells, the α-SMA-expressing fibroblasts are termed "myofibroblasts" (Darby et al., 2014). Myofibroblasts can be found abundantly in the granulation tissues of wounds, playing a central role in wound closure (Hinz et al., 2001). Besides tissues of injury, myofibroblasts are also frequently present in the cancer stroma, possibly because wound healing and cancer development share many mechanistic similarities (Schafer & Werner, 2008). The transdifferentiation of quiescent fibroblasts to myofibroblasts is regulated by extrinsic factors. Transforming growth factor-beta (TGF-β), a well-known pleiotropic cytokine often secreted by cancer cells, for example, is one of the cytokines that can induce *de novo* α-SMA expression in fibroblasts (Casey et al., 2008; Zheng et al., 2016b) and is thought to be a potent inducer of the CAF phenotype. It was shown in a recent study that cancer-derived TGF-β was transferred to fibroblasts primarily through exosomes in bladder cancer (Ringuette Goulet et al., 2018). In addition to TGF-β, tumour-derived microRNAs (mi-RNAs) have also been shown to upregulate α-SMA expression in normal fibroblasts. In breast cancer, tumour-derived miR-125b which had previously been shown to promote cancer cell proliferation and survival, was shown to upregulate α-SMA expression in normal fibroblasts by suppressing TP53INP1 and TP53 (Vu et al., 2019).

α-SMA expression may induce a pro-tumoural phenotype in CAFs by altering the morphology and function of the cells. For example, α-SMA expression induces contractile activities in CAFs (Casey et al., 2008), which facilitates their ability to promote cancer cell dissemination through ECM remodelling (Casey et al., 2008).

Moreover, studies have also shown that α-SMA expressing CAFs are highly proliferative and have enhanced secretory capabilities, supporting tumour growth through cytokine secretion (Kalluri, 2016). However, these phenotypic changes might not be direct downstream effects of α-SMA expression in CAFs and the mechanisms of how this contractile protein might promote fibroblast proliferation and secretory capabilities have not been elucidated.

The expression of α-SMA by CAFs is also a prognostic indicator. For example, higher expression of α-SMA in the stromal compartment correlated with a shorter overall survival and relapse-free survival in patients with invasive ductal breast cancer (Surowiak et al., 2007) and indicated an invasive growth type of colorectal cancer (Takatsuna et al., 2016). In OSCC, stromal α-SMA expression has been shown to be the strongest independent risk factor of early OSCC death (Marsh et al., 2011). Although α-SMA is usually associated with poor prognosis, in pancreatic ductal adenocarcinoma (PDAC), patients with lower stromal levels of α-SMA had worse survival (Özdemir et al., 2014). The biological mechanism underlying these contradictory observations, however, remains to be determined.

2.3.1.2 PDPN

PDPN is a transmembrane glycoprotein receptor with a molecular mass that varies from 38 to 50kDa depending on the length of serine and threonine amino acid chains attached to the extracellular domain (Pula et al., 2013). It is a common marker for lymphatic endothelial cells and its expression is critically important for lymphatic vessel development (Pula et al., 2013). The expression of PDPN, however, is not only restricted to lymphatic epithelial cells as the protein is also expressed by cancer cells and CAFs, particularly at the invasive front of some tumours (Li et al., 2014; Pula et al., 2013). Lung (Kato et al., 2005) and OSCC (Yuan et al., 2006), mesothelioma (Ordóñez, 2005) and

certain brain cancers (Mishima et al., 2006) are examples of malignancies in which PDPN expression has been reported in cancer cells. Cancer cell upregulation of PDPN often leads to enhanced tumour invasion and metastases, as well as poor prognosis (Li et al., 2014; Xie et al., 2018). Similarly, the presence of PDPN-positive CAFs in the stroma of various cancers such as oesophageal (Schoppmann et al., 2013), pancreatic (Shindo et al., 2013), lung (Hu et al., 2018; Kawase et al., 2008; Kubouchi et al., 2018), and breast (Pula et al., 2011) cancers was associated with lymphangiogenesis, cancer metastasis, unfavourable disease outcome and shorter survival time (Kitano et al., 2010).

The mechanisms by which PDPN-positive CAFs can promote tumour progression have started to be elucidated. For example, it was shown that PDPN-positive CAFs from pancreatic cancers expressed higher levels of matrix metalloproteinase (MMPs) such as CD10, MMP-2 and MMP-3 than PDPN-negative CAFs and these facilitated soluble factor-mediated cancer cell migration and invasion (Shindo et al., 2013). Moreover, PDPN expression in CAFs also promoted motility of the fibroblasts (Neri et al., 2015). In a collagen invasion assay model, PDPN positive CAFs with enhanced motility were shown to "lead" lung cancer cells to invade into the collagen matrix, suggesting a possible role for PDPN positive CAFs in contributing to local invasion of cancer cells (Neri et al., 2015). In addition, the enhanced motility of fibroblasts with upregulation of PDPN was also shown in a 3D co-culture study involving breast cancer cells, whereby the PDPN positive fibroblasts were observed to move towards the cancer cells and eventually formed scaffolds to aid cancer cell aggregation and coalescence. Interestingly, it has been shown that conditioned media from breast and pancreatic cancer cells dramatically induced PDPN expression in fibroblasts (Shindo et al., 2013; Wessels et al., 2019). These results suggest that PDPN upregulation in CAFs is most probably dependent on tumour-derived factors. In fact, miRNA dysregulation (Cortez et al., 2010) and cytokines such as epidermal growth factor (EGF), fibroblast growth factor (FGF2), and TGF-β (Wicki et

al., 2006) have been shown to induce PDPN expression in cancer cells. However, the mechanisms of PDPN acquisition in CAFs have not been well investigated. CAFs expressing PDPN are usually also positive for α-SMA, similar to myofibroblasts at wound sites (Kitano et al., 2010), suggesting that PDPN might be also a protein associated with fibroblast activation.

Although PDPN expression has primarily been associated with worse disease outcome, the function of PDPN expressing CAFs is cancer type-dependent. In colorectal and cervical cancers, PDPN expression in CAFs was an indicator of favourable outcome (Carvalho et al., 2010; Choi et al., 2013; Yamanashi et al., 2009). With regards PDPN expression in NPC, it was recently reported that a NPC cell line, TWO1, expressed PDPN and the knockdown of the gene expression resulted in attenuated proliferation, migration, and invasion of the cells (Hsu et al., 2019). However, in NPC tissues, PDPN expression was apparently restricted to vessel structures (Wakisaka et al., 2012). The expression of PDPN by NPC-CAFs is unknown.

2.3.1.3 Other CAF markers

FAPα is a membranous protein in the serine protease family which have dipeptidyl peptidase and collagenolytic activity. The expression of FAPα is common in stroma of more than 90% of epithelial cancers (Zi et al., 2015) with activated CAFs being the predominant cell type expressing the protein. Besides activated CAFs, immune cells and cancer cells sometimes express FAPα. Due to the frequent expression of FAPα in cancer stroma, the protein has also often been used to identify CAFs either alone or concurrently with α-SMA or other CAF markers (Feig et al., 2013; Yang et al., 2016a). It has also been shown that FAPα co-expression in α-SMA positive CAFs was associated with the acquisition of an inflammatory phenotype in the CAFs as demonstrated by the upregulation of some inflammation-related genes including CCL2 and that FAPα induced

inflammatory cytokine secretion in CAFs is through the activation of STAT3 with its intracellular domain. Stromal FAPα expression in intrahepatic cholangiocarcinoma (ICC) was associated with large tumour size, disease recurrence, and poor patient survival (Yang et al., 2016a). FAPα expressing myofibroblast-like CAFs are also present in colorectal, ovarian, breast, bladder, lung and pancreatic cancers (Fan et al., 2016).

PDGFRα and PDGFRβ are tyrosine kinase cell surface receptors for platelet-derived growth factors (PDGFs), a potent mitogen of fibroblasts and various other mesenchymal cells (Heldin, 2013). Many pathological conditions have been associated with PDGF-PDGFR pathway hyperactivation and these include fibrosis in the lung, kidney and heart (Klinkhammer et al., 2018), atherosclerosis, pulmonary artery hypertension, and cancers (Papadopoulos & Lennartsson, 2018). In cancers, PDGFR expression can be found in pericytes, CAFs and cancer cells that have undergone EMT (Heldin, 2013). PDGFs are mostly secreted by cancer cells and autocrine activation of PDGFR signalling in cancer cells enhances proliferation, survival and invasion of cancer cells (Heldin, 2013). On the other hand, the paracrine action of PDGFs on PDGFRs expressed on stromal cells results in fibroblast recruitment and accumulation (Anderberg et al., 2009; Cadamuro et al., 2013; Heldin, 2013). In addition, PDGFRβ signalling has also been shown to mediate tumour interstitial hypertension, a condition which hinders tumour transvascular transport of chemotherapeutic drugs (Pietras et al., 2002). In fact, increased PDGFRβ expression in CAFs has been observed in various cancers such as breast, prostate, colon and pancreatic cancers and was usually associated with chemoresistance and poor treatment outcome (Kitadai et al., 2006; Nordby et al., 2017; Paulsson et al., 2017; Yuzawa et al., 2012).

CAV-1 is a major structural protein of caveolae, complexes that are located at the cell membrane of many differentiated cell types including fibroblasts, epithelial cells and

endothelial cells. CAV-1 downregulation has been observed in CAFs of some malignancies such as skin, breast, gastric, prostate cancers and it was usually associated with a more aggressive cancer phenotype and unsatisfactory treatment outcome (Chen & Che, 2014; Witkiewicz et al., 2009). CAV-1 downregulation in CAFs may be induced by cancer cells probably at the post transcriptional or translational level (Mercier et al., 2008). For example, it is believed that an oxidative stress environment created by cancer cells may induce autophagic loss of CAV-1 (Chen & Che, 2014). CAFs with CAV-1 loss have been shown to be more proliferative due to functional inactivation of the retinoblastoma tumour suppressor protein (Mercier et al., 2008; Sotgia et al., 2009). In addition, CAV-1 loss also has been shown to enhance the ability of CAFs to induce breast cancer cell invasiveness (Simpkins et al., 2012).

2.4 Pro-tumorigenic functions of CAFs

A large body of existing literature has highlighted a range of tumour-promoting roles for CAFs. Specifically, CAFs have been shown to contribute to tumorigenesis in a variety of ways, such as promoting cancer initiation, invasion, metastasis, drug resistance, cancer stemness, as well as immune evasion. The influence of CAFs on these processes will be described in more detail below.

2.4.1 Tumourigenesis

Tumourigenesis used to be thought of as a process whereby epithelial cells with accumulated genetic alterations proliferated rapidly to form a solid mass. However, it is now widely accepted that genetic mutations *per se* are insufficient to drive tumourigenesis, as the carcinogenic process has to be supported by a permissive microenvironment (Catalano et al., 2013). In various *in vivo* studies, CAFs have been shown to facilitate tumourigenesis. This has been demonstrated by the observation that mice injected with a mixture of CAFs and cancer cells developed tumours faster than mice injected with a mixture of cancer cells and normal fibroblasts or with cancer cells alone (Lin et al., 2017; Olumi et al., 1999). The CAF-specific tumourigenic properties could partly be explained by the capacity of CAFs in inducing angiogenesis as microvasculature formation is essential in tumourigenesis to allow a more efficient nutrient supply to the tumour cells (Lin et al., 2017). Indeed, CAFs in breast cancer have been shown to induce angiogenesis by secreting stromal cell-derived factor-1 (SDF-1/CXCL12) to promote bone marrow-derived endothelial cells recruitment (Orimo et al., 2005). Besides SDF-1, vascular endothelial growth factor A (VEGF A), is another potent angiogenic factor mainly secreted by CAFs (Sewell-Loftin et al., 2017). Furthermore, it was recently shown that VEGF A stimulation of angiogenesis in the TME may be

essentially coupled to the enhanced mechanical activity of CAFs in deforming the ECM (Sewell-Loftin et al., 2017).

The ability of CAFs to promote cancer cell proliferation also results in a more rapid formation of the tumour mass. Insulin-like growth factor (IFG), hepatocyte growth factor (HGF), EGF, FGF and SDF-1 are the examples of cytokines secreted by CAFs to promote cancer cell proliferation (Bhowmick et al., 2004). For example, HGF secretion by CAFs derived from breast cancers promoted cancer colony formation in soft agar, as well as tumour formation in immunocompromised mice, effects that were abrogated by the use of a neutralising antibody (Tyan et al., 2011). In addition to cytokines, CAF-derived micro-RNAs can also modulate cancer cell proliferation. For example, micro-RNA 7 (mi-RNA 7) secreted by head and neck cancer CAFs could induce cancer cell growth *in vitro*, an effect that was reduced following silencing of the mi-RNA (Shen et al., 2017)

2.4.2 Cancer progression: invasion and metastasis

A role for CAFs in promoting cancer cell invasion and metastasis has also clearly been demonstrated. With enhanced secretory capabilities, CAFs are known to influence the invasiveness and metastatic potential of cancer cells, at least in part through aberrant secretion of pro-metastatic soluble factors. At primary sites, cytokines such as Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES or CCL5), monocyte-chemotactic protein-3 (MCP-3 or CCL7), and hepatocyte growth factor (HGF) are chemotactic factors highly secreted by CAFs that enhance the motility of cancer cells. For example, RANTES secreted at high level by gastric cancer CAFs promoted the migration of gastric cancer cells in transwell migration assays, an effect that could be diminished by anti-RANTES neutralising antibody (Yang et al., 2017). Similarly, the secretion of RANTES was also elevated in hepatocellular carcinoma (HCC) CAFs that also had enhanced secretion of MCP-3; both cytokines were shown to promote HCC cell

migration and invasion (Liu et al., 2016). HGF, on the other hand, enhances cancer cell motility by inducing lamellipodia and filopodia formation in oral cancer cells through c-Met signalling (Yasui et al., 2017). In gastric cancer, HGF derived from CAFs promoted the migration as well as proliferation of gastric cancer cells (Ding et al., 2018). Notably, HGF and c-MET have been reported to be upregulated in NPC and associated with lymph node metastasis (Luan & Yu, 2014).

EMT is thought to play a role in cancer metastasis as it allows cancer cells to detach from primary tumours and acquire migratory and invasive properties (Jolly et al., 2017). The role of CAFs in inducing EMT in cancer cells was clear when breast cancer cells co-cultured with CAFs, but not normal fibroblasts, were shown to have downregulation of the epithelial adhesion molecule E-cadherin, but upregulation of N-cadherin, a mesenchymal adhesion protein (Angelucci et al., 2012). Several CAF-derived factors such as SDF-1 (Jung et al., 2013), IL-6 (Wu et al., 2017), and TGF-β (Yu et al., 2014) can induce cancer cell EMT. IL-6, for example, was secreted a higher amount by CAFs derived from gastric cancer and was shown to induce EMT and metastasis of gastric cancer cells through JAK2/STAT3 signalling (Wu et al., 2017).

Furthermore, CAFs can facilitate cancer cell invasion by altering ECM stiffness and degradation (Najafi et al., 2019). ECM degradation and remodelling ease the motility of cancer cells in the ECM and this usually involves MMPs such as MMP2 and MMP9 (Najafi et al., 2019). Additionally, CAFs may also guide local invasion creating extended tracks in the ECM for cancer cells to follow. This CAF-mediated cancer cell invasion model has been experimentally proven using PDPN-expressing CAFs derived from lung adenocarcinoma (Neri et al., 2015).

2.4.3 Immunosuppression

In addition to the direct tumour-promoting roles of CAFs described above, the immunosuppressive potential of CAFs has also been explored. CAFs do not only interact with cancer cells but also cross-talk with immune cells, such as monocytes, macrophages, neutrophils, natural killer (NK) cells, dendritic cells (DCs) and lymphocytes to create an immunosuppressive TME that favours tumour survival (Ziani et al., 2018). In general, CAFs attenuate the function of effector immune cells like NK cells and cytotoxic T cells directly through cytokine secretion or cell-cell contact and indirectly through immunosuppressive cell recruitment and differentiation. Examples of CAF-derived factors that have immunosuppressive functions include IL-1β, IL-6, IL-10, MCP-1, MCP-3, RANTES, VEGF, TGF-β, indoleamine-2,3-dioxygenase (IDO), tumor necrosis factor (TNF) and nitric oxide (NO) (Ziani et al., 2018). On the other hand, membrane-bound immunomodulatory molecules such as PD-L1 and PD-L2 are also expressed by some CAFs (Ziani et al., 2018). In addition, CAFs may also attenuate anti-tumour immunity by disrupting dendritic cell function (Ziani et al., 2018).

2.2.1.1 Direct cytotoxic T cell inhibition

Cytotoxic T cells or CD8 T cells are a subpopulation of T lymphocytes in the adaptive immune system that can execute antigen-specific cytolysis on cancer cells or cells infected with viruses through secretion of IFN-γ, granzyme B and perforin (Janas et al., 2005; Tewari et al., 2007). A higher ratio of tumour-infiltrating CD8 T cells to Tregs, therefore, usually correlates with improved prognosis and better overall survival in cancer patients (Nakano et al., 2001; Schumacher et al., 2001; Zhang et al., 2003). TGF-β has been shown to promote apoptosis in CD8 T cells by downregulating the expression of Bcl-2, a pro-survival protein expression (Sanjabi et al., 2009). In addition, TGF-β also interferes with CD8 T cell cytolytic function by inhibiting the expression of perforin, granzyme A, granzyme B, Fas ligand, and IFN-γ in a smad-dependent pathway (Thomas

& Massague, 2005). An inhibitory effect of CAFs on T cell proliferation was demonstrated by Takahashi et al. (2015) whereby PBMCs stimulated with anti-CD3/anti-CD28, when incubated with CAFs and CAF-derived conditioned media, produced a lower proportion of T cells than those incubated with normal fibroblasts. The inhibition of T cell proliferation was mediated by TGF-β and VEGF present in the CAF conditioned media, as well as by PD-L1 and PD-L2 expressed on the CAFs (Takahashi et al., 2015).

Immune checkpoint molecules are crucial in maintaining immune homeostasis in order to prevent uncontrolled and excessive immune responses that could lead to autoimmunity. PD-L1 and PD-L2 are the examples of immunoregulators that can be upregulated by cancer cells (Lipson et al., 2015) and CAFs (Nazareth et al., 2007; Takahashi et al., 2015) of certain cancer types. In a recent study, CAFs were shown able to induce antigen-specific deletion of CD8 T cells by having the capacity to process and cross-present tumour-derived antigens and expressing PD-L2 (Lakins et al., 2018). PD-L1 and PD-L2 expression is generally regulated by IFN-γ, a cytokine that is usually released by activated T cells and some other immune cells in the TME. IL-1 usually secreted by cancer cells can also upregulate PD-L1 in CAFs, as demonstrated in melanoma (Khalili et al., 2012). In addition, in pancreatic adenocarcinoma, CXCL12 released by pancreatic stellate cells (CAFs in pancreatic tumours) has been found to impede the infiltration of CD8 T cell into tumours. CAFs were also shown to suppress T cell proliferation through nitric oxide production (Cremasco et al., 2018)

2.4.3.2 Recruitment and differentiation of tumour-associated macrophages (TAMs)

Macrophages are phagocytes that function to identify, engulf and destroy pathogens or apoptotic cells. Two distinct types of macrophages have been found, the classically activated M1 macrophages and the tumour-associated M2 macrophages (also known as

TAMs). In contrast to M1 macrophages that support Th1 immune responses, TAMs tend to produce more immunosuppressive factors such as IL10, IDO and TGF-β but less IL-12, an immunostimulatory cytokine that promote and maintain NK cells, cytotoxic T cell and T helper cell function (Ostuni et al., 2015). TAMs are frequently found around tumours and CAFs have been shown to play a part in the differentiation of macrophages into M2 phenotype (Gok Yavuz et al., 2019; Takahashi et al., 2017; Zhang et al., 2019). In addition, CAFs can recruit monocytes, the precursors of macrophages through chemokine secretion. Breast cancer-derived CAFs which were α-SMA positive, for example, were shown to be more effective in recruiting monocytes than normal fibroblasts by secreting elevated levels of MCP-1 and SDF-1 to recruit monocytes, (Gok Yavuz et al., 2019).

2.4.3.3 Interaction with NK cells, neutrophils and mast cells

CAFs were also found to influence various other innate immune cells like natural killer (NK) cells, neutrophils and mast cells. NK cells function to mount rapid immune response against pathogens or infected cells as well as to activate the adaptive immune system. Similar to cytotoxic T cells, NK cell cytolytic effects are perforin- and granzyme-mediated (Paul & Lal, 2017). TGF-β has been shown to suppress NK cell activation, cytotoxic function as well as IFN-γ secretion (Donatelli et al., 2014). Hence, being one of the main producers of TGF-β in the TME, the contribution of CAFs in NK cell dysfunction is undeniable. In addition, CAFs were also shown to inhibit the killing activity of NK cells by downregulating its expression of cell-surface poliovirus receptor (PVR/CD155), a ligand to activate NK cells (Inoue et al., 2016). Neutrophils are one of the most abundant tumour-infiltrating immune cells and have been shown to have tumour-promoting roles. Similar to TAMs, tumour-associated neutrophils (TANs) often exhibit N2 pro-tumoural phenotype in the TME and TGF-β is a potent inducer to TANs

(Fridlender et al., 2009). The presence of TANs often relates to patient poor prognosis. CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, SDF-1 and MCP-1 are examples of chemokines secreted by CAFs that can recruit neutrophils (Cheng et al., 2018). Mast cells are another type of innate immune cells that interact with CAFs. It was shown that secretion from CAFs in hepatocellular carcinoma (also known as hepatic stellate cells) could activate mast cells. The activated mast cells in turn differentiated the stellate cells into hyperproliferative CAFs (Ma et al., 2013). In addition, mast cells have also been shown to mediate myeloid-derived suppressor cells (MDSCs) and Tregs recruitment, exacerbating immunosuppression in the TME (Yang et al., 2010).

2.4.3.4 Recruitment of Tregs and MDSC

Tregs are a subpopulation of T cells which function to suppress excessive immune response and prevent autoimmunity. They are mostly CD4 T cells expressing IL-2 receptor (CD25) and having high expression of transcription factor Forkhead box P3 (FoxP3) (Zorn et al., 2006). TGF-β has been shown to upregulate FoxP3 in Tregs (Chen et al., 2003; Zhang et al., 2006). The accumulation of Tregs in the TME has been observed in various cancers such as lung adenocarcinoma, colorectal and breast cancers (Bohling & Allison, 2008). CAFs have been shown to recruit and differentiate Tregs, as well as promote Treg survival and proliferation (Givel et al., 2018; Jacobs et al., 2018; Kinoshita et al., 2013). For example, ovarian tumours with the presence of FAPα and SDF-1β positive CAFs were found to have a higher accumulation of Tregs (Givel et al., 2018). MDSCs are another immunosuppressive population of cells in the TME. They are derived from immature myeloid cells under pathological condition like cancer. The presence MDSCs is associated with the recruitment of CAFs, Tregs and TAMs (Tesi, 2019). Reciprocally, CAFs can also facilitate MDSC accumulation in the TME (Chen et al., 2017b; Liu et al., 2017a; Yang et al., 2016b).

2.5 CAFs in the NPC tumour microenvironment

Many studies have demonstrated a pro-tumorigenic role for the TME in NPC (Huang et al., 2018). The most extensively investigated cellular components in the NPC TME are the TILs (Huang et al., 2018). This is because tumours of undifferentiated NPC are invariably heavily infiltrated with lymphocytes and despite the abundant presence of TILs, tumour development and progression continues unabated. Indeed, TILs have been reported to be crucial for primary tumour development in NPC because without the presence of TILs, xenograft establishment from primary tumour tissues was difficult in immunocompromised mice (Gourzones et al., 2012). The fact that most NPC patients are immunocompetent and CD8 T cells from TILs of NPC patients could be functionally restored *in vitro* strongly suggests there is local immune tolerance within the TME in NPC (Li et al., 2007a). In contrast to TILs, the role of CAFs in the pathogenesis of NPC has received little attention. Only until recently, the presence of CAFs within the NPC TME was confirmed and the levels of α-SMA expression in CAFs was associated with metastasis (Chen et al., 2017a) and poor prognosis (Yu et al., 2018). However, to date there have been no functional studies using NPC-derived CAFs. Therefore, studies to examine the influence of CAFs on tumour cell proliferation and migration are warranted. Furthermore, a possible role for CAFs in contributing to an immunotolerant TME in NPC has never been investigated. The association of NPC with EBV infection makes the disease a good candidate for EBV-specific adoptive T cell therapy or vaccine-based immunotherapies that enhance T cell functions. Thus, the identification of possible mechanisms by which CAFs might inhibit T cell activity would be a significant advance and may be helpful in improving the efficacies of immunotherapeutic interventions.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell Lines

3.1.1 Fibroblasts

3.1.1.1 Human NPC-derived CAF strains (NPC-CAFs)

Five NPC-CAF strains (NPC50F, NPC55F, NPC58F, NPC65F, and NPCJDF) were used in this study. NPC50F, NPC55F, NPC58F, NPC65F were kindly provided by Prof George Tsao (University of Hong Kong) and NPCJDF was obtained from Dr Alan Khoo (Institute for Medical Research, Malaysia). The CAF strains were isolated from primary cultures of NPC biopsies.

3.1.1.2 Normal human oral fibroblast strains (NHOFs)

Two NHOF strains (NHOF2 and NHOF4) that were derived from normal oral mucosa were used in this study as normal controls. The derivation of these normal fibroblast strains has been described previously (Lim et al., 2011) and were a kind gift from Professor Ken Parkinson (Queen Mary University of London).

3.1.2 NPC cell lines

Three NPC cell lines (C17, SUNE-1 and HONE-1) were used in this study. C17 is EBV-positive (Yip et al., 2018), while SUNE-1 and HONE-1 are EBV-negative (Teng et al., 1996). The C17 cell line was established from a poorly differentiated EBV-positive NPC xenograft and the process of establishment has been detailed previously (Yip et al., 2018). Similarly, the HONE-1 and SUNE-1 cell lines were derived from poorly differentiated EBV-positive primary NPC cultures and were initially EBV-positive, but lost their EBV genomes after long term culture (Teng et al., 1996). The C17 cell line was kindly provided by Prof George Tsao (University of Hong Kong).

3.1.3 EBV-specific CD8 T cell clones and target cells

Two EBV-specific CD8 T cell clones (SSC and CLG CD8 T cells) were used in this study. The SSC and CLG CD8 T cell clones specifically targeted two different LMP2 epitopes, which were HLA-A11 restricted SSCSSCPLSKI (subsequently referred to as "SSC antigen") (Midgley et al., 2003) and HLA-A2 restricted CLGGLLTMV (subsequently referred to as "CLG antigen") (Lee et al., 1993), respectively. These T cell clones were named based on the first three letters of the peptide sequences they specifically recognise and were kind gifts from Dr Graham Taylor (University of Birmingham).

Two types of target cells were used to stimulate the SSC and CLG CD8 T cells. They were (i) EBV-infected B cells (also known as lymphoblastoid cell line, LCL) and (ii) antigen-pulsed NPC cells. Two LCLs with HLA-A11 and HLA-A2 phenotypes were used to stimulate SSC and CLG CD8 T cells, respectively. The NPC cell lines, NPC43 that was stably transfected with HLA-A11 (NPC43-A11) and C17 with endogenous expression of HLA-A2 were used as target cells for SSC and CLG CD8 T cells, respectively. The LCLs and NPC43-A11 were kind gifts from Dr Graham Taylor (University of Birmingham).

3.2 Cell culture

3.2.1 Cell maintenance

CAFs and NHOFs were cultured in Dulbecco's Modified Eagle's medium (DMEM) (HyClone Laboratories Inc, USA) plus 10% fetal bovine serum (FBS) (Gibco Life Technologies, USA) and were not used above passage 15. The NPC cell lines (HONE-1, SUNE-1, C17 and NPC43-A11) and LCLs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Life Technologies, USA) containing 10% FBS (Gibco Life Technologies, USA), 1% PenStrep (Gibco Life Technologies, USA) and

25mM HEPES (Gibco Life Technologies, USA). Additionally, Y-27632 (Sigma-Aldrich, USA), a selective inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK), was added to the culture media for C17 and NPC43-A11 (4μM final concentration) to prevent lytic cycle induction in the cells. The T cell clones were cultured in 24-well plates using RPMI media supplemented with 10% FBS, 1% human serum, 30% gibbon leukaemia line (MLA 144) supernatant and 50U/ml recombinant IL2 in the presence of non-proliferating feeder cells and peptide-loaded LCLs. The protocol to culture the T cell clones is detailed in section 3.3.4.

All the cells were cultured in a humidified 37°C CO2 incubator (Binder, Germany) ventilated with 5% CO2.

3.2.2 Sub-culturing

Adherent cells were sub-cultured at 80-90% confluency. Culture media were discarded and the cells were rinsed with phosphate buffered saline (PBS) (Gibco Life Technologies, USA) before cells were dissociated or detached from the cell culture flasks with the use 0.25% trypsin-EDTA (Gibco Life Technologies, USA). The protease activity of trypsin was then neutralised with 3-5ml of complete growth medium and the cell suspension was collected in a universal tube. Cells were pelleted by centrifugation at 1,000 rpm for 8 minutes. LCLs and T cells were cultured in suspension and hence, the cells were straightway harvested by centrifugation for sub-culture. Cell pellets were then re-suspended carefully in 500μl of fresh media using a 1000μl micropipette in order to obtain a single cell suspension. The cell suspension was then diluted with an appropriate volume of fresh media to ease cell counting. To determine the cell number, a small volume of cell suspension was first mixed 1:1 with trypan blue (Gibco Life Technologies, USA) and eventually, 10μl of the mixture was sampled for cell counting using a Luna automated cell counter (Logos Biosystems, Korea). Cells were then seeded at the desired densities

in new cell culture flask(s) for sub-culturing or in various cell culture plates for specific experiments as indicated.

3.2.3 Cryopreservation and recovery of cells

$1x10^6$ cells were re-suspended in 1ml of cryopreserving reagent which was made up of 10% DMSO (Sigma-Aldrich, USA) and 90% FBS and transferred to a cryovial (Nunc, USA). The cells were then gradually cooled to -80ºC in a MrFrosty™ Cryo Container (Nalgene, USA) overnight. The frozen cells were then kept in liquid nitrogen for long term storage.

Cryopreserved cells were thawed rapidly in a 37ºC water bath and transferred immediately to a universal bottle containing 9mL of complete growth medium to minimise the cytotoxic effect of DMSO. The cell suspension were subsequently centrifuged at 1000rpm for 8 minutes. Supernatant was discarded whilst the cell pellet was re-suspended with 5ml or 10ml of complete media and the cell suspension was then transferred to a cell culture flask.

3.2.4 Culture and stimulation of EBV-specific CD8 T cell clones

3.2.4.1 PBMC extraction

The feeder cells used to grow the T cells were a mix of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) isolated from 2-3 healthy donors. The unwanted blood fractions following leucodepletion were obtained from the University Malaya Medical Centre blood blank and PBMCs isolated using Ficoll-Paque PLUS density gradient media (GE Healthcare Life Sciences, USA). Briefly, 15ml of Ficoll was added to a 50ml Falcon tube and 30ml of blood diluted at 1:1 in serum free media then layered carefully on top of the Ficoll, followed by 30 minutes of centrifugation at 1900 rpm without braking. The buffy coat (a whitish layer in between the plasma and the Ficoll) was collected in a universal bottle and serum free media added to give a final

volume of 20ml before the mononuclear cells were harvested at 1600 rpm for 10 minutes. The supernatant was removed and the cell pellet resuspended with another 20ml of serum free and then centrifuged at 1300 rpm for 10 minutes. The cells were then resuspended in 10% DMSO FBS and cryopreserved at $40x10^6$ cells per vial for further use.

3.2.4.2 Feeder cell and LCL preparation

PBMCs from two or three different donors were revived in 30ml of complete RPMI supplemented with 10µg/ml of phytohaemagglutininone (PHA) in separate T75 flasks one day before the T cell clones were revived or stimulated with LCLs. The following day, the PBMCs were collected in universal bottles harvested by centrifugation. The cell pellets were then pooled and resuspended in 10ml complete RPMI. Meanwhile, appropriate amounts of LCLs were incubated at 37°C with 5µM of SSC or CLG peptides (First BASE Laboratories, Malaysia) in RPMI for 1 hour. The PBMC mix and LCLs were then irradiated at 40 Grays in a gamma irradiator (Gammacell® 3000 Elan, Best Theratronics, Canada) for approximately 10 minutes. After the irradiation, the PBMC mix and LCLs were rinsed with 5ml of complete media three times to remove excess PHA and peptides and then resuspended in RPMI complete media at cell densities of $1x10^7$ cells/ml and $1x10^6$ cell/ml, respectively.

3.2.4.3 Reviving and stimulating the T cell clones with LCLs

Approximately $1x10^6$ T cells were resuspended in 2ml of T cell media in a 24-well plate and 100µl of PBMC suspension prepared earlier added to each well. 100µl of SSC or CLG peptide-loaded LCLs was then added to SSC or CLG CD8 T cell clones, respectively. The T cells were allowed to grow for 7 days before they were again stimulated with LCLs or used in experiments.

3.3 Collection and concentration of fibroblast conditioned media (CM)

NHOFs and CAFs were grown in T75 flasks in complete culture media to 90% confluence. The culture media were removed and the fibroblasts rinsed with PBS and subsequently with serum-free DMEM. The fibroblasts were then incubated with 8mL of serum-free DMEM. After 72 hours of incubation, the CM were collected and centrifuged at 3000 rpm to remove cell debris. The fibroblasts were then trypsinised and the number of cells per flask counted. The volume of CM was then adjusted relative to the total cell number using fresh serum free DMEM. For cytokine analysis, CM were concentrated 7.5 times using an Amicon Ultra-15 Centrifugal Filter Unit with a 3kDa molecular weight cut-off (Merck Millipores, Germany). Briefly, 7.5 mL of CM was loaded to the upper chamber of the filter units and then centrifuged in a 4^{o}C pre-chilled centrifuge at 9500 rpm for approximately 90 minutes to finally obtain 1mL of concentrated CM. The CM were stored in -80^{o}C in small aliquots to avoid repeated freeze-thaws.

3.4 In Vitro Assays

3.4.1 Senescent-associated β-galactosidase (SA β-gal) staining

SA β-gal activity in senescent cells was detected using a commercial Senescence Detection Kit (BioVision Milpitas, CA). Briefly, 7x10^{3} fibroblasts were seeded in a 12-well plate and cultured until 60-70% confluence. The culture media were removed and the cells were rinsed with PBS before incubation with fixation solution at room temperature for 15 minutes. The proprietary staining solution was added with 1mg/ml X-gal dissolved in DMSO and staining supplement. The fixed cells were rinsed twice with PBS and then incubated overnight in the supplemented staining solution in dark at 37^{o}C. The following day, the cells were rinsed twice with PBS and then counterstained with Eosin for 10 minutes at room temperature. Excessive Eosin was removed by rinsing the cells three times with PBS. Fresh PBS was added to the cells, which were then observed under a microscope at a magnification of 20x using brightfield illumination and images

were taken from 6 random fields. The percentage of senescent cells in each of the random fields was determined and averaged.

3.4.2 Cell proliferation assay

Cell proliferation was examined using MTT assays. 100µl of HONE-1 ($1.5x10^4$ cells/ml), SUNE-1 ($1.5x10^4$ cells/ml) and C17 ($1.5x10^5$ cells/ml) were added to each well of a 96-well plates. After 24 hours of incubation, without removing the existing culture media, 100µl of serum-free DMEM, NHOF CM or CAF CM were added to the appropriate wells. The cells were allowed to proliferation in the presence of fibroblast CM for 72 hours and then, 20µl of 5mg/ml MTT (Merck, Germany) dissolved in PBS was added to the wells for another 4 hours in order to allow the formation of formazan blue. To dissolve the water insoluble formazan crystals formed in the cells, the culture media was first removed, followed by the addition of 150µl DMSO to the cells. DMSO solubilised the formazan crystals in the cells and the absorbances of the formazan solution were measured at a wavelength of 575nm with 650nm as a reference wavelength using an Infinite 200 Pro NanoQuant microplate reader (Tecan, Switzerland).

3.4.3 Transwell migration assay

Transwell inserts with 8µm pores polycarbonate membranes were coated with 200µl of 10µg/ml of fibronectin (Gibco Life Technologies, USA) in a 24-well plate while the proliferation of NPC cells was inhibited with mitomycin c (Merck, Germany) treatment at 10µg/ml for 2 hours. The cells were rinsed three times with PBS to remove traces of mitomycin c before they were trypsinised, and resuspended in serum free media. Next, 500µl of CM from NHOFs and NPC-CAFs were added to wells of a 24-well plate and the fibronectin-coated inserts were then placed in the wells filled with CM, creating the upper and lower chambers. 200µl of serum-free media containing $3x10^4$ cells was then added to the upper chamber. The cells were allowed to migrate to the fibronectin-coated

lower chamber for 18 hours in a humidified 37°C CO_2 incubator. At the end of the experiment, the non-migrated cells in the upper chamber were swabbed away using cotton buds while the cells migrated to the other side of the membrane were fixed and stained with 0.1% crystal violet (Merck, Germany) containing 20% methanol for 1 hour. The inserts were then repeatedly washed in ultrapure water to remove any excess crystal violet staining solution and then allowed to dry completely. The number of cells migrated was counted from five random fields at 20X magnification.

3.4.4 T cell activation assay

3.4.4.1 Effect of cell-cell contact

T cell activation assays were performed in 96-well microplates. A triple co-culture system consisting of CAFs, EBV-specific CD8 T cells and target cells (LCLs, NPC43-A11 or C17) was used to investigate the possible influence of cell-cell contact on T cell activation. Briefly, $5x10^3$ or increasing numbers ($2.5x10^3$, $5x10^3$, $1x10^4$ and $2x10^4$) of fibroblasts were seeded per well in 100µl of complete DMEM and allowed to attach for 3-4 hours. For controls without fibroblasts, only DMEM was added to the wells. Once the fibroblasts had attached, $5x10^3$ T cells resuspended in 50µl complete RPMI were then added to the fibroblasts, followed by $1x10^5$ of target cells n 50µl complete RPMI. The cells were co-cultured for 18 hours. The following day, the amount of IFN-γ produced by the T cells was determined using enzyme-linked immunosorbent assay (ELISA).

In experiments where NPC43-A11 and C17 were used as target cells, the cells were pre-loaded with SSC and CLG antigen peptide, respectively, before they were used to stimulate the T cells. Briefly, NPC43-A11 and C17 were trypsinised and resuspended in complete RPMI containing 1µg/ml of SSC or CLG antigen peptides and incubated with the antigen peptides for one hour. The cells were then harvested by centrifugation after

three rounds of rinsing to remove the unbound antigen peptides. The peptide-loaded cells were then resuspended in appropriate volumes of complete RPMI prior to use.

3.4.4.2 Effect of fibroblast CM

To examine the effect of NHOF and CAF CM in inhibiting T cell activation, EBV-specific CD8 T cells were co-cultured with target cells in CM from NHOFs or CAFs. Briefly, 140µl of fresh serum-free DMEM or CM from NHOFs or CAFs were added to wells in a 96-well plate. $5x10^3$ T cells resuspended in 20µl of complete RPMI were added to wells containing serum-free DMEM or CM, followed 20µl of $1x10^5$ target cells. The cells were incubated for 18 hours before IFN-γ secretion by the T cells was determined by ELISA.

3.4.4.3 IFN-γ ELISA

ELISA was used to determine IFN-γ concentrations in the supernatants. A flat-bottom Nunc Maxisorp 96-well plate (Thermo Fisher Scientific, USA) was coated overnight at 4ºC with 50µl of 0.75µg/ml anti-human IFN-γ monoclonal antibody (2G1) in 1x coating buffer. Primary antibody solution was removed and replaced with 200µl of blocking buffer. The plate was incubated with the blocking buffer at room temperature for 1 hour. Meanwhile, IFN-γ standards were prepared by performing two-fold serial dilution from recombinant human IFN-γ (Invitrogen, USA) stock solution starting at 20000ng/ml down to 3.125ng/ml with culture medium. The blocking buffer was removed and the wells were washed 6 times with phosphate buffer saline with Tween 20 (PBST). 50µl of the supernatants from the T cell activation assays and the IFN-γ standards were transferred to the ELISA plate and incubated for a 2 hours. The media were then removed and the wells washed 6 times with PBST prior to the addition of 50µl of biotinylated anti-human IFN-γ antibody (diluted 1:1333 in blocking buffer) and incubation for 90 minutes. The secondary antibody was removed by washing 6 times with PBST and 50µl of 1:1000

diluted Extravidin-peroxidase (Sigma-Aldrich, Germany) in blocking buffer were then added the wells, followed by 30 minutes of incubation. 8 times of washes using PBST were performed to remove excess Extravidin-peroxidase. 100µl of TMB solution (Thermo Fisher Scientific, USA) was then added to the wells and allowed to react with peroxidase for 5-15 minutes, depending on the intensity of the soluble blue reaction product. The reaction was stopped using 500µl of 1M HCl which produced a soluble yellow reaction product. The absorbance of the soluble yellow reaction product was measured at 450nm using an Infinite 200 Pro NanoQuant microplate reader (Tecan, Switzerland). Background signal reduction was performed and the absorbance values of the IFN-γ standards were used to construct a standard curve. The concentration of IFN-γ in each sample was determined from the standard curve.

3.5 RNA Analysis

3.5.1 Total RNA isolation

Cell pellets were collected from fibroblasts (80% confluence) cultured in T75 flasks and total RNA was extracted from the cell pellets using an RNeasy® mini kit (Qiagen, Germany). Cells were first lysed with 350µl of RLT buffer containing 1% β-mercaptoethanol and the lysates then transferred to QIAshredder spin columns and centrifuged at 13,000 rpm for 2 minutes in order to homogenise the lysates (Qiagen, Germany). Then, 350µl of freshly prepared 70% ethanol (Merck, Germany) was added to each homogenised lysate, mixed well and then transferred to RNeasy® spin columns for centrifugation at 10,000 rpm for 1 minute. The flow-through was discarded and the spin columns bound with RNA were rinsed once with RW1 wash buffer. DNase I (RNase-Free DNase Set; Qiagen, Germany) was then added to each column and DNase digestion was allowed to carry out for 15 minutes. This was followed by multiple washing using RPE buffer. The columns were then thoroughly dried by means of high speed centrifugation of 13,000 rpm for 2 minutes. Finally, 50µl of RNase-free water was used

to elute the RNA from each column. The concentrations and purity of RNA isolated were then determined using a spectrophotometer (Nanodrop 2000, Thermo Scientific, USA).

3.5.2 cDNA synthesis

Total was used to synthesise single-stranded complementary DNA (cDNA) by reverse transcription using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The reverse transcription master mix was prepared according to manufacturer's protocol and 10µl of the master mix was then added to 750ng of RNA in 10µl in a 0.2ml PCR tube. cDNA was synthesised in a thermal cycler (Applied Biosystems, USA) using the followings settings: 25^{o}C for 10 minutes, 37^{o}C for 2 hours, and 85^{o}C for 5 minutes. cDNA synthesized was then kept at -30 ^{o}C for further use.

3.5.3 Real-time quantitative polymerase chain reaction (QPCR)

The mRNA expression of CAF markers and PD-L1 was determined by SYBR Green-based QPCR using a QuantiNova® SYBR® green PCR kit (Qiagen, Germany). The primer sequences used are shown in Table 3.1. Briefly, cDNA samples were diluted at 1:15 in nuclease-free water (Gibco Life Technologies, USA) and then loaded onto a 96-well optical reaction plate in triplicate. The reaction mixture was prepared following the manufacturer's protocol whereby 1µl each of forward and reverse primers at 10uM, 10µl of 2x QuantiNova SYBR Green PCR Master Mix, 0.1µl QN ROX reference dye, and 2.9µl of sterile distilled water (total volume to 15µl) were needed per reaction. The reaction mix was then added to the wells pre-loaded with diluted cDNA or nuclease free water (non-template control; NTC). The plate was sealed with an adhesive film (Applied Biosystems; UK), subjected to brief centrifugation and the reaction was carried out using an ABI 7500 fast real-time PCR system (Applied Biosystems; UK). GAPDH was used as the endogenous control to normalise the expression of each target gene. The 7500 software v2.0 was used to determine relative gene expression based on the delta-delta Ct

(ΔΔCt) method. Melting curve analyses were also carried out at the end of the PCR cycles to confirm the specificity of the amplification reactions.

Table 3.1: Primer sequences for CAF markers and PD-L1

Gene	Sequence
CAV-1	Forward primer: GAGCTGAGCGAGAAGCAAGT Reverse primer: TCCCTTCTGGTTCTGCAATC
FAPα	Forward primer: TGAACGAGTATGTTTGCAGTGG Reverse primer: GGTCTTTGGACAATCCCATGT
PDGFRα	Forward primer: GTGCCAGACCCAGATGTAGC Reverse primer: GTACCACCCCCTCACTGTTG
PDGFRβ	Forward primer: CGTCAAGATGCTTAAATCCACAGC Reverse primer: TGATGATATAGATGGGTCCTCCTTTG
PDPN	Forward primer: TGACTCCAGGAACCAGCGAAG Reverse primer: GCGAATGCCTGTTACACTGTTGA
PD-L1	Forward primer: TATGGTGGTGCCGACTACAA Reverse primer: TGCTTGTCCAGATGACTTCG

3.6 Western Blotting

3.6.1 Protein extraction and quantification

Cell pellets of NHOFs and CAFs were collected by trypsinisation (as described in Section 3.3.2) when the fibroblasts were approximately 80% confluent in T75 flasks. Cells were then lysed with 30-50µl of NP40 lysis buffer supplemented with Protease Inhibitor Cocktail Set III (Calbiochem, Germany) and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific, USA) by pipetting up and down for several times, followed by a quick 2 minutes of sonication. The lysates were then left on ice for 30 minutes before they were centrifuged at 13200rpm at 4ºC for 30 minutes to remove cell debris. The

supernatants (debris-removed lysates) were aspirated and transferred to Eppendorf tubes pre-chilled on ice.

Bradford Protein Assay was carried out to determine the concentration of protein in each lysate. Briefly, 1.5µl of lysate was first diluted in 15µl of PBS. Bradford 1x dye reagent (Bio-rad, USA) was then added at 250µl per well into a 96 well plate. Next, 5µl of bovine serum albumin standards (Bio-Rad BSA standard set 0.125-2mg/ml, CA, USA), diluted lysates or PBS were then added to the well containing dye reagent in duplicates. The reaction mixtures were incubated for 5 minutes at room temperature before the absorbances of all the samples were measured at 595nm using an Infinite® 200 PRO microplate reader (Tecan Trading, Switzerland). A standard linear curve was plotted from the absorbances of the BSA standards and finally the protein concentration of each lysate was estimated based on the standard curve. Protein lysates collected were then kept at -80 oC until use.

3.6.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In order to prepare protein samples for SDS-PAGE, the total protein concentrations of the lysates were first normalised before they were mixed 3:1 with 4x Laemmli sample buffer (Bio-rad, USA) supplemented with 5% β-mercaptoethanol (Bio-basic, Canada) The mixtures were then denatured at 95^{o}C for 5 minutes. To prepare a 1.5mm thick polyacrylamide gel, a gel-casting apparatus (Bio-rad, USA) was first filled with 7.4ml of 10% resolving gel and left to polymerise for 20 minutes. Next, 6% stacking gel was added on top of the resolving gel and a gel comb was inserted. After polymerisation for at least 45 minutes the gel comb was removed and the gel placed in the electrophoresis apparatus (Bio-rad, USA) filled with cold running buffer (Thermo Scientific, USA). Appropriate volumes of denatured protein lysates (20µg or 30µg of total proteins) or 5µl of Precision Plus Protein All Blue standard (Bio-rad, USA) were loaded into the wells before

electrophoresis was first carried out at 70V for 20 minutes within the stacking gel, followed by 100V for 1.5 hours in the resolving gel.

3.6.3 Transferring and detection of protein

The proteins separated on gels were transferred to polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Germany) using a Mini Trans-Blot® Cell (Bio-Rad, USA) following the manufacturer's protocol. The hydrophobic PVDF membrane was activated prior to protein transfer. Briefly, the membrane was first immersed in 100% methanol for a few seconds, then rinsed with ultrapure water for 2 minutes, and finally equilibrated in transfer buffer (10x Tris/Glycine Buffer, Bio-Rad, USA) added with 20% methanol for at least 5 minutes. Semi-dry protein transfer was then carried out at 25V for 30 minutes.

The membrane was then blocked in 5% non-fat milk in Tris-buffered Saline [150mM NaCl, 50mM Tris-HCl (pH7.6)] with 0.1% Tween-20 (TBST) for 1 hour before it was incubated overnight with diluted primary antibody at 4^{o}C. The next day, the primary antibody was removed and any excess primary antibody on the membrane was rinsed away thoroughly in TBST (3 x 5 minutes). The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich, USA) or goat anti-mouse IgG (Sigma-Aldrich, USA) at 1:5000 dilution in 5% non-fat milk/TBST for 1 hour. After that, the membrane was again washed in TBST (3 x 5 minutes) and finally rinsed with TBS. WesternBright Sirius enhanced chemiluminescence (ECL) (Advansta, USA) was then added to the membrane and the proteins were visualised using an Odyssey Fc Imaging System (LI-COR Biosciences, USA) The primary antibodies and working dilutions are listed in Table 3.2. The intensity of the bands was determined by densitometry using Image Studio Lite v5.2.

Table 3.2: Details of primary antibodies and dilutions

Antibody	Species	Dilution	Manufacturer
Anti-α-SMA	Mouse monoclonal	1:2500	Dako, Japan
Anti-PDPN (D2-40)	Mouse monoclonal	1:1000	Dako, Japan
Anti-GAPDH	Rabbit polyclonal	1:3000	Abcam, USA

3.7 Cytokine array

The RayBio Human Cytokine Antibody Array G-Series 5 (RayBiotech, USA) which allowed simultaneous detection of 80 cytokines was used to examine the cytokine secretory profile of NHOFs and NPC-CAFs. Following the manufacturer's instructions, the antibody arrays were first blocked with 100µl of blocking buffer for 30 minutes. 50µl of concentrated CM from fibroblasts (see section 3.3) were then added to the arrays, followed by an overnight incubation at 4^{o}C. The following day, CM were removed and the arrays were rinsed thoroughly using the proprietary wash buffers. The arrays were incubated with 70µl of biotin-conjugated anti-cytokines antibody cocktail at room temperature for 2 hours and then washed thoroughly using the wash buffers provided. 70µl of Streptavidin-Fluor was added to the arrays and incubated at room temperature for 2 hours. After multiple times of thorough washing, the arrays were rinsed with distilled water and then air-dried in a laminar flow hood for 20 minutes. The glass chip was then stored at -20^{o}C in dark prior to laser scanning using cy3 or green channel at an excitation frequency of 532nm, which was performed by the manufacturer. For data analysis, background subtraction was allowed, and all the signal intensity data were normalised against the positive control signal intensity of the reference array which was NHOF2.

3.8 Statistical Analysis

All the statistical analyses were carried out using GraphPad PRISM 5.0 software (GraphPad, USA). For in vitro assays, statistical differences between experimental groups were evaluated by one-way analysis of variance (ANOVA) and post-hoc Dunnett's test. p values <0.05 were considered as statistically significant.

CHAPTER 4: RESULTS

4.1 Preliminary *in vitro* characterization of NPC-CAFs

4.1.1 Cellular senescence

Activated CAFs are generally highly proliferative and have enhanced secretory capability compared to normal or quiescent fibroblasts (Kalluri, 2016). However, a sub-population of prematurely senescent CAFs has been found in some head and neck tumours, such as OSCC, and they were reported to be pro-tumorigenic due to the secretion of tumour-promoting senescence-associated soluble factors (Hassona et al., 2014). In the present study, 3 out of 5 CAF strains (NPC55F, NPC58F, and NPC65F) proliferated at a similar rate to normal fibroblasts (NHOF2 and NHOF4), whilst 2 CAF strains (NPC50F and NPCJDF) proliferated very slowly (data not shown). NPC50F and NPCJDF cells were larger and morphologically appeared flatter than other fibroblast strains, characteristics that are typical of senescent cells (Hassona et al., 2014). In view of these preliminary findings and the fact that CAF senescence has not been examined in the context of the pathogenesis of NPC, I determined the levels of senescence in NPC-CAFs and NHOFs by SA β-gal staining. The results showed that NPC50F and NPCJDF were highly senescent (65% and 72% of cells showed positive staining, respectively), while the other three CAF strains showed low degrees of senescence that were similar to NHOFs (less than 10% of positive staining) (Figure 4.1)

Due to the high level of senescence in NPC50F and NPCJDF, the long-term culture of these cells was impossible. However, sufficient cell numbers were obtained to examine the expression of several CAF markers in a single experiment. Subsequent functional experiments were performed using NPC55F, NPC58F and NPC65F only.

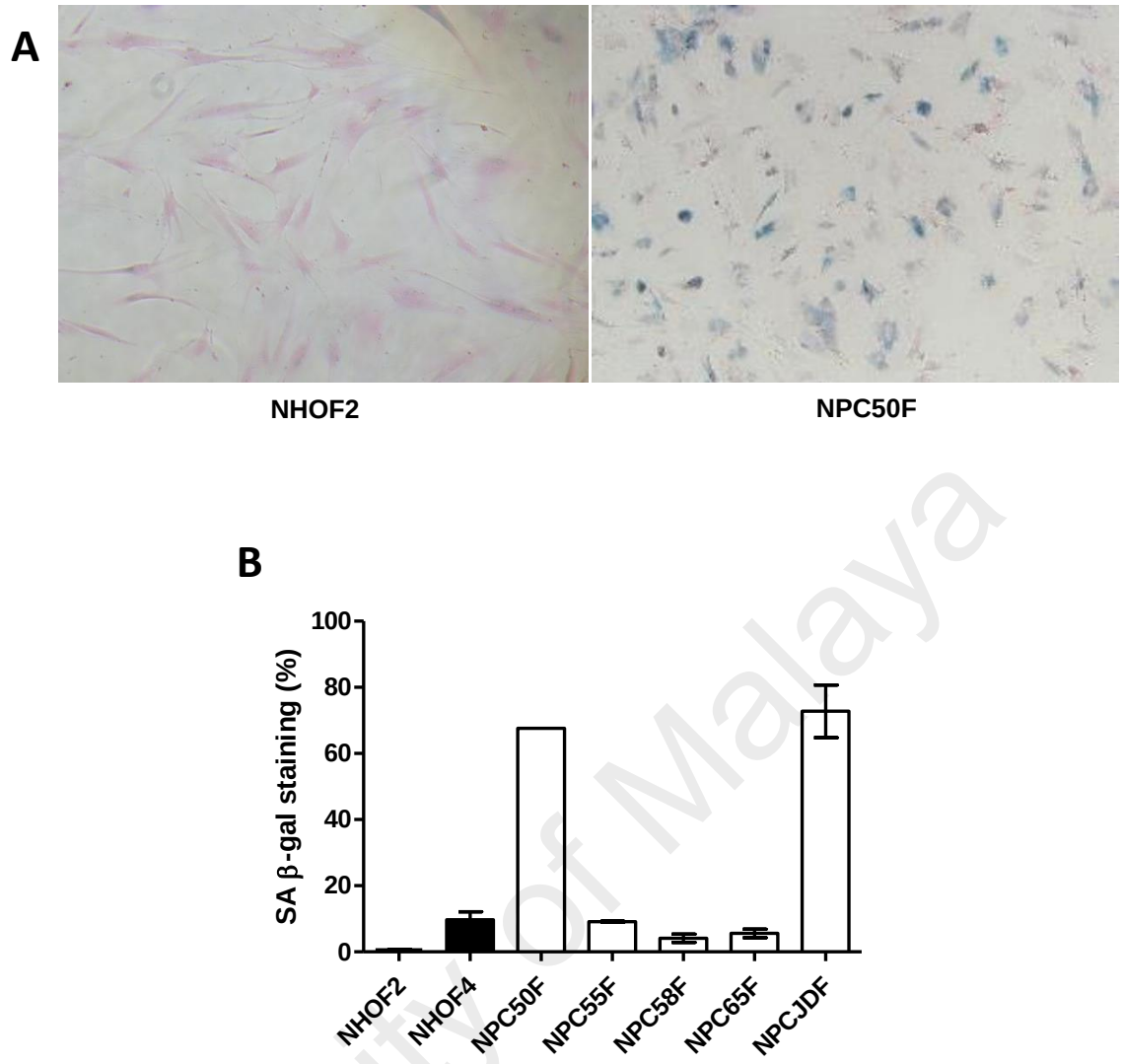

Figure 4.1: Senescence-associated β-galactosidase (SA β-gal) staining in normal fibroblasts and NPC-CAFs. (A) Representative images of fibroblasts showing negative (left panel) and positive (right panel) cytochemical SA β-gal staining. Cells with blue staining were senescent (B) Quantitative analysis of SA β-gal positive staining. Senescent cells were counted in 6-9 fields containing at least 250 cells in total. The results are expressed as the percentage of stained cells. Bars, mean of duplicates; error bars, SD of duplicates.

4.1.2 Expression of CAF markers

CAFs are recognised as being a highly heterogeneous population of cells present abundantly in the tumour microenvironment. As a result of this heterogeneity, there is currently no single specific marker that can identify all CAFs. Although α-SMA is the most widely used CAF marker, it can only detect a sub-population of activated CAFs having *de novo* expression of α-SMA. The expression of FAPα, PDGFRα, PDGFRβ, and PDPN has also been reported to be upregulated in CAFs, whilst the expression of CAV-1 shown to be down-regulated in some CAF populations (Vitale et al., 2019). However, these proteins have not been so widely used as CAF markers and less is known about how deregulated expression of these proteins affects CAF function. There have been only a limited number of reports examining the presence of CAFs in NPC biopsies using α-SMA as biomarker and the expression of the other possible markers has not been examined in NPC-derived CAFs. Therefore, the expression levels of these markers in NPC-CAFs and normal fibroblasts were examined and compared.

All the fibroblast cultures expressed detectable levels of α-SMA protein, as determined by Western blotting (Figure 4.2). The level of expression was heterogeneous but NPC-CAFs generally expressed higher levels of α-SMA than NHOFs. Amongst the NPC-CAFs, the senescent CAF strains (NPC50F and NPCJDF) expressed substantially higher levels of α-SMA than NPC55F, NPC58F and NPC65F (Figure 4.2). Whilst the GAPDH (loading control) levels amongst the fibroblast strains were a little inconsistent, densitometric analyses were carried out to normalize α-SMA expression to the GAPDH level in of NPC50F in order to obtain a more accurate interpretation of the expression of α-SMA in the fibroblast strains.

Next, the expression of CAV-1, FAPα, PDGFRα, PDGFRβ, and PDPN in NPC-CAFs and NHOFs was examined by QPCR. The results showed that FAPα, PDGFRα and

PDGFRβ were expressed by both NPC-CAFs and NHOFs, and there was no consistent upregulation in NPC-CAFs compared to NHOFs (Figure 4.3). Similarly, CAV-1 was expressed by all the fibroblast strains at similar levels, indicating that loss of CAV-1 is not a feature of NPC-CAFs. By contrast, all the CAF strains were found to express PDPN, whilst the expression of PDPN mRNA was undetectable in NHOFs (Figure 4.4A) and this was confirmed by Western blotting (Figure 4.4B). The exclusive expression of PDPN in NPC-CAFs demonstrates that the NPC-CAFs were phenotypically different than NHOFs.

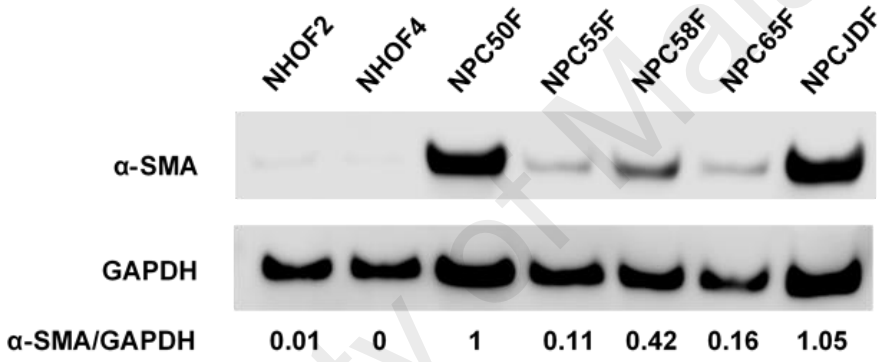

Figure 4.2: α-SMA protein expression is higher in NPC-CAFs than NHOFs. Proteins were extracted from two NHOF and five NPC-CAF strains grown in complete medium and subjected to Western blot analyses to determine the protein levels of α-SMA and GAPDH (loading control). α-SMA expression was normalised to GAPDH and shown relative to NPC50F.

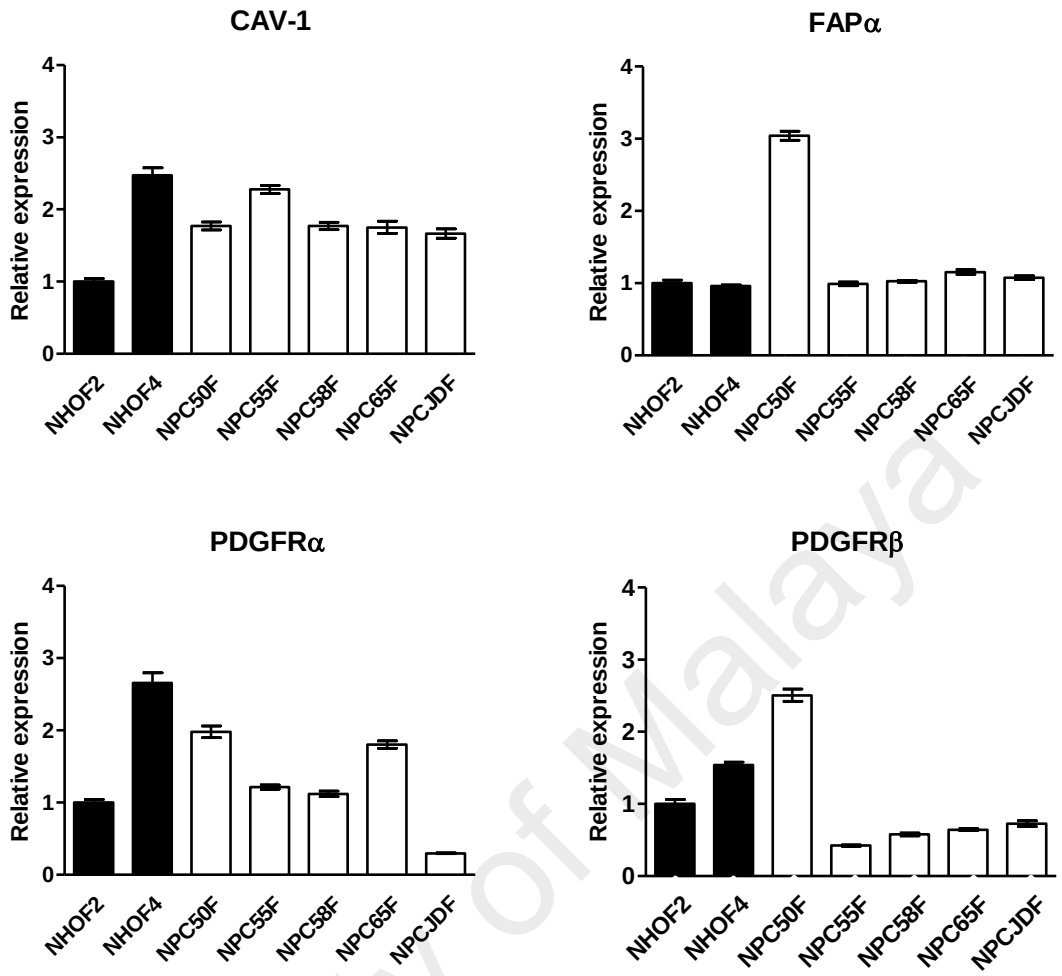

Figure 4.3: Expression of CAV-1, FAPα, PDGFRα and PDGFRβ in NHOFs and NPC-CAFs. The expression of CAV-1, FAPα, PDGFRα and PDGFRβ in NPC-CAFs and NHOFs was examined by QPCR and expression was shown relative to NHOF2. Bars, mean of triplicates; error bars, SD of triplicates.

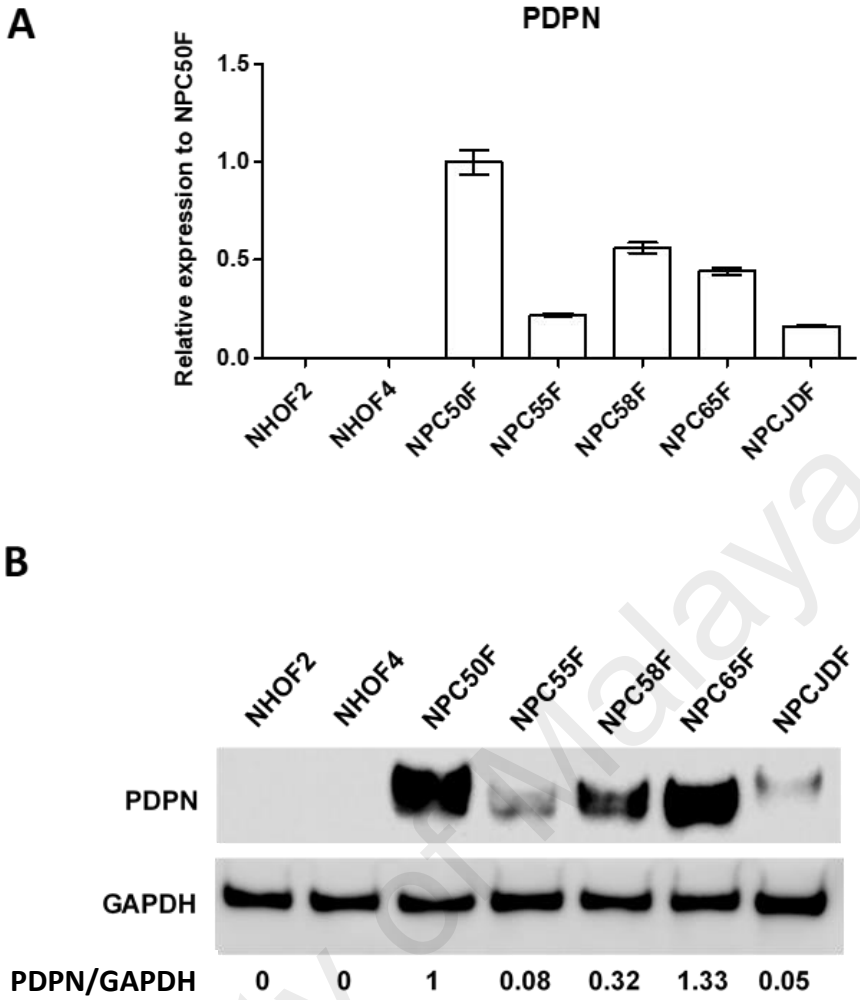

Figure 4.4: PDPN was exclusively expressed by NPC-CAFs. (A) The mRNA expression of PDPN in CAFs and NHOFs was determined by QPCR and expression was shown relative to NPC50F as PDPN expression in NHOFs was undetermined. Bars, mean of triplicates; error bars, SD of triplicates (B) Western blotting confirmed the expression of PDPN protein in NPC-CAFs. α-SMA expression was normalised to GAPDH and shown relative to NPC50F.

4.2 Effect of the CAF secretome on NPC cell proliferation

To examine whether NPC-CAFs secreted factors that influenced NPC cell growth, CM were collected from the NHOFs and NPC-CAFs. The NPC cell lines HONE-1, SUNE-1 and C17 were then cultured in the presence of serum-free DMEM, NHOF CM or CAF CM for 72 hours before MTT assays were carried out to determine the number of viable cells as an indicator of cell proliferation. The results showed that cell growth was similar when the NPC cells were cultured in serum-free DMEM and CM from both NHOFs and CAFs, indicating that the CAF secretome had no effect on NPC cell proliferation (Figure 4.5).

4.3 Effect of the CAF secretome on NPC cell migration

Cancer cell migration and invasion are the initial steps of the metastatic cascade. A cancer cell has to acquire a motile phenotype in order to escape from the primary tumour and intravasate into the bloodstream in order to spread to distal organs. CAFs have been shown to promote cancer cell motility in a number of cancer types in part via the secretion of promigratory soluble factors. In the present study, transwell migration assays were used to study the effect of the NPC-CAF secretome on the migration of NPC cells. Given that C17 is unable to migrate in transwell assays, this cell line was not used in these experiments. The migration of HONE-1 and SUNE-1 cells towards CM from all three NPC-CAFs was significantly greater than migration towards NHOF CM (Figure 4.6), demonstrating that promigratory factors are secreted by NPC-CAFs.

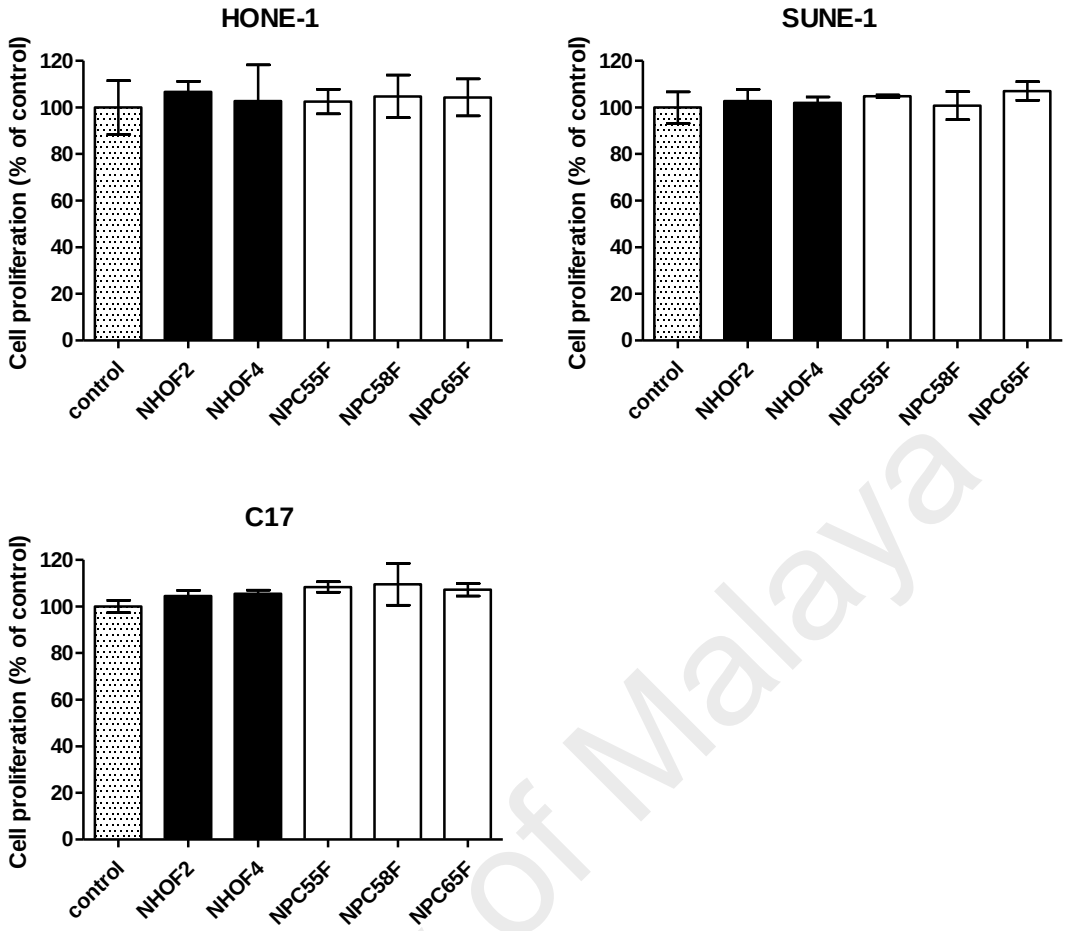

Figure 4.5: CM from NPC-derived CAFs did not enhance NPC cell proliferation. MTT assays showed that the proliferation of HONE-1, SUNE-1 and C17 cells treated with CM from NHOFs and CAFs was similar to controls (serum free media) after 72 hours. Results shown are the percentage of cell proliferation relative to vehicle controls. The data are representative of two independent experiments. Bars, mean of triplicates; Error bars, SD of triplicates.

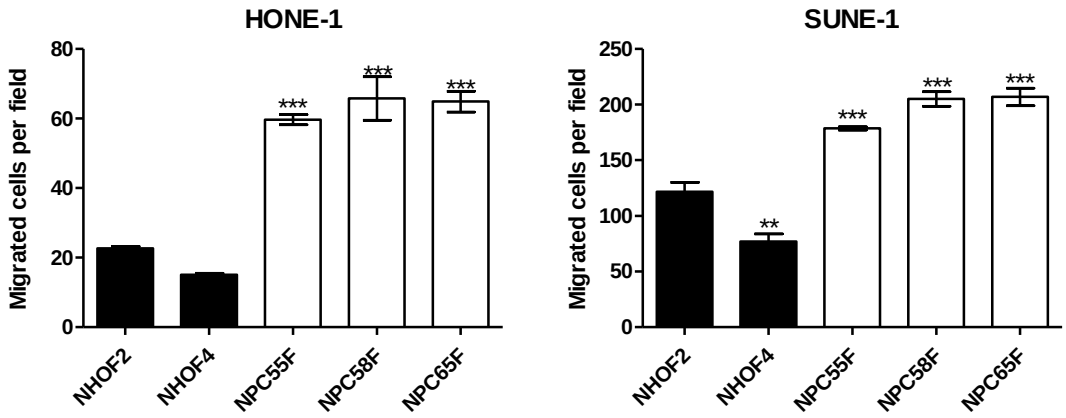

Figure 4.6: CM from NPC-derived CAFs enhanced NPC cell migration. NPC cells were pre-treated with mitomycin C to induce cell cycle arrest. The non-proliferating NPC cells were then seeded in the upper chambers while CM from NHOFs and CAFs were used as a chemoattractant in the lower chambers. CAF CM significantly enhanced the migration of HONE-1 and SUNE-1 cells. The total number of migrated cells was counted in 5 random fields using an inverted microscope and the results expressed as the average number of cells per field. The data are representative of two independent experiments. Bars, mean of triplicates; Error bars, SD of triplicates. *** = $p<0.001$; ** = $p<0.01$ (Dunnett's test)

4.4 Effect of NPC-CAFs on the activity of EBV-specific CD8 T cells

The co-existence of TILs and unrestrained tumour growth suggests local immune tolerance within the NPC TME. CAFs could potentially contribute to this phenomenon, as immunosuppressive roles for CAFs have been documented in other tumour types. Therefore, I investigated whether NPC-CAFs could inhibit the activity of EBV-specific CD8 T cells. CD8 T cells, also known as cytotoxic T cells, are the predominant subpopulation of T cells that can eliminate cancerous or infected cells by secreting IFN-γ, perforin, and granzyme B upon antigen encounter. In the present study, T cell activation assays were performed to examine whether IFN-γ production by two EBV-specific CD8 T cell clones named "SSC" and "CLG" when co-cultured with target cells would be affected by NPC-CAFs. The SSC and CLG CD8 T cell clones recognise two different epitopes of LMP2, an EBV latent protein. Two different target cells were used to stimulate the CD8 T cells to produce IFN-γ: 1) EBV-infected B cells, also known as lymphoblastoid cell lines (LCLs), which expressed almost all EBV latent antigens endogenously, including LMP2; 2) NPC cells loaded with appropriate peptides. T cell activation was determined by quantifying the amount of IFN-γ released by the T cells into the culture media using ELISA.

4.4.1 LCLs as target cells

4.4.1.1 Cell-cell contact

The possibility that NPC-CAFs might affect T cell activation by direct cell-cell contact was investigated by co-culturing NPC-CAFs with SSC CD8 T cells and LCLs. The results showed that the presence of NPC-CAFs inhibited SSC CD8 T cell activation and this effect was highly significant (Figure 4.7). The use of NHOFs in place of NPC-CAFs did not result in a similar inhibition of T cell activity (Figure 4.7). To further confirm the T cell inhibitory effects of NPC-CAFs, similar T cell activation assays were performed in the presence of increasing numbers of fibroblasts. A cell density-dependent inhibition on

T cell activity was observed following the co-culture of NPC-CAFs with SSC CD8 T cells and LCLs (Figure 4.8A-C). A similar effect was observed with NHOF2, particularly at higher cell densities, although the effects were generally weaker than those caused by NPC-CAFs (Figure 4.8D). No inhibition of T cell activity was observed with NHOF4 at any cell density (Figure 4.8E). These observations demonstrate that NPC-derived CAFs can inhibit CD8 T cell function to a greater extent than normal fibroblasts. This effect could be due to the expression of cell surface immunomodulatory molecules, but the possibility of the T cell inhibition was mediated by CAF-derived soluble factors could not be excluded, as the fibroblasts would secrete cytokines during the co-culture.

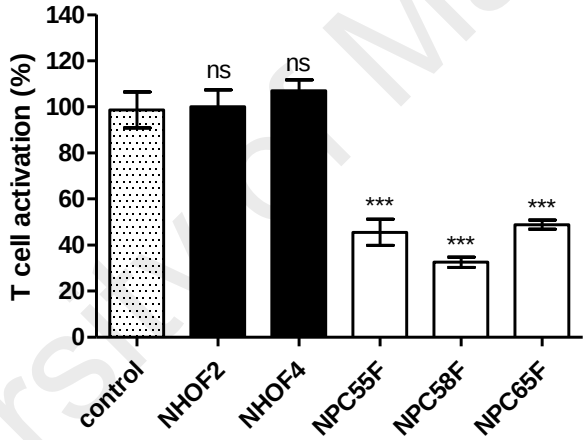

Figure 4.7: NPC-CAFs inhibited the activation of SSC CD8 T cell clones stimulated by LCLs. The presence of NPC-CAFs in the co-culture of SSC CD8 T cells and LCLs resulted in a drastic attenuation of T cell activation (control: without fibroblast = 100% T cell activation). The data are representative of three independent experiments. Bars, mean of triplicates; Error bars, SD of triplicates. ns= not significant; *** = $p<0.001$ (Dunnett's test)

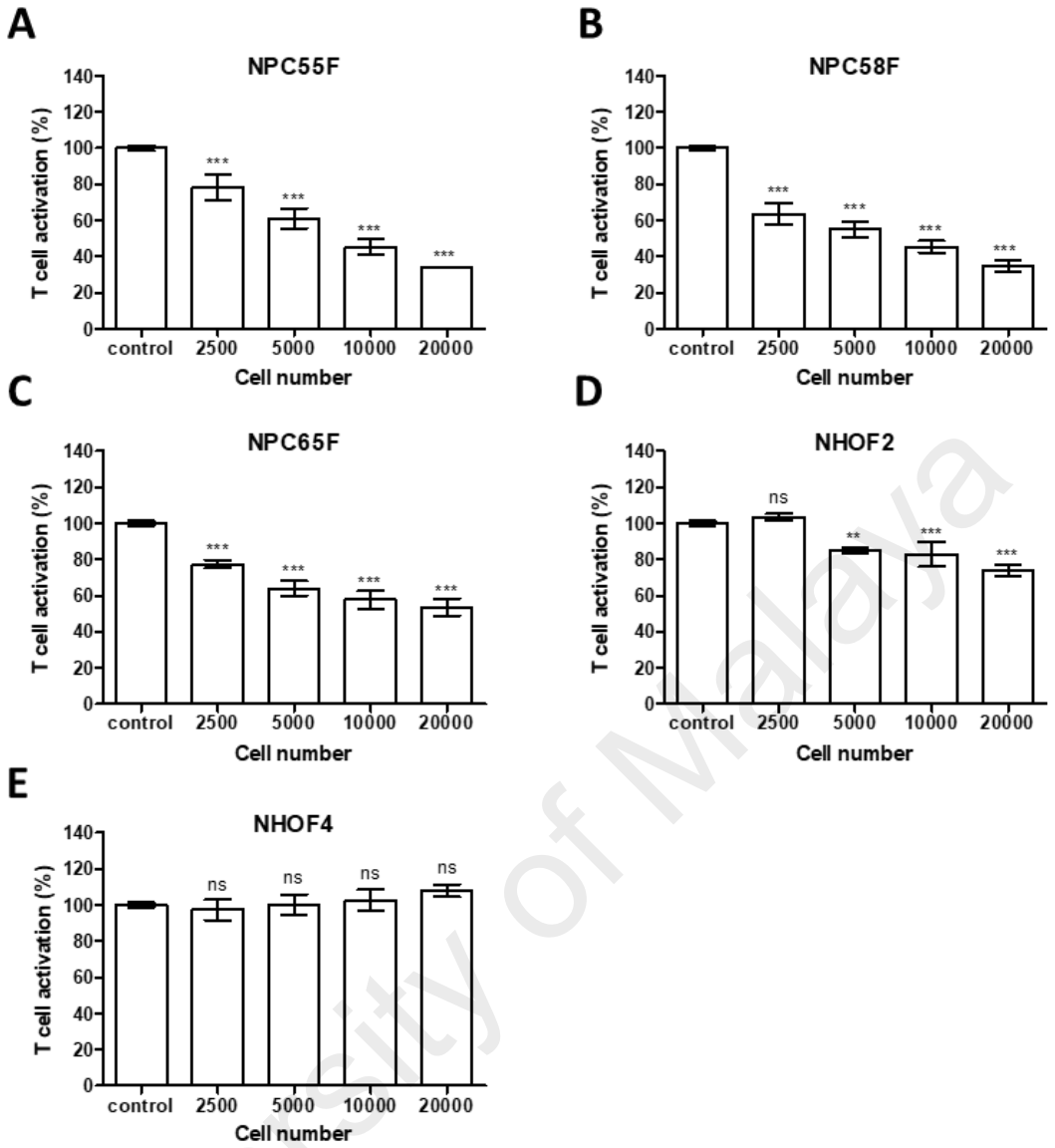

Figure 4.8: The inhibitory effect of NPC-CAFs on LCL-stimulated SSC CD8 T cells is cell density-dependent. CAFs or NHOFs were co-cultured together with SSC CD8 T cells and LCLs at increasing cell densities. (A) NPC55F, (B) NPC58F, and (C) NPC65F inhibited SSC CD8 T cell activation in a cell density-dependent manner. A similar effect was observed with (D) NHOF2 but to a lesser extent, while (E) NHOF4 did not inhibit the T cells at any cell density. The data are representative of two independent experiments. Bars, mean of triplicates; Error bars, SD of triplicates. ns= not significant; *** = $p<0.001$; ** = $p<0.01$ (Dunnett's test).

4.4.1.2 CM

Next, I investigated if soluble factors secreted by NPC-CAFs alone could inhibit T cell activation by co-culturing SSC CD8 T cells and LCLs in the presence of CM from CAFs and NHOFs. The results showed that CAF CM significantly inhibited SSC CD8 T cell activation, whilst NHOF CM had no effect on T cell function (Figure 4.8). These data suggest that CAFs may also contribute to the immunosuppressive TME of NPC by secreting cytokines that inhibit CD8 T cell activity.

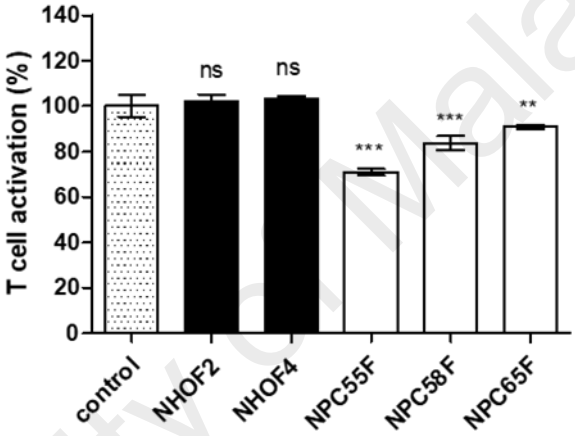

Figure 4.9: CM from NPC-CAFs inhibited the activation of SSC CD8 T stimulated by LCLs. CAF CM inhibited LCL- stimulated SSC CD8 T cell activation (vehicle control = 100% T cell activation). NHOF-CM did not inhibit SSC CD8 T cells. The data are representative of three independent experiments. Bars, mean of triplicates; Error bars, SD of triplicates; ns= not significant; *** = $p<0.001$; ** = $p<0.01$ (Dunnett's test)

4.4.2 NPC cells as targets

The observation that NPC-CAFs could inhibit EBV-specific CD8 T-cell activity when co-cultured with LCLs prompted the use of NPC cells as target cells. Two NPC cell lines with suitable HLA types were used as target cells in these experiments. Firstly, NPC43 cells transduced with *HLA-A11* (NPC43-A11), a gene that encodes a variant of the major histocompatibility complex (MHC) class I antigen processing and presenting molecules, were used as a target for SSC CD8 T cells. The ectopic expression of HLA-A11 was necessary because SSC CD8 T cells specifically recognise the peptide sequence SSCSSCPLSK (referred to subsequently as "SSC peptide") of the LMP2 epitope through HLA-A11 (Midgley et al., 2003) and NPC43 cells did not express this protein endogenously. Secondly, C17 were used to activate CLG CD8 T cells, another LMP2-specific T cell clone targeting epitope sequence CGLGLLTMV (referred to subsequently as "CLG peptide") that was restricted through HLA-A2 (Lee et al., 1993). C17 expresses endogenous HLA-A2, hence, ectopic expression of HLA-A2 was not necessary. To intensify IFN-γ secretion by the T cells, NPC43-A11 and C17 were loaded with SSC and CLG peptides before they were used to stimulate the T cells.

The co-incubation of SSC CD8 T cells and SSC peptide-loaded NPC43-A11 in CAF CM did not result in reduced secretion of IFN-γ from the T cells (Figure 4.10A). Similarly, when another T cell clone, CLG CD8 T cells, were co-cultured with CAFs, CLG CD8 T cells activity following stimulation with LCLs was inhibited (Figure 4.10B), but this was not seen with peptide-loaded C17 (Figure 4.10C)

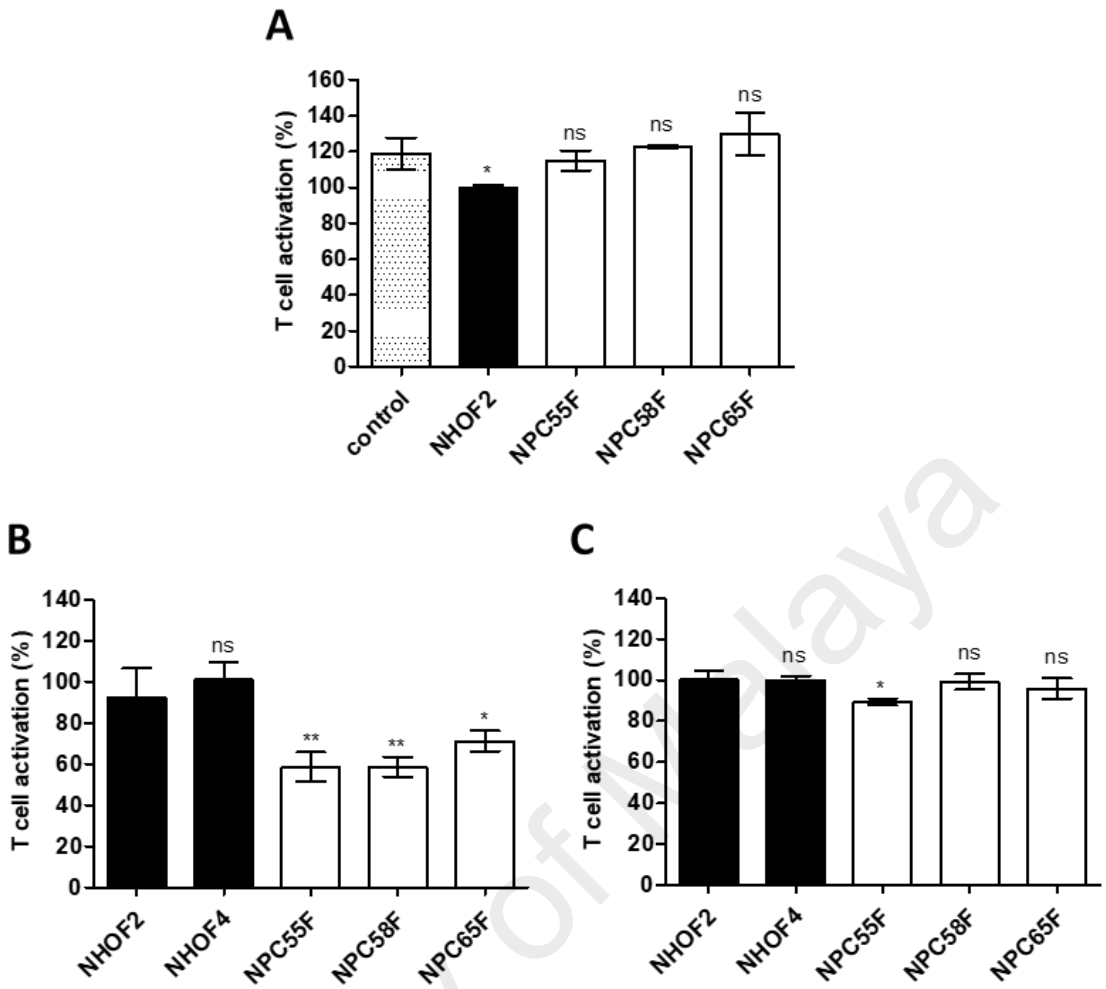

Figure 4.10: NPC-CAFs and CM did not inhibit the activation of SSC and CLG CD8 T cells stimulated by NPC cells (A) CAF CM failed to inhibit SSC CD8 T cell response towards peptide-loaded NPC43-A11 (control = without fibroblast). (B) NPC-CAFs inhibited CLG CD8 T cell activation in response to LCL stimulation. (C) However, similar inhibitory effects were not seen when the T cell target was changed to peptide-loaded C17. Bars, mean of triplicates; Error bars, SD of triplicates. ns= not significant; ** = $p<0.01$; * = $p<0.05$ (Dunnett's test)

4.4.3 CAF contact-dependent T cell inhibition is not mediated by PD-L1

PD-L1, an immune checkpoint inhibitor, has been reported to be present on CAFs from a number of different cancer types (Nazareth et al., 2007; Takahashi et al., 2015; Ziani et al., 2018). The interaction of PD-L1 with PD-1 expressed on T cells dampens T cell activity (Ziani et al., 2018). In the present study, PD-L1 was found to be expressed by all fibroblasts and NPC-CAFs did not consistently express higher levels of PD-L1 (Figure 4.11). Only NPC50F expressed a higher mRNA level of PD-L1 compared to NHOFs and the other four NPC-CAF strains. The fact that NPC55F, NPC58F and NPC65F (NPC-CAFs used in the T cell assays described above) expressed similar levels of PD-L1 mRNA as NHOF4 (Figure 4.11), suggests that PD-L1 is unlikely to be responsible for the contact-dependent T cell inhibition by NPC-CAFs.

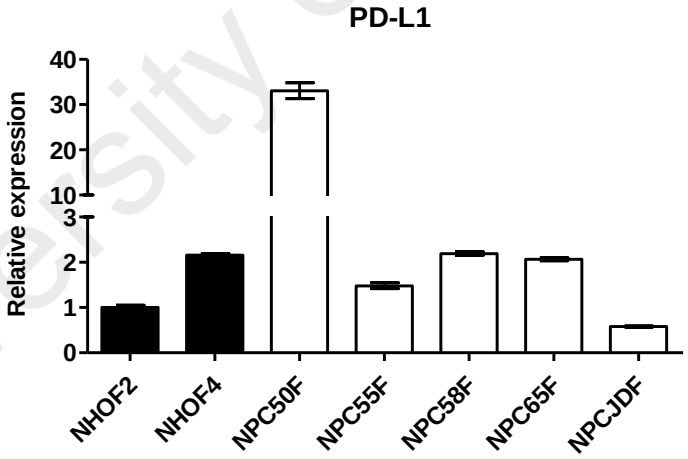

Figure 4.11: PD-L1 is not consistently expressed at higher levels by NPC-CAFs. The expression of PD-L1 in CAFs and NHOFs was examined by QPCR and expression was shown relative to NHOF2. The data are representative of two independent experiments. Bars, mean of triplicates; error bars, SD of triplicates.

4.5 Cytokine expression in NPC-CAFs

The results of the present study have shown that factors secreted by CAFs enhanced NPC cell migration and inhibited T cell activity but the specific factors involved need to be identified. Therefore, cytokine arrays were used to determine the differences in the cytokine secretory profiles between NHOFs and NPC-CAFs.

The RayBio Human Cytokine Antibody Array G-Series, which allows the simultaneous detection of 80 cytokines, was used and the antibody array map is shown in Figure 4.12. Among the 80 cytokines analysed, the levels of only VEGF A and Flt-3 ligand were found to be elevated more than 2-fold in the CM from all three CAFs compared to NHOF CM. Further, another 4 cytokines were detected at levels at least 2 times higher in the CM from two of three NPC-CAFs compared to NHOFs, namely IGFBP-3, eotaxin, RANTES, and MCP-3. The scanned array results are shown in Figure 4.13 and the quantified data shown in Figure 4.14.

	1	2	3	4	5	6	7	8	9	10	11	12	13
A	POS1	POS2	POS3	NEG	NEG	NEG	ENA-78	GSCF	GM-CSF	GRO	GRO-α	I-309	IL-1α
B	POS1	POS2	POS3	NEG	NEG	NEG	ENA-78	GSCF	GM-CSF	GRO	GRO-α	I-309	IL-1α
C	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-γ
D	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-γ
E	MCP-1	MCP-2	MCP-3	CSF-1	MDC	MIG	MIP-1β	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1
F	MCP-1	MCP-2	MCP-3	CSF-1	MDC	MIG	MIP-1β	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1
G	TNF-α	TNF-β	EGF	IGF-1	ANG	OSM	THPO	VEGFA	PDGF-BB	LEPTIN	BDNF	BLC	CKβ 8-1
H	TNF-α	TNF-β	EGF	IGF-1	ANG	OSM	THPO	VEGFA	PDGF-BB	LEPTIN	BDNF	BLC	CKβ 8-1
I	Eotaxin	Eotaxin-2	Eotaxin-3	FGF-4	FGF-6	FGF-7	FGF-9	FIt-3 lg	CX3CL1	GCP-2	GDNF	HGF	IGFBP1
J	Eotaxin	Eotaxin-2	Eotaxin-3	FGF-4	FGF-6	FGF-7	FGF-9	FIt-3 lg	CX3CL1	GCP-2	GDNF	HGF	IGFBP1
K	IGFBP-2	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3	NT-4
L	IGFBP-2	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3	NT-4
M	OPN	OPG	PARC	PLGF	TGF-β2	TGF-β3	TIMP1	TIMP2	NEG	NEG	NEG	NEG	NEG
N	OPN	OPG	PARC	PLGF	TGF-β2	TGF-β3	TIMP1	TIMP2	NEG	NEG	NEG	NEG	NEG

Figure 4.12: RayBio Human Cytokine Antibody Array G-Series 5 Map

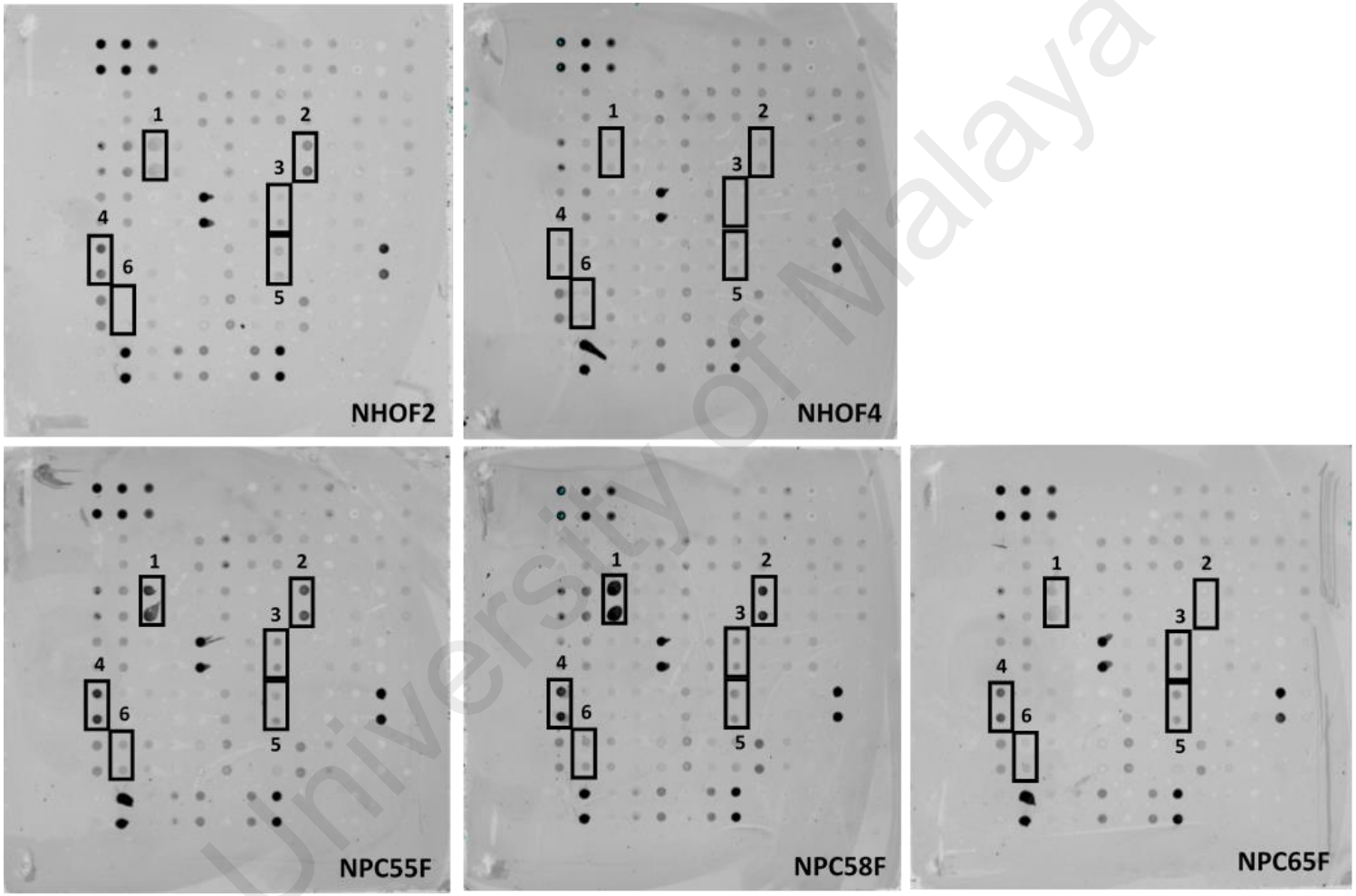

Figure 4.13: Cytokine array images showing the expression 80 cytokines by NHOFs and CAFs. The cytokines that were elevated by more than 2-fold in at least two CAFs compared to NHOFs are (1) MCP-3, (2) RANTES, (3) VEGF A, (4) eotaxin, (5) Flt-3 ligand, and (6) IGFBP-3.

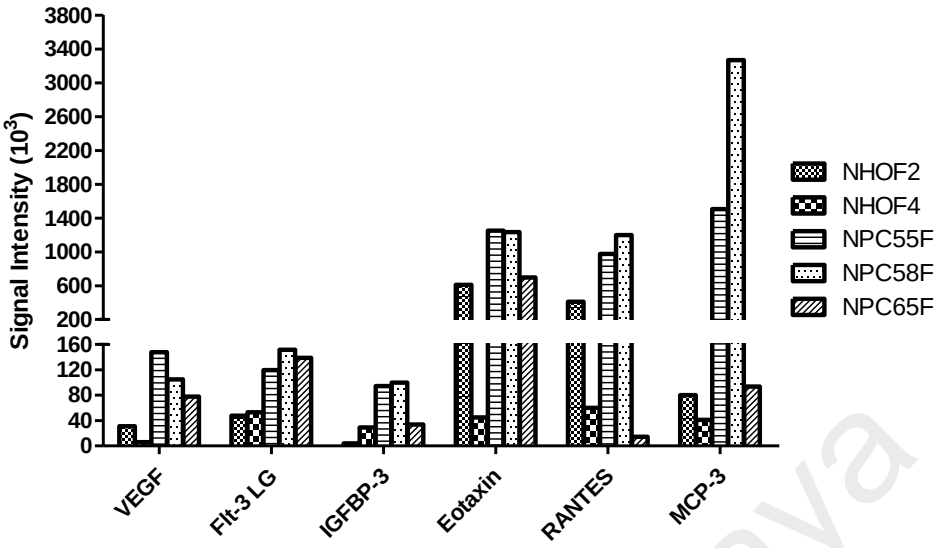

Figure 4.14: Levels of VEGF A, Flt-3 ligand, IGFBP-3, Eotaxin, RANTES (CCL5) and MCP-3 (CCL7) in CM from NHOFs and CAFs. Cytokine levels were assessed using RayBio Human Cytokine Antibody Array G-Series. The levels of VEGF A and Flt-3 ligand secretion were elevated in all CAFs tested. The levels of Eotaxin, RANTES, and MCP-3 were only elevated in in the CM from NPC55F and NPC65F.

CHAPTER 5: DISCUSSION

There is now clear evidence that the TME is essential for tumour growth and progression (Bremnes et al., 2011; Huang et al., 2018). Various non-cancerous cells are present in the TME, communicating with each other and the tumour cells to form a permissive and supportive microenvironment for tumorigenesis. CAFs are a prominent cell type within the TME, and have been shown to be pro-tumourigenic and associated with poor prognosis in a number of cancer types (Hu et al., 2018; Kawase et al., 2008; Kellermann et al., 2007; Kubouchi et al., 2018; Schoppmann et al., 2013; Shindo et al., 2013; Surowiak et al., 2007; Takatsuna et al., 2016). In NPC, the presence of CAFs in the TME has also been reported and it was shown that NPC-CAFs expressed α-SMA *in vivo* and the level of expression was associated with metastasis (Chen et al., 2017a) and poor prognosis (Yu et al., 2018). However, CAFs from NPC have not been well-characterised to date and there have been no studies on the functional roles of NPC-CAFs on tumour development and progression. Therefore, the present study was initiated in order to provide the first characterization of NPC-derived CAFs *in vitro* and to examine some of their pro-tumorigenic properties. I first examined the senescence status of a series of NPC-CAFs, together with the expression of known CAF markers in these cells and NHOFs (as normal controls). Next, functional experiments were performed to examine whether NPC-CAFs could promote NPC cell proliferation and migration, as well as contribute to immunosuppression by suppressing CD8 T cell function. Lastly, the differences in the cytokine secretory profiles between NHOFs and NPC-CAFs were examined. The results showed that NPC-CAFs were phenotypically different from NHOFs, as a subset of CAFs was prematurely senescent and all CAFs expressed higher levels of α-SMA and PDPN than their normal counterparts. The functional studies further revealed that soluble factors secreted by NPC-CAFs enhanced the motility of NPC cells and inhibited the activation of antigen-stimulated EBV-specific CD8 T cells, an effect

that also occurred as a result of direct cell-cell contact. Cytokine profiling revealed that NPC-CAFs secreted higher levels of VEGF A, RANTES and MCP-3 compared to NHOFs, suggesting that these factors may contribute to the tumour promoting effects of CAFs.

5.1 CAF heterogeneity and expression of CAF markers

Activated CAFs have been shown to be highly proliferative and to have an enhanced secretory capability which supports tumour growth and progression. However, a sub-population of activated CAFs which are prematurely senescent has been identified in a number of cancer types, including head and neck cancer. For example, CAFs derived from oral (Hassona et al., 2014), oesophageal (Mellone et al., 2017) and pancreatic ductal carcinomas (Wang et al., 2017b) have been shown to be highly senescent in culture. Cellular senescence in the context of NPC-derived CAFs has never been investigated. In the present study, two of five NPC-CAF strains (NPC50F and NPCJDF) were found to contain a high percentage of senescent cells, which suggests that NPC-CAFs are heterogeneous in nature. Senescent CAFs have been shown to support tumour progression by secreting various senescence-associated pro-inflammatory and ECM remodelling proteins (Alspach et al., 2013). It would have been interesting, therefore, to investigate the functional significance of senescence in NPC-derived CAFs in the present study. However, due to difficulties in propagating highly senescent CAFs in *in vitro* cultures, it was technically not possible to investigate the functional roles of NPC50F and NPCJDF CAFs.

Various proteins have been used as markers to distinguish CAFs from normal fibroblasts, but unfortunately, none are ubiquitously or specifically expressed by CAFs. α-SMA is the marker most widely used to identify CAFs. The expression of α-SMA in fibroblasts enhances the contractile and secretory capabilities of the cells, indicating

fibroblast activation. α-SMA positive CAFs have been identified in many cancer types including NPC (Chen et al., 2017a; Yu et al., 2018). Consistent with previous studies, the NPC-CAFs examined in the present study invariably expressed α-SMA, although the level of expression varied between the CAF strains. This observation confirms the presence of α-SMA positive CAFs in NPCs and further highlights the fact that CAF populations in NPCs are heterogeneous, as shown by the different degrees of activation seen in these CAF strains. Although the expression of α-SMA in NPC-CAFs was generally higher than in NHOFs, low levels of α-SMA were detected in one normal fibroblasts strain, NHOF2. This phenomenon can be explained because α-SMA expression has also been observed in non-cancer-associated fibroblasts from benign tumours and peritumoral fibroblasts (Berdiel-Acer et al., 2014; Jia et al., 2013). Moreover, some cultured normal fibroblasts have also been reported to express basal levels of α-SMA as TGF-β, a potent inducer of α-SMA expression, is present in serum-containing culture media (Hinz et al., 2001; Shinde et al., 2017). It is interesting to note that the senescent CAF strains (NPC50F and NPCJDF) were also found to express the highest levels of α-SMA. This observation is in accordance with studies reported by Mellone et al. (2017) which showed that prematurely senescent CAFs in head and neck and oesophageal cancers are mostly α-SMA positive and induction of senescence in normal fibroblasts by different stimuli invariably resulted in increased α-SMA expression.

It is important to recognise that α-SMA expression is not a universal and specific marker of CAFs, as α-SMA is not ubiquitously expressed by all CAFs and α-SMA positive fibroblasts can also be found in tissues undergoing fibrosis and wound healing (Darby et al., 2014). In addition to α-SMA, various other markers have been used to identify CAFs and these are, for examples, FAPα, PDGFRα, PDGFRβ, and PDPN. In addition, CAV-1 loss has been reported in some CAFs (Vitale et al., 2019). The

expression of these markers in NPC-derived CAFs, however, has never been reported. In the present study, QPCR analyses showed that there were no consistent differences in the expression of CAV-1, FAPα, PDGFRα, and PDGFRβ between NHOFs and CAFs. These data can be explained by the marked heterogeneity observed in CAFs, as well as the plasticity of fibroblasts in which their transcriptional profiles are altered in response to multiple stimuli in the TME. An example to illustrate the high heterogeneity in CAFs comes from a study by Park et al. (2016) showing that the stromal expression of CAF-related proteins such as FAPα, PDGFRα, PDGFRβ and fibroblast specific protein-1 (FSP-1) was different between two histologic subtypes of invasive breast cancer. This suggests the transcriptional profiles of CAFs are highly context-dependent. In contrast to the expression of CAV-1, FAPα, PDGFRα, and PDGFRβ, the present study highlighted that PDPN expression was specifically upregulated in NPC-CAF strains at both the mRNA and protein levels; neither PDPN mRNA or protein were detected in normal fibroblasts. Secretion from breast and pancreatic cancer cells have been shown to drastically induce PDPN expression in normal fibroblasts (Shindo et al., 2013; Wessels et al., 2019). Hence, it is very likely that NPC cells could secrete soluble factor(s) or extracellular vesicles to upregulate PDPN expression in adjacent fibroblasts through paracrine signalling. In fact, NPC cells may also express PDPN as well because TWO1, an established NPC cell line, has recently been shown to express PDPN (Hsu et al., 2019). The expression of PDPN by NPC cells *in vivo* needs further investigation. However, the expression of the protein in transformed epithelial cells of many other malignancies such as lung (Kato et al., 2005), oral (Yuan et al., 2006) and brain (Mishima et al., 2006) cancers have been evident and it was usually associated with tumour invasion and EMT (Li et al., 2014; Xie et al., 2018). Hence, if NPC cells express PDPN, it could also be possible that the PDPN expressing CAFs originated from PDPN positive NPC cells following EMT.

PDPN is a common marker for lymphatic endothelial cells but its expression has also been observed in both the stroma and tumour cells of various cancer types. For example, PDPN expressing CAFs have been reported in lung squamous cell carcinoma and adenocarcinoma (Kubouchi et al., 2018; Yurugi et al., 2017), melanoma (Kan et al., 2014), pancreatic ductal carcinoma (Hirayama et al., 2018) and oesophageal squamous cell carcinoma (Chuang et al., 2014). Furthermore, the expression of PDPN in CAFs was reported to be associated with tumour stage, lymph node metastasis, angiogenesis and patient poor prognosis in 14 common malignancies (Kitano et al., 2010). However, there is little information regarding the expression of PDPN in NPC apart from a very recent study showed that it was expressed in a primary NPC tumour cell line, TWO1 (Hsu et al., 2019). Taken together with the observations of the present study that showed PDPN expression in NPC-derived CAFs, these data suggest that PDPN might be generally upregulated in NPC. A thorough investigation of the functional roles of PDPN in the pathogenesis of NPC is warranted.

5.2 Effect of CAFs on NPC cell behaviour and T cell activity

5.2.1 Cell proliferation

The functional role of CAFs in the development and progression of NPC has not been investigated, despite the fact that these cells are often predominant in the tumour stroma. This represents a significant gap in our understanding of the pathogenesis of NPC. Activated fibroblasts have been shown to support cancer cell proliferation by secreting increased amount of various mitogens such as fibroblast growth factor (FGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), epithelial growth factor (EGF) and stromal cell-derived factor 1 (SDF-1) (Bhowmick et al., 2004). More specifically, FGF-1 present in the conditioned media of activated CAFs derived from ovarian cancer promoted cancer cell proliferation (Sun et al., 2017). The secretion of HGF, a member epidermal growth factor family, was elevated in activated CAFs isolated from

hepatocellular carcinoma (HCC) and the growth factor enhanced proliferation of two HCC cell lines (Jia et al., 2013). Additionally, the secretion of CAFs from endometrial cancer, but not of normal fibroblasts from hyperplastic tissues, activated PI3K/Akt and MAPK/Erk signaling pathways in endometrial cancer cells, which subsequently promoted the tumour cell proliferation (Subramaniam et al., 2013). Interestingly, these CAFs secreted elevated levels of macrophage chemoattractant protein (MCP)-1, IL-6, IL-8, and RANTES (Subramaniam et al., 2013). However, in the present study, treatment of HONE-1, SUNE-1 and C17 cells with CM from NPC-CAFs did not result in enhanced proliferation. Through cytokine profiling analysis in the present study, CAF CM was shown to contain significantly higher concentrations of RANTES and eotaxin than CM from NHOFs and these cytokines have been shown to enhance the proliferation of various cancer cells in previous studies. The importance of eotaxin in tumour progression was manifested in ovarian cancer whereby patients with ovarian cancer were found to have lower serum levels of eotaxin than those postmenopausal patients with other cancers, because the circulating cytokine had been absorbed by the endometrial tumour cells expressing high levels of eotaxin receptors (Levina et al., 2009). Exogenous eotaxin was shown to potently stimulate the proliferation of two ovarian cancer cell lines in a dose dependent manner (Levina et al., 2009). RANTES was shown to promote breast cancer cell proliferation by augmenting glucose uptake and catabolism in the cells in a mTOR-dependent manner, a regulatory signalling pathway of several pivotal enzymes involved in the pentose phosphate pathway (Gao et al., 2016). Nevertheless, my data are supported by the observation that treatment of NPC cell lines (BM1, TW02, TW04) with recombinant RANTES did not result in enhanced cell proliferation (Lin et al., 2013), suggesting that the effects of these cytokines could be cancer type-dependent. Indeed, the expression of cognate receptors on cancer cells is essential for the normal function of cytokines. In addition, paracrine cytokines in CAF conditioned media might not be the

only factors influencing cancer cell proliferation. In fact, other contents in CAF secretion like microRNAs (miRNAs) can also affect the behaviour of cancer cells in proliferation. For example, it was shown that CAFs secreted tumour-suppressive miR-320a, but in a lower amount than normal fibroblasts, to deregulate hepatocellular carcinoma cell proliferation (Zhang et al., 2017). The reason(s) why CM from NPC-CAFs did not enhance NPC cell proliferation remains to be determined, but it is important to bear in mind that CAFs might secrete anti-proliferative factors which might counteract the pro-proliferative factors present in CAF-CM.

5.2.2 Cell migration

Although CM from NPC-CAFs had no stimulatory effect on HONE-1, SUNE-1 and C17 cell proliferation, the CM significantly enhanced the motility of HONE-1 and SUNE-1 cells. In the present study, significantly more cells were attracted towards CAF-CM compared to CM from NHOFs in transwell migration assays. These data indicate that CAFs secrete pro-migratory and/or chemotactic factors, which might be relevant in driving metastasis. Data from the cytokine array analysis in the present study indicated that some pro-migratory cytokines were indeed secreted by CAFs. For example, RANTES, MCP-3, and Eotaxin have been shown to have pro-migratory effects in a number of previous studies. RANTES has been associated with the metastasis of several cancers including breast (Khalid et al., 2015), hepatocellular (Liu et al., 2016), lung (Huang et al., 2009), and gastric cancers (Ding et al., 2016). In gastric cancer, CAFs were found to secrete higher levels of RANTES than peritumoural fibroblasts and the secretion had a promoting effect on cancer cell motility which could be inhibited with a RANTES neutralising antibody (Yang et al., 2017). Similarly, HCC-derived CAFs secreted higher levels of RANTES and MCP-3 than peritumoural fibroblasts and through the activation of hedgehog and TGF-β signalling respectively, RANTES and MCP-3 enhanced the migration of HCC cells (Liu et al., 2016). Notably, the migration of NPC cells was also

shown to be stimulated by RANTES (Lin et al., 2013). CAFs co-cultured with OSCC were found to secrete high levels of MCP-3 and the pro-migratory effect of MCP-3 on OSCC cells was demonstrated using transwell migration and wound healing assays (Jung et al., 2010). Eotaxin-1 was found to bind specifically to CCR3 on prostate cancer cells to exert its pro-migratory effect which was mediated by ERK1/2 signalling pathways and the subsequent upregulation of MMP-3 (Zhu et al., 2014). Collectively, these previous studies suggest that cytokines identified to be elevated in the CM from NPC-CAFs in the present study may potentially promote NPC cell migration but further studies, for example with neutralising antibodies, are required to test this experimentally.

5.2.3 T cell function

In addition to having reciprocal interactions with cancer cells, CAFs have also been reported to communicate with other non-malignant cell types within the TME, such as immune cells. A number of studies have shown that CAFs are involved in cancer immune evasion. Some known mechanisms by which CAFs are thought to counteract anti-tumour responses are through the recruitment and differentiation of immune suppressive cells involving in both innate and adaptive immune responses, suppression of antigen presentation, direct impairment of effector T cell function and expression of immune regulatory proteins, in addition to forming a physical barrier that hinders immune cells infiltration to the tumour site (Ziani et al., 2018). In the present study, NPC-CAFs were shown to exert inhibitory effects on the activity of EBV-specific CD8 T cells stimulated with LCLs. The presence of NPC-CAFs in co-cultures was shown to markedly reduce IFN-γ secretion by the SSC CD8 T cell clone in response to LCLs and these inhibitory effects were cell number dependent. These results suggest that NPC-CAFs may express immunoregulatory molecules that can inhibit T cell function through cell-cell contact. However, it is also possible that the inhibitory effects were due to cytokine interference, as CAFs would secrete cytokines during the co-culture experiment. To further investigate

the underlying mechanisms by which NPC-CAFs inhibit T cell activity, CAF CM was used in place of CAFs in the co-culture system. Interestingly, CAF CM also reduced IFN-γ secretion by SSC CD8 T cells. The suppression of T cell activity appeared marginal but the effects were statistically significant and comparatively weaker than inhibition observed with the direct co-culture with NPC-CAFs. Collectively, these results imply that NPC-CAFs can possibly influence CD8 T cell function through cell-cell contact and, perhaps to a lesser extent, through paracrine signaling. VEGF A, which was shown to be secreted at higher levels by CAFs in the present study, could potentially be involved in the cytokine-mediated T cell inhibition. Despite its prominent function as a pro-angiogenic factor, VEGF A has been shown to upregulate inhibitory checkpoints, such as PD-1, TIM-3 and CTLA-4 on CD8 T cells (Lapeyre-Prost et al., 2017). In addition to VEGF A, RANTES has also been reported to selectively recruit immunosuppressive immune cells to the tumour site in breast cancer (Velasco-Velazquez et al., 2014), thereby creating local immune tolerance in the TME. However, RANTES has not been shown to exert any direct inhibitory effects on T cells. It was noticeable that NHOF2 also slightly inhibited SSC CD8 T cell activity at high cell densities. One possible explanation could be due to the detectable levels of α-SMA protein expression in NHOF2, which implies a low level of fibroblast activation and possible elevated cytokine secretion, but this is hypothetical only at the present time.

Next, I examined the expression of PD-L1 in NPC-CAFs in order to determine whether the contact-dependent T cell inhibition by NPC-CAFs might be mediated by the activation of the PD-1 checkpoint. PD-L1 is a potent membrane-bound immunoregulatory molecule that interacts with PD-1, its cognate receptor expressed on T cells, resulting in the suppression of T cell function. PD-L1 is often upregulated in cancer cells but in certain cancers, such as head and neck, pancreatic and non-small cell lung cancers, PD-L1 was also found to be expressed by CAFs (Gorchs et al., 2019;

Nazareth et al., 2007; Takahashi et al., 2015). In the present study, QPCR analysis revealed that NPC-CAFs (NPC55F, NPC58F and NPC65F) did express PD-L1, but the levels of expression were not higher than NHOF4, suggesting that the contact-dependent inhibition on T cell activity exerted by NPC-CAFs may not be mediated by PD-L1. However, this implication may not be true because IFN-γ has been shown to be a potent inducer of PD-L1 and, therefore, PD-L1 upregulation in CAFs may only occur in co-cultures when IFN-γ was secreted by the T cells (Abiko et al., 2015; Garcia-Diaz et al., 2017). This hypothesis needs to be proven experimentally and it will be interesting to determine whether CAFs have a greater propensity to express PD-L1 upon IFN-γ stimulation compared to normal fibroblasts. Furthermore, as observed in pancreatic cancer, CAFs could also induce the expression of various co-inhibitory receptors including PD-1 on T cells through paracrine signalling or cell-cell contact (Gorchs et al., 2019). VEGF A has been reported to induce the upregulation of PD-1 and some other inhibitory checkpoints on CD8 T cells in mouse colorectal tumours (Voron et al., 2015) and since NPC-CAFs were shown to have elevated secretion of VEGF A in the present study, it is reasonable to postulate that when NPC-CAFs were co-cultured with T cells and LCLs in the T cell activations assays, VEGF A secreted by NPC-CAFs might induce the expression of PD-1 on the T cells, resulting in PD-L1/PD-1-dependent T cell inhibition. Whilst PD-L1 can be expressed by various cell types, PD-L2 is another ligand for PD-1 which expression is mainly restricted to antigen-presenting cells (Philips et al., 2018). Interestingly, the expression of the protein has also been reported in CAFs derived from lung (Lakins et al., 2018; Nazareth et al., 2007), oral and hypopharynx cancers (Takahshi et al., 2015). Hence, it is also possible that the contact-dependent T cell inhibition induced by NPC-CAFs was mediated by PD-L2 but this has yet to be confirmed.

Although CM from CAFs significantly inhibited IFN-γ production by SSC CD8 T cells stimulated with LCLs, a similar effect was not seen when the T cells were stimulated with peptide-loaded NPC cells (NPC43-A11). Similarly, when another T cell clone, CLG CD8 T cells, were co-cultured with CAFs, CLG CD8 T cells activity following stimulation with LCLs was inhibited, but this was not seen with peptide-loaded NPC cells (C17). A possible explanation for these apparently contradictory observations could be that the T cell activation that occurred as a result of loading NPC cells with excessive peptides might override any inhibitory effects of CAFs. Loading the NPC cells with lower concentrations of peptides might resolve this problem. Alternatively, NPC cells might be intrinsically immunosuppressive to T cells and hence mask the inhibitory effect from CAFs.

As discussed in section 5.1, PDPN, a transmembrane glycoprotein, was exclusively expressed by NPC-CAFs. The key pro-tumoural roles of PDPN are thought to be the promotion of cancer invasion and metastasis. However, PDPN expression by CAFs has recently been reported to result in cancer immune evasion. The presence of PDPN-positive CAF was found to be associated with the immunosuppressive TME of lung adenocarcinoma, as indicated by a lower frequency of intratumoural CD8 T cells and a higher infiltration of TAMs and Tregs (Sakai et al., 2018). It was shown that CAFs with high PDPN expression had upregulated expression of several immunosuppressive cytokines such as macrophage colony-stimulating factor (M-CSF), IDO, galectin-1, VEGF A and TGF-β1 than CAFs with low expression of PDPN (Sakai et al., 2018). Furthermore, Cremasco et al. (2018) showed that PDPN-expressing CAFs suppressed T cell proliferation through nitric oxide (NO) production, an effect that could be reversed by inhibiting CAF NO synthase with NG-Monomethyl-L-arginine, monoacetate salt (L-NMMA). These studies raise the possibility that PDPN may in part mediate the inhibition of CD8 T cell function by NPC-derived CAFs.

This could have major clinical implications for the development of future immunotherapy protocols to treat patients with NPC.

5.3 CAFs as therapeutic targets

The results of the present study have shown that CAFs from NPCs likely contribute to tumour progression by promoting NPC cell migration and creating an immune tolerant microenvironment. This suggests that targeting CAFs from a therapeutic perspective, possibly by developing approaches to inhibit or reverse fibroblast activation might have some clinical benefit. Indeed, it has been proposed that cancer might be a reversible disease and that tumour cells could be reprogrammed to a more normal phenotype if the TME could be "normalized" (D'Anselmi et al., 2013; Kalluri, 2016).

It is widely accepted that TGF-β1 is a potent inducer of fibroblast activation (Hassona et al., 2013; Mellone et al., 2017). Interestingly, it has been reported that TGF-β1 is upregulated in EBV-positive NPCs (Hu et al., 2012) and that serum TGF-β1 levels are higher in NPC patients compared to controls (Xu et al., 1999). Collectively, these data point to the possibility that inhibitors of TGF-β signaling might inhibit or reverse CAF activation *in vivo*. Therefore, in parallel with the main focus of my study on NPC-derived CAFs, I have shown that TGF-β1 can activate NHOFs and this could serve as an *in vitro* model to test TGF-β inhibitors. Further, in collaboration with Dr Wang Hao (Minzu University, Beijing) our group has identified an inhibitor of TGF-β signaling and I demonstrated that this compound (CJJ300) inhibits TGF-β receptor dimerization (Appendix I). Future work will examine the ability of CJJ300 to inhibit fibroblast activation.

5.4 Limitations of the study

The results of the present study revealed that NPC-derived CAFs could promote NPC cell migration and inhibit the activity of EBV-specific CD8 T cells, suggesting that CAFs may contribute to the pathogenesis of NPC, at least in part, by facilitating cancer cell metastasis and immune evasion. However, the experiments performed in the present study were carried out *in vitro* and should only be considered as preclinical proof of concept studies. Whilst an attempt was made to use CM and co-culture experiments wherever possible to investigate intercellular communication, it is impossible *in vitro* to totally recapitulate the complexity of the TME in vivo. Further work using animal models would be needed to unequivocally demonstrate the functionality of CAFs.

5.5 Future work

By showing that CAFs derived from NPC tumours could promote NPC cell migration and inhibit EBV-specific CD8 T cell responses, the present study is the first to suggest that CAFs in NPC tumours have tumour-promoting roles in facilitating invasion/metastasis and immune evasion. A number of areas for future research have been identified and that would enhance our understanding of the pathogenesis of NPC and ultimately might lead to the identification of new therapeutic targets.

Cultured CAFs derived from NPC tumours were shown to have elevated expression of α-SMA and PDPN. However, it is necessary to confirm the expression of these proteins in NPC tissues. Immunohistochemical staining of α-SMA and PDPN should be carried out to confirm the presence of α-SMA and PDPN positive CAFs in NPC tissues, to understand the distribution and localisation of CAFs expressing the proteins within the tumours, as well as to relate the expression of these proteins to clinical data. In this regard, I have currently optimised the immunohistochemistry protocols in order to examine the expression of α-SMA and PDPN within the same tissue section. Preliminary results

indicate that both α-SMA and PDPN positive CAFs are present in NPC tissue specimens, but that there is heterogeneity between cases (Appendix II).

Cytokine profiling revealed a number of cytokines which were secreted more by CAFs than normal controls, but the cytokines responsible for promoting cell migration and inhibiting T cell responses have yet to be identified. In order to know which cytokine(s) is/are responsible for these effects, the function of these cytokines would need to be investigated individually. For example, recombinant proteins and/or neutralising antibodies could be added to migration and T cell assays in the absence/presence of CAF CM to test the function of individual cytokines.

CAFs also significantly inhibited CD8 T cell activity through cell-cell contact. PD-L1 is a membrane-bounded immunoregulatory protein that could inhibit CD8 T cells when it binds to PD-1 on the T cells (Ziani et al., 2018) and it has been shown to be upregulated in some CAFs (Nazareth et al., 2007; Takahashi et al., 2015; Ziani et al., 2018). However, the present study showed that the mRNA expression of PD-L1 was not upregulated in NPC-CAFs, suggesting that the contact-dependent T cell inhibition was not due to PD-L1. Therefore, a more complete investigation of the expression of other immunoregulatory molecules by NPC-CAFs is warranted.

The functional roles of α-SMA and PDPN should be investigated as the pro-migratory and the T cell inhibitory capabilities of NPC-CAFs might be associated with α-SMA and PDPN upregulation. Gene silencing using techniques such as CRISPR-CAS9 or siRNAs to knockout/knockdown α-SMA and PDPN could be employed prior to their inclusion in assays of cell migration and T cell activity. In addition, changes in the transcriptional profiles of NPC-CAFs following α-SMA or PDPN knockdown can be investigated by DNA microarray analyses in order to identify gene cluster(s) that are associated with α-SMA or PDPN upregulation.

Lastly, CD4 T cells, also regarded as helper T cells, generally function to maintain CD8 T cell immunity by secreting cytokines, such as IL-2, to promote CD8 T cell proliferation. In addition, there is also a subset of CD4 T cells which has been found to be cytotoxic, secreting granzyme B and perforin to kill target cells in an MHC class II-restricted fashion (Takeuchi & Saito, 2017). Since NPC cells also express EBV antigens via MHC class II molecules and EBV epitope-specific CD4 memory T cells have been found in the blood of NPC patients (Lin et al., 2008), it would be informative to investigate whether NPC-CAFs also inhibit EBV-specific CD4 T cells. A fuller understanding of how CAFs contribute to immune tolerance in NPC is likely to have major implications to the design of more effective immunotherapy protocols in the future.

CHAPTER 6: CONCLUDING REMARKS

The present study shows for the first time that CAFs derived from NPCs were phenotypically different from normal fibroblasts, as PDPN expression was observed exclusively in CAFs. Furthermore, NPC-CAFs expressed heterogenous levels of α-SMA and the CAF strains with the highest levels of α-SMA were also highly senescent in culture. The present study is also the first to show that NPC-CAFs are pro-tumourigenic by promoting cancer cell migration and inhibiting EBV-specific CD8 T cell activity. These tumour promoting effects of CAFs could occur via the secretion of cytokines, such as RANTES, MCP-3 and VEGF A, as well as via the expression of as yet unidentified cell surface molecules. A model can be proposed to illustrate how CAFs contribute to the pathogenesis of NPC (Figure 5.1).

Taken together, the results of this study indicate that NPC-CAFs might help to drive carcinogenesis by promoting a more motile and possibly metastatic phenotype in NPC cells, as well as by contributing to the creation of an immunosuppressive microenvironment. The development of strategies to target or reverse CAF phenotypes in NPC might have some therapeutic benefit. Additionally, the observation that CAFs likely contribute to immune tolerance in NPC is expected to have major implications to the design of more effective immunotherapy protocols in the future.

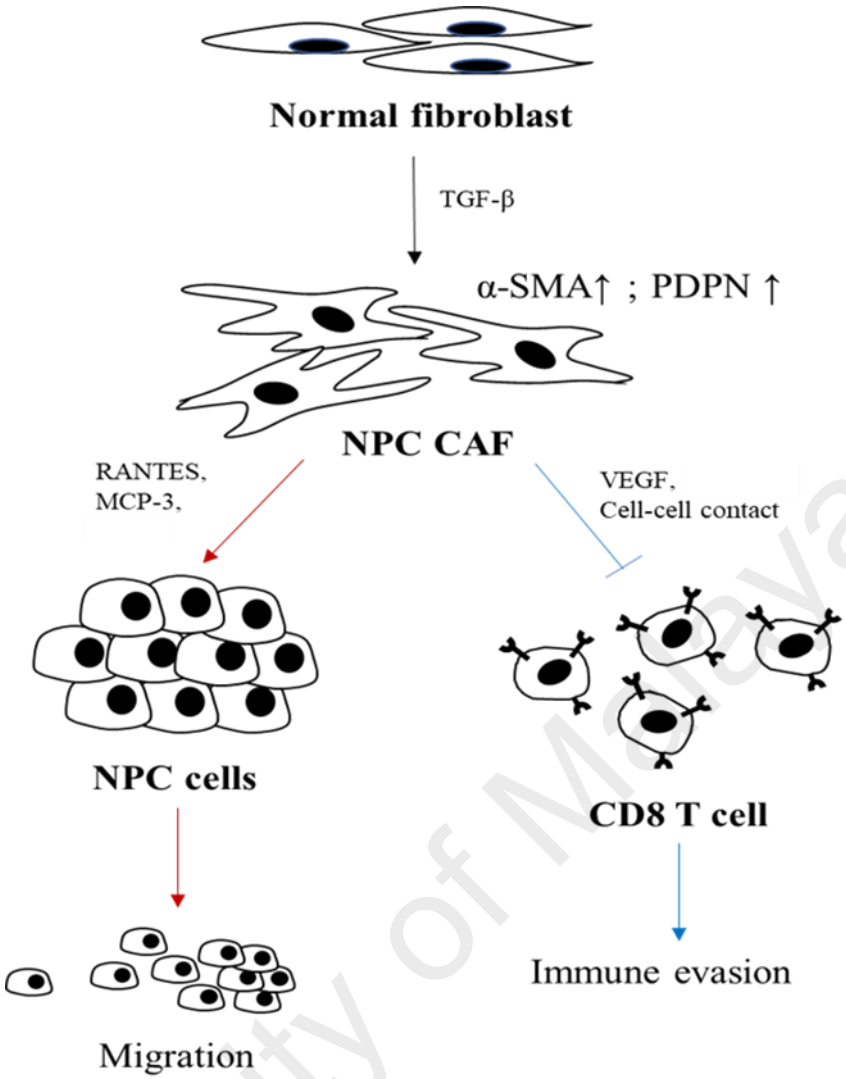

Figure 6.1: NPC-derived CAFs promote cancer cell migration and inhibit EBV-specific CD8 T cell activity. TGF-β can transdifferentiate normal fibroblasts to CAFs. CAFs derived from NPC, which are α-SMA and PDPN positive, might promote NPC metastasis by inducing a more motile phenotype in NPC cells through secretion of cytokines such as RANTES and MCP-3. Also NPC-CAFs are likely to contribute to NPC immune evasion as they can interfere with cytotoxic CD8 T cell function through the expression of immunoregulatory cell surface molecule(s) and cytokines such as VEGF A and RANTES.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Wu H*, Sun Y*, Wong WL*, Cui J, Lie J, You X, Yap LF, Huang Y, Hong W, Yang X, Paterson IC, Wang H. (2020). The development of a novel transforming growth factor-β (TGF-β) inhibitor that disrupts ligand-receptor interactions. European Journal of Medicinal Chemistry, 112042. *Equal contribution by these authors.
2. Lee HM, Wong WL, Taylor G, Paterson IC & Yap LF. Immunomodulatory cytokine expression in NPC. (Manuscript in preparation)
3. Wong WL, Tsao SW, Taylor G, Murray PG, Khoo AS, Yap LF, Paterson. Characterization of cancer-associated fibroblasts from NPC. (Manuscript in preparation)

LIST OF PRESENTATION

1. Wong WL, Lee HM, Tsao GSW, Haigh T, Murray PG, Taylor G, Khoo AS, Yap LF, Paterson IC. Influence of Cancer-Associated Fibroblasts on Immune Evasion in Nasopharyngeal Carcinoma. Presented: Gordon Research Conference on Nasopharyngeal Carcinoma. The Hong Kong University of Science and Technology in Hong Kong, China (2018)
2. Wong WL. Functional characterisation of cancer-associated fibroblasts from nasopharyngeal carcinoma. Presented: Master candidature defence. University of Malaya, Kuala Lumpur (2019)
3. Wong WL. Functional Characterisation of Cancer-Associated Fibroblasts from Nasopharyngeal Carcinoma. Presented: Master proposal seminar. University of Malaya (2019)