Epigenetic Targeting of CD1d Increases Cytolytic Activity of Invariant Natural Killer T Cells Against Non-Small Cell Lung Cancer Cells

A dissertation submitted to Trinity College Dublin as requirement for the degree of Doctor of Philosophy (PhD)

BY

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Signed: Éilis Fong Dockry   November 2015

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For my mother.

Who made me believe that anything is possible and, more importantly, helped me achieve it.

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Lists of Publications

Gray, S. G.; Baird, A. M.; O'Kelly, F.; Nikolaidis, G.; Almgren, M.; Meunier, A.; Dockry, E.; Hollywood, D.; Ekström, T. J.; Perry, A. S.; O'Byrne, K. J. (2012) "Gemcitabine reactivates epigenetically silenced genes and functions as a DNA methyltransferase inhibitor" *International Journal of Molecular Medicine* 30(6): 1505 – 1511

Baird, A. M.; Dockry, E.; Daly, A.; Stack, E.; Doherty, D. G.; O'Byrne, K. J.; Gray, S. G. (2013) "IL-23R is epigenetically regulated and modulated by chemotherapy in non-small cell lung cancer" *Frontiers in Oncology* 3: 162

Manuscript in preparation: Dockry, E.; Ghnewa, Y.; Doherty; D. G.; Gray, S. G. "Epigenetic induction of CD1d in non-small cell lung cancer cells increases their susceptibility to lysis by invariant killer T cells".

Lists of Courses and Placements

As part of my PhD program I had to undertake various courses and research placements that are outlined below.

Year	Modules and Placements	ECTS
1	Communication, Teamwork and Research Ethics Integrated Pharmacology Fundamental Biological Imagaing Molecular Mechanisms of Disease Pathogenesis Molecular Medicine: Human Disease Academic Lab Placement 1: Effects of gemcitabine on DNA CpG methylation in cancer Clinical Research Facility Placement Biostatistics Neonatal Brain Injury Cardiovascular Biology Translational Research in Haematology and Oncology Stem Cells and Gene Therapy Academic Lab Placement 2: The influence of Vγ9Vδ2 T cells on antigen presentation by human B cells Industry Placement: Innovation in New Product & Business Development in Oral Vaccines, Gastroenterology and Orphan Drugs – Concept through Phase II	70 credits
2	Project Management/Intellectual Property Project Management in the Research Context Epidemiology Regulation of Medicines and Medical Devices	10 credits
3	Case Studies in Drug Development and Design Case Studies in Research Ethics Skills and Professional Development Principles of Medical Device Design	10 Credits
Total ECTS		90 credits

Table of Abbreviations

°C	degrees Celsius
A549	Adenocarcinoma cell line
APC	Antigen-presenting cell
Bcl-2	B Cell Lymphoma 2
bp	Base Pairs
CD	Cluster of Differentiation
cfcDNA	Cell-free circulating DNA
CIK	Cytokine-Induced Killer cells
cRPMI	complete RPMI
CTL	Cytotoxic T Lymphocyte
CTLA-4	CTL-associated antigen 4
COPD	Chronic Obstructive Pulmonary Disease
DAC	Decitabine (5-Aza-2'-Deoxycytidine)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DNMTi	DNMT inhibitor
DPBS	Dulbecco's Phosphate Buffered Saline

EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked ImmunoSpot
FACS	Fluorescence Activated Cell Sorting
FMO	"Fluorescence minus one" control
FSC	Forward Scatter
GC	Germinal centre
GEM	Gemcitabine hydrochloride (2'-Deoxy-2',2'-difluorocytidine hydrochloride)
HDAC	Histone Deacetylase
HDACi	HDAC inhibitor
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate
HMG CoA	3-Hydroxy-3-Methyl-Glutaryl Coenzyme A
HLA	Human Leukocyte Antigen
HPTM	Histone Post-Translation Modifications
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IPP	Isopentenyl Pyrophosphate
iNKT	Invariant Natural Killer T cells
KRAS	Kirstan Rat Sarcoma Viral Oncogene Homolog

LAK	Lymphokine-Activated Killer cells
LAMP-1	Lysosomal-associated membrane protein -1
LPS	Lipopolysaccharides
mAb	Monoclonal antibody
MAPK	Ras-Mitogen-Activated Protein Kinase
Mb	Megabase
MDSC	Myeloid-Derived Suppressor Cell
MeOH	Methanol
MHC	Major Histocompatibility Complex
Min	Minutes
mRNA	Messenger RNA
NaN_2	Sodium Azide
NF-KB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NK	Natural Killer
NSCLC	Non-Small Cell Lung Cancer
PBA	PBS containing BSA and NaN_2
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PHA	Phytohaemahglutinin
qPCR	Quantitative PCR

RNA	Ribonucleic Acid
RT-PCR	Real Time PCR
SAHA	Suberanilohydroxamic acid or Vorinostat
SCID	Severe Combined Immunodeficiency
SCLC	Small Cell Lung Cancer
SCNA	Somatic Copy Number Alteration
SEM	Standard Error of the Mean
SK-MES-1	Squamous Cell Carcinoma Cell Line
SNP	Single Nucleotide Polymorphism
SOX2	Sex Determining Region Y Box 2
SSC	Side Scatter
STAT	Signal Transducer and Activator of Transcription
TAA	Tumour-Associated Antigen
TAM	Tumour-Associated Macrophage
TCGA	The Cancer Genome Atlas Research Network
TCR	T Cell Receptor
TERT	Telomerase Reverse Transcriptase
T_{FH}	Follicular helper T cells
TGF	Transforming Growth Factor
TIL	Tumour-Infiltrating Lymphocyte

TKI	Tyrosine Kinase Inhibitor
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
TP53	Tumour Protein p53
TRAF3	TNF-Receptor-Associated Factor 3
TRAIL	TNF-related apoptosis-inducing ligand
TSA	Trichostatin A
US(A)	United States (of America)
VEGF	Vascular Endothelial Growth Factor

Table of Contents

I.   Abstract

Lung cancer is the most common form of cancer-related death in the world, accounting for approximately 19.4% of all cancer mortalities. Currently platinum-based therapies are the gold-standard of care for non-small cell lung cancer (NSCLC), however the prognosis of advanced NSCLC remains bleak. As such there is a drive to identify novel therapeutics that can combat the disease more effectively. Widespread epigenetic alterations are observed in NSCLC, such as aberrant DNA methylation and histone acetylation patterns. The reversible nature of these modifications makes them an attractive prospect as novel therapeutic targets.

Lymphocyte subpopulations were enumerated in blood and bronchial lavage (BAL) samples from NSCLC patients and control subjects. Invariant natural killer T (iNKT) cells were found to be significantly depleted from both tissues. iNKT cells can recognize and kill cancer cells in a CD1d-dependent manner, however, we found that NSCLC cell lines do not express CD1d. We hypothesized that CD1d expression in NSCLC is epigenetically regulated and that increasing CD1d expression in NSCLC would increase their susceptibility to iNKT cell lysis. NSCLC cell lines were treated with a panel of DNA methyltransferase inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi) and quantitative polymerase chain reaction amplification of reverse transcribed mRNA and flow cytometry were performed to detect changes in CD1d expression. Both DNMTi and HDACi significantly induced CD1d expression in NSCLC cell lines, and this increase in CD1d expression was associated with increased susceptibility to iNKT cell-mediated cytolytic degranulation.

These results indicate that epigenetic targeting therapies can up-regulate CD1d expression in NSCLC, and that over-expression of CD1d leads to increased susceptibility to iNKT cell cytotoxicity. These results indicate that epigenetic manipulation of CD1d is a viable therapeutic option in combatting NSCLC.

II. Introduction

II.I. Lung cancer

Cancer is one of the leading causes of death worldwide, accounting for 14.1 million new cases, as well as 8.2 million deaths in 2012 (Torre *et al.*, 2015). Over the next two decades it is predicted that new cancer cases will rise by 70%. Increasing cancer occurrence is due to the growth of an ageing population, as well as an increasing prevalence of established risk factors, such as smoking and obesity (Bray and Møller, 2006). Among lung, colorectal, prostate and female breast cancer, incidence is generally higher in developed countries; in some cases the incidence rate can be several times higher (Torre *et al.*, 2015).

Lung cancer accounts for approximately 19.4% of cancer mortalities, and is the most common form of cancer-related death globally (Siegel *et al.*, 2014). It is also the most commonly diagnosed cancer, and while gender variations have existed previously, lung cancer is predicted to surpass breast cancer as the most common form of cancer-related death in women in 2015. An estimated 1.8 million new cases of lung cancer were diagnosed in 2012, accounting for about 13% of total cancer diagnoses (Torre *et* al., 2015). For newly diagnosed patients the five year net survival is 15.7%.

Smoking continues to be a high risk factor for developing lung cancer, with the causal relationship well-established at a 10 − 20 fold increased risk (Brownson *et al.,* 1998). Nevertheless, lung cancer in never smokers accounts for 25% of cases, and if considered a separate disease would rank as the seventh most common form of cancer related death in the world (Sun *et al.*, 2007). Interestingly the incidence of lung cancer in Chinese women is higher than in some European countries despite a lower prevalence of smoking. This is believed to be as a result of other lifestyle habits. Other known risk factors for lung cancer include asbestos, arsenic, polycyclic aromatic hydrocarbons, and indoor air pollution such as unventilated coal-
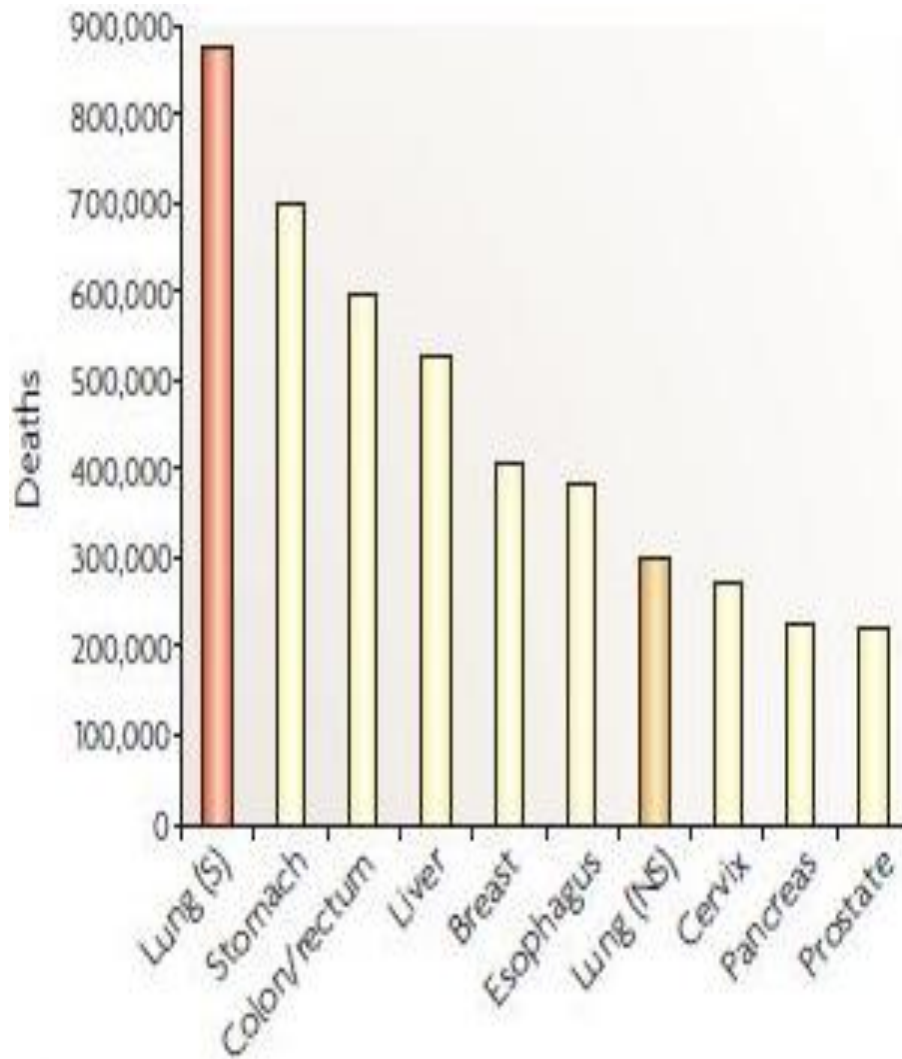
Figure 2.1: Schematic of estimated cancer deaths worldwide. Lung cancer is the leading cause of cancer-related death in the world. An estimated 15% of lung cancer deaths in men and 53% in women are not attributable to smoking (S) (Parkin *et al.*, 2005). If considered as a separate disease, lung cancer in never smokers (NS) would rank as the seventh most common form of cancer related death in the world. Adapted from Sun *et al.*, 2007.

fuelled stoves and cooking fumes. 50% of all lung cancer cases attributable to ambient fine particles were estimated to come from East Asian countries (Loomis *et al*., 2013).

II.I.I.   Epidemiology of lung cancer

While lung cancer incidence and mortality rates have continued to rise over the last few decades there has been a major shift in the global distribution of lung cancer. In the United States more than a quarter of all cancer deaths are due to lung cancer. The United States is believed to have reached the peak of its epidemic, with incidence and mortality rates (1.9%) declining in men and plateauing in women (Siegel *et al*., 2012). This decrease is believed to be the result of a successful tobacco control measures that have been implemented there in the last thirty years resulting in a reduction of smoking prevalence (Chu and Tarone, 2001).

Globally, lung and breast cancer are the most frequently diagnosed cancers as well as the leading causes of deaths in males and females (Torre *et al*., 2015). Both incidence and mortality rates of lung cancer are higher in developed countries. This is largely the result of a younger population, immaturity of tobacco epidemic and competing causes of diseases in developing countries. It is estimated that the cancer burden will shift to developing countries unless they can implement smoking cessation and prevention strategies quickly (Torre *et al*., 2015). Indeed, over the past twenty years the proportion of lung cancer cases in developing countries has risen by 18.9% (Parkin *et al*., 2005).

Lung cancer is the leading cause of cancer-related death in females in developed countries, as well as the second leading cause of death in developed countries. Incidence rates were highest in Europe, North America, East Asia and Australasia. In males it is the leading cause of cancer death worldwide, as well as the most frequently diagnosed cancer. Male incidence rates were highest in North America, East Asia and Europe, and lowest in sub-Saharan Africa (Torre *et al*., 2015).

In never smokers, lung cancer occurs more frequently in women than in men. Regional variations have been observed among female never smoker lung cancer cases. In the United States only 15% of female lung cancer patients are never smokers (Wakalee *et al*., 2007), compared with 83% in South Asia (Bader *et al*., 2006). In male lung cancer cases the proportion of never smokers is lower with less regional variation (Sun *et al*., 2007).

II.I.II.   Histopathological features of lung cancer

Lung cancer can be broadly divided into two categories, non-small cell lung cancer (NSCLC), and small cell lung cancer (SCLC). The former can be further classified into three major types, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. NSCLC accounts for approximately 85% of lung cancer cases. Of these, 40% of new cases are diagnosed with advanced disease (Goldstraw *et al*., 2007). This thesis will focus on the adenocarcinomas and squamous cell carcinoma, the two most commonly diagnosed subtypes of NSCLC.

All major histological types of lung cancer are attributable to smoking, although there is a stronger association between smoking and SCLC and squamous cell carcinoma than adenocarcinoma (Khuder *et al*., 2001). Adenocarcinoma is the most common form of lung cancer in never smokers, and a major global trend has emerged where the incidence of adenocarcinoma has risen sharply (Gabrielson, 2006). This change in global incidence has occurred over a number of decades and is believed to be the result of changes to cigarette make-up such as lowered tar and nicotine content. Liu *et al.* found that adenocarcinoma was the commonest subtype found in patients under forty; and that of the 45% of female lung cancer patients studied, 27% had never smoked (Liu *et al.*, 2000). Tobacco-associated carcinogens appear to target both the peripheral and central compartments of the lung, whereas the relatively unknown carcinogens causing lung cancer in never smokers selectively affect the peripheral airways.

II.I.I.i.　　　Adenocarcinoma

Lung adenocarcinoma a malignant tumour that is formed from the glandular structures in lung epithelial tissue. It is the most commonly diagnosed histological subtype of NSCLC (Travis, 2011). Nearly 40% of all lung cancers diagnosed in the United States are adenocarcinoma; this tumour usually originates in the peripheral lung. As mentioned previously, adenocarcinoma is significantly associated with smoking, with an odds ratio of 6.18 for current smokers (Khuder, 2001). However, this is also the most common histological subtype diagnosed in lifelong 'never smokers', defined as individuals who have smoked <100 cigarettes in their lifetime (Kreuzer *et al.*, 1999 and Brownson *et al.*, 1998).

Lung cancer is characterised by a high mutational burden when compared to many other types of cancer studied previously (Devarakonda *et al.*, 2015). After whole-exome sequencing of 230 lung adenocarcinoma samples, lung adenocarcinoma has been found to have a mean somatic mutation rate of 8.87 per megabase of DNA (Cancer Genome Atlas Research Network, 2014), as well as a non-synonomous mutation rate of 6.86 per megabase. The mutagenic effect of carcinogenic exposure, e.g. cigarette smoke, is the cause of the high mutational burden of lung cancer (Govindan *et al.*, 2012).

Nucleotide sequencing studies have established the molecular heterogeneity of lung cancer. Adenocarcinoma has a considerably different mutational landscape from either squamous cell carcinoma or SCLC. An example of this is the near exclusivity of *EGFR* (Epidermal growth factor) mutations to lung adenocarcinoma (Sharma *et al.*, 2007 and Sequist *et al.*, 2006). In addition lung cancer in never smokers has been seen to be both molecularly and demographically distinct to lung cancer in smokers (Subramanian and Govindan, 2007), as well as being primarily adenocarcinoma.

II.I.I.ii.　　　Squamous cell carcinoma

Squamous cell carcinoma of the lung is a form of malignant tumour that develops in squamous cells that line the inside of the bronchial tubes and bronchioles. A systematic analysis of 3281 tumour types across 12 tumour types sequenced by The Cancer Genome Atlas (TCGA) identified squamous cell carcinoma of the lung as the cancer with the highest mutational burden (Kandoth *et al*., 2013). Squamous cell carcinoma is composed of tumours that arise from multi-layered squamous lining cells. These cells are generally not present in the respiratory epithelium, but can arise from glandular or secretory cells as a result of exposure to metaplastic change through exposure to tobacco products, inflammation etc. Squamous cell carcinoma accounts for between 20 – 30% of all NSCLC cases (Travis, 2011).

A study by Govindan and colleagues found that smokers had a 10-fold higher mutation average than never smokers, and that cytosine to adenine transversions were found predominantly in smokers (Govindan *et al*., 2012). Squamous cell carcinoma shows a predominance of cytosine to adenine mutations suggesting an exposure to tobacco smoke. This correlates with evidence that identifies squamous cell carcinoma and SCLC as having the strongest association with cigarette smoking (Khuder, 2000), with an odds ratio of 25.5 in current smokers with squamous cell carcinoma compared with 6.18 for adenocarcinoma.

It has been established that squamous cell carcinoma carries a large number of protein altering mutations (Kan *et al*., 2010). The study by TCGA reported an average of 228 non-silent mutations in 178 squamous cell carcinoma samples and a mutation rate of 8.1/Mb of DNA (Hammerman *et al*., 2012). As mentioned previously, *EGFR* mutations are typically not present in squamous cell carcinoma, and the few that are vary from those found in lung adenocarcinoma (TCGA, 2012).

II.I.III.   Molecular alterations in NSCLC

Many studies have examined the pathways that contribute to lung tumorigenesis. These molecular alterations differ between subtypes of NSCLC, as well as other factors such as smoking status, making them an important target of novel therapeutics.

II.I.III.i.   Copy number variations

Copy number variations (CNVs) are a type of structural variation. They consist of regions of repeated DNA sequences of greater than 1000 base pairs in size that are present among individuals within a population in a variable amount. These structural aberrations take the form of large deletions or duplications, as well as balanced inversions and translocations. These variations were once considered quite rare but are now accepted as a form of inter-individual variation and account for approximately 13% of the human genome (Stankiewicz and Lupski, 2010). Analysis of this variation is typically done by a genome-wide mutation discovery approach that allows scientists to search for disease association. CNVs can occur either as a single gene, or can include adjacent genes. CNVs have been increasingly linked to disease, including lung cancer.

Somatic copy number alterations (SCNAs) are sequences that are found at different copy numbers in an individual's germline DNA and in the DNA of a clonal sub-population of cells. SCNAs are ubiquitous in cancer and can be associated with particular cancers, patient prognosis, and tumour aggressiveness. They affect a larger part of cancer genomes than any other type of somatic genetic alteration (Beroukhim *et al.*, 2010), and play critical roles in activating oncogenes and deactivating tumour suppressors (Stratton *et al.*, 2010; TCGA, 2012 and Weir *et al.* 2007). Beroukim and Bignell, and their colleagues, have ascertained that positively selected SCNAs tend to reoccur across cancers at elevated rates. Other studies have illustrated that SCNAs can also reoccur in the absence of positive selection as a result of decreased negative selection or higher rates of generation (Nijhawan *et al.*, 2012 and Solimini *et al.*, 2012).
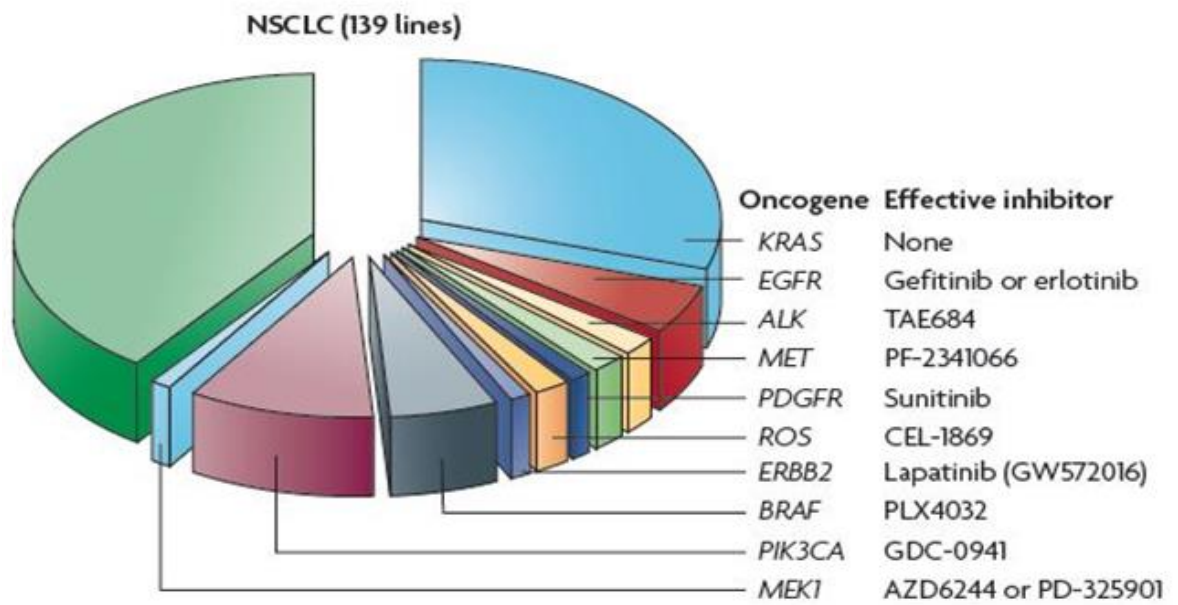
Figure 2.2: Pie chart showing the distribution of various oncogenic mutations that have been reported in NSCLC. 139 NSCLC-derived cell lines were studied to identify activating mutations. Also shown are the inhibitors that selectively target the activating oncoproteins. KRAS has no known inhibitor. The dark green sections represents the cell lines that have no known oncogenic mutations. Adapted from Sharma *et al.*, 2010.

As mentioned previously, SCNAs affect the cancer cell genome to a greater extent than any other somatic genetic alteration and are widely prevalent across lung cancer (TCGA, 2014 and Zack *et al.*, 2013). Weir and colleagues identified a total of 57 SCNAs in a panel of 528 lung adenocarcinoma samples, and illustrated how copy number gain of the short arm of chromosome 5 (5p) was the most frequent alteration in this histological subtype. They suggested that the telomerase catalytic subunit gene, telomerase reverse transcriptase (TERT) on 5p15 was the target of this amplification, indeed 18% of lung adenocarcinoma samples showed *TERT* amplification (TCGA, 2014). Other types of amplification found in lung adenocarcinoma include *Myc* transcription factors which are downstream targets of several pathways and regulate many important cellular processes. *Myc* amplification was reported in 9% of TCGA samples (TCGA, 2014).

Analysis of squamous cell carcinoma samples using single nucleotide polymorphism (SNP) arrays identified an average of 323 SCNAs per sample (TCGA, 2012). The sex determining region Y (SRY)-box 2 gene (2) is a potentially critical oncogene that participates in lung organogenesis. Almost 20% of lung squamous cell carcinoma samples have been found to have Sex Determining Region Y Box 2(SOX2) amplification (Hussenet *et al.*, 2010); this was also observed in the TCGA study where SOX2 was activated in 21% of samples, 44% of samples had an altered squamous differentiation pathway.

Another family of proteins, FOX, are highly conserved and have well-defined roles in cancer. The *FOXP1* gene is located in the short arm of chromosome 3 (3p); this region is often deleted in lung squamous cell carcinoma (Banham *et al.*, 2001). Overexpression of *FOXP1* in NSCLC is associated with better survival for lung cancer patients (Feng *et al.*, 2012). Inactivation of *FOXP1* was observed in 4% of samples in the TCGA study, and Dmitriev and colleagues found that *FOXP1* messenger RNA (mRNA) levels were significantly lower in squamous cell carcinoma than

adenocarcinoma. Understanding how SCNAs remodel the cancer genome is important to develop novel therapeutics to combat NSCLC.

II.I.III.ii. Mutations

Several sequencing studies have indicated that lung cancer is a molecularly heterogeneous disease. The mutational landscape of lung squamous carcinoma is considerably different from that of SCLC or lung adenocarcinoma, and lung cancer in smokers and never smokers is both demographically and molecularly distinct (Subramanian and Govindan, 2007). Three major genes involved in lung tumorigenesis that differ between histological subtypes as well as smoking status and gender, are epidermal growth factor receptor (*EGFR*), *KRAS* and *TP53*.

Lung cancer in never smokers is diagnosed more frequently in women than in men, and is primarily associated with adenocarcinoma histology. Adenocarcinoma and squamous cell carcinoma in smokers show an increased frequency of cytosine to adenine transversions, suggesting exposure to tobacco smoke, whereas non-smokers are more generally characterised by cytosine to thymidine mutations (Govindan *et al.*, 2012 and Imeilinski *et al.*, 2012).

II.I.III.ii.i. EGFR mutations

EGFR is one of four tyrosine kinase receptors that make up the ERBB family. It is overexpressed in many cancers, including approximately half of lung cancers (Sharma *et al.*, 2007). Upon activation these receptors form homodimers or heterodimers, initiating three major signalling pathways that trigger proliferation, anti-apoptotic signalling, angiogenesis, invasion and metastasis (Sharma *et al.*, 2007). The AKT pathway inhibits apoptosis leading to tumour survival; increased proliferation is achieved through activation of the Ras-mitogen activated protein kinase (MAPK) pathway; while the STAT signalling pathway affects many other functions including growth and survival.

Following on from these observations was the development of small molecule tyrosine kinase inhibitors (TKIs) including gefitinib and erlotinib (Thatcher *et al.*, 2005 and Shepherd *et al.*,

2005). While these inhibitors had little or no effect in most patients with NSCLC, dramatic results were observed in some patients. It was found that mutations in the tyrosine kinase domain of *EGFR* correlated with treatment response and drug sensitivity (Lynch *et al*., 2004 and Paez *et al*., 2004). *EGFR* mutations appear in both squamous cell carcinoma and adenocarcinoma, although they are more common in the latter with mutations present in approximately 16% of non-squamous NSCLC (Rosell *et al*., 2009). *EGFR* mutations are usually more common in women and non-smokers.

Analysis of TCGA's lung adenocarcinoma samples found that known oncogenic mutations were present in nearly 11% of samples (TCGA, 2014), while Imielinski and colleagues found *EGFR* mutations in 17% of samples. *EGFR* mutations can occur in squamous cell carcinoma, but are nearly four times more frequent in non-smokers than smokers (An *et al*., 2012). Only a limited number of studies have examined the frequency of *EGFR* mutations in lung squamous cell carcinoma and although an association has been seen between *EGFR* mutations and superior outcomes with TKIs, outcomes are generally inferior when compared to lung adenocarcinoma (Shukaya *et al*., 2011).

II.I.III.ii.ii.   KRAS mutations

Ras proteins function downstream of the EGFR signalling pathway and facilitate cell proliferation (Downward, 2003). Oncogenic missense mutations in *KRAS* disrupt the negative-feedback control of Ras activity leading to a constitutive activation (Downward, 2003). *KRAS* mutations are not present in SCLCs, only NSCLC, and are predominantly found in adenocarcinoma (Shigematsu *et al*., 2005 and Tam *et al*., 2006). Typically, these mutations predict poor survival (Mascaux *et al*., 2005) and resistance to EGFR TKI therapy (Pao *et al*., 2005). *KRAS* mutations are more common in smokers, and less frequent in lung cancers in East Asia (Shigematsu *et al*., 2005). Both *EGFR* and *KRAS* mutations target the peripheral airways and can give rise to lung adenocarcinoma, however, these two mutations are almost completely mutually exclusive

(Shigematsu *et al*., 2005). *KRAS* mutations were identified in just under a third of TCGA lung adenocarcinoma samples, and are rarely present in squamous cell carcinoma (TCGA, 2012).

II.I.III.ii.iii.   TP53 gene

The tumour suppressor p53 generally acts as a transcription factor for a large number of target genes (Sengupta and Harris, 2005). It is located on chromosome 17 and its downstream effects include DNA damage responses and the regulation of apoptosis. Changes in the *TP53* gene are one of the most commonly identified mutations in cancer and can occur in lung cancer in both smokers and non-smokers, although they are less common in the latter (Le Calvez *et al*., 2005). TP53 is significantly mutated in adenocarcinoma, but interestingly this mutation is differentially enriched between genders and smoking status. A study by TCGA reported a mutation rate of about 90% in lung squamous cell carcinoma.

II.I.IV.   Mortality rates and treatment

Mortality rates in lung cancer remain high because of difficulties in early detection and resistance to current therapeutics. Surgical resection is well-recognised as a treatment for localised NSCLC, but only 15% of patients are considered candidates for surgery at diagnosis (Freise *et al*., 1978). In advanced NSCLC cases, overall survival is only marginally improved by standard chemotherapy, which causes substantial morbidity. The median overall survival for a patient with advanced NSCLC is approximately one year, and only 3.5% of patients with advanced disease survive five years following diagnosis. Platinum-based therapy is the current gold standard of care for lung cancer patients (Pujol *et al*., 2006). In spite of this, most patients do not profit from this treatment, and many tumours become resistant to this therapy type. In light of this there has been a drive to develop novel therapeutics to overcome this platinum-based resistance.

II.II. Tumour Immunology

In a seminal review in 2000, Hanahan and Weinberg described the six hallmarks of cancer; functional capabilities acquired by cancer cells to allow them to survive and proliferate. Eleven years later they published another review listing two enabling characteristics that allowed cancer cells to acquire those functional capabilities, tumour-promoting inflammation and genome instability and mutation, as well as two novel hallmarks of cancer, namely reprogramming energy metabolism, and avoiding immune destruction (Hanahan and Weinberg, 2011). While the idea of tumour immunology dates back over a century, this review highlighted a renewed focus and a growing body of evidence that gave credence to earlier hypotheses. Tumour immunology is a rapidly growing area that is focused on understanding the mechanisms whereby the immune system can both enable and suppress tumorigenesis, and on the novel cancer immunotherapies that have arisen from these studies.

The immune system is comprised of the innate and adaptive immune systems. The innate immune system is the so-called "first line of defence" against microbial attack. It is characterised by a rapid and non-specific response to pathogens. The adaptive immune system is characterised by a slower, yet highly specific response, and the memory cells that develop remain in circulation far after the primary immune response has subsided.

II.II.I. Tumour-promoting inflammation

It has long been recognised that certain tumours are densely infiltrated by cells from both the innate and adaptive immune systems, which can play both pro-tumorigenic and anti-tumorigenic roles. Tumour-promoting inflammatory cells include macrophages, mast cells, and lymphocytes (Hanahan and Weinberg, 2011) which can promote tumour angiogenesis, proliferation, invasion and metastasis.

Figure 2.3: The hallmarks of cancer. Schematic of the eight hallmarks of cancer, and the two enabling characteristics. The hallmarks of cancer denote capabilities that a normal cell acquires during its progression to a neoplastic state. The acquisition of these hallmarks is made possible by two enabling characteristics: Genome instability & mutation, and tumour-promoting inflammation. . Adapted from Hanahan and Weinberg, 2011.

Research over the past two decades has demonstrated how inflammation and immune cells, namely those of the innate immune system, promote tumorigenesis (Colotta *et al*., 2009, DeNardo *et al*., 2010, Grivennikov *et al*., 2010, Qian and Pollard, 2010). Inflammation aids tumorigenesis by supplying bioactive molecules such as growth factors, survival factors, proangiogenic factors, etc., to the tumour microenvironment that facilitate growth, survival and metastasis (DeNardo *et al*., 2010, Grivennokov *et al*., 2010, Qian and Pollard, 2010). NF-KB (Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells) is a central mediator of innate immunity and inflammation, and aberrant NF-KB activity has been reported in many cancers (Karin, 2006). NF-KB is a transcription factor that promotes gene expression that leads to the production of inflammatory cytokines, adhesion molecules and angiogenic factors. It can also stimulate anti-apoptotic genes such as Bcl-2 (B cell lymphoma 2) which promotes survival in tumour cells as well as epithelial cells targeted by carcinogens (Colotta *et al*., 2009).

Tumour necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that plays a big role in supporting tumorigenesis. The first genetic evidence linking TNF-α-mediated inflammation and cancer came from a murine study which showed that TNF-α-deficient mice were protected from skin tumorigenesis (Moore *et al*., 1999). Another study of ovarian cancer cell lines found that constitutive TNF-α expression was associated with increased secretion of IL-6, macrophage migration-inhibitory factor (MIF) and the angiogenic factor, vascular endothelial growth factor (VEGF) (Kulbe *et al*., 2007). Ovarian cancer cell lines with a stable knockdown of TNF-α showed decreased secretion of IL-6, MIF and VEGF compared with wild-type cells; these cells were non-invasive and had high levels of apoptosis (Kulbe *et al*., 2007).

Furthermore, inflammation can also be present at the initiation of tumorigenesis and is capable of abetting the development of neoplasms into cancer (Qian and Pollard, 2010). Tumour-promoting lymphocytes can release actively mutagenic chemicals to nearby cancer cells that aid in their evolution (Grivennikov *et al*., 2010). NF-KB is involved in tumour initiation and

progression in tissues prone to chronic inflammation (Karin, 2006, Greten *et al.*, 2004). Inhibition of the NF-KB pathway in epithelial and myeloid cells prevented inflammation-associated tumour formation in a mouse model of colitis (Greten *et al.*, 2004). Another mouse model showed that inhibition of NF-KB activity in the later stages of tumour formation led to the apoptosis of transformed cells and failure of tumour progression (Pikarsky *et al.*, 2004). A murine study of colitis-associated colon carcinoma found that inducing colitis in wild-type mice fed with a carcinogen developed severe colonic inflammation and subsequent tumour formation (Popivanova *et al.*, 2008). These mice exhibited increased TNF-α expression, as well as increased numbers of leukocytes expressing the major TNF-α receptor, p55. Administration of a TNF-α inhibitor markedly decreased tumour numbers and formation, and decreased colonic infiltration by macrophages and neutrophils (Popivanova *et al.*, 2008).

Chronic inflammation is linked to an increased risk of tumorigenesis through angiogenesis and tissue remodelling, as well as DNA and protein damage through oxidative stress. Epidemological studies have shown that chronic inflammation predisposes to a variety of cancers (Colotta *et al.*, 2009). Carcinogens such as asbestos and cigarette smoke are known to promote tumorigenesis through chronic inflammation (Mantovani *et al.*, 2008). In addition, pulmonary diseases such as pulmonary fibrosis and chronic obstructive pulmonary disease (COPD) are characterised by deregulated and profuse inflammation, and are linked to increased risk of lung cancer (O'Donnell *et al.*, 2006 and Lee *et al.*, 2008).

Tumour-associated macrophages (TAMs) are the primary leukocyte subset that amplify the inflammatory response in the tumour microenvironment. Macrophages are split into two phenotypes – M1 and M2. M1 macrophages or pro-inflammatory macrophages are characterised by their ability to release pro-inflammatory cytokines and chemokines such as IL-6, IL-12 and TNF-α, and to present antigens to T cells. M2 macrophages or anti-inflammatory macrophages are characterised by the release of anti-inflammatory mediators such as IL-10 and

IL-1 receptor antagonist, and the ability to promote angiogenesis and extracellular matrix remodelling. A variety of studies have demonstrated that an increase of TAMs is associated with poor prognosis and angiogenesis (Mantovani *et al*., 2008, Pollard, 2006, Balkwill *et al*., 2005 and Bingle *et al*., 2002). TAMs also contribute to tumorigenesis through secretion of cytokines, matrix-degrading enzymes and growth factors (Yang *et al*., 2008, Wyckoff *et al*., 2007, Condeelis and Pollard, 2006 and Mantovani *et al*., 2006).

II.II.II.   Anti-tumour immunity and evasion of immune destruction by tumours

The immune system also plays a role in tumour detection and elimination. The incidence of certain cancers is greater in immunocompromised individuals than in immunocompetent individuals (Vajdic and van Leeuwen, 2009). Genetic mice deficient in various components of the immune system have been shown to develop tumours more frequently and rapidly than immunocompetent mice. Deficiencies in CD8$^+$ cytotoxic T lymphocytes (CTL), CD4$^+$T$_h$1 helper T cells, and natural killer (NK) cells led to substantial increases in tumour incidence. In addition, mice with combined deficiencies in both T cells and NK cells were even more susceptible to tumorigenesis. These results indicate that both the innate and adaptive immune cells are critical to tumour surveillance and elimination (Kim *et* al., 2007, Teng *et al*., 2008).

In spite of this, there is been some evidence that immunosuppression is a risk factor for developing lung cancer. HIV-positive patients have an estimated incidence of lung cancer that is 2 to 4 times higher than that of the general population. Smoking, viral load, immunosuppression and CD4$^+$ T cell count are some of the factors that have been linked to the development of lung cancer in HIV-positive patients (Engsig *et al*., 2011, Pakala and Ramalingam, 2010 and Engels *et al*., 2008). In addition, both tobacco and immunosuppression are risk factors for developing lung cancer after liver transplant (Jiménez *et al*., 2007), and lung cancer risk is reportedly higher in patients following solid organ transplantation (Engels *et al*.,

2011). These studies suggest that the immune system might control some aspect of lung tumorigenesis

CTL and NK cells play central roles in anti-tumour immune responses (Bindea *et al*., 2010, Ferrone and Dranoff, 2010, Nelson, 2008). CTLs and NK cells can kill tumour cells *in vitro* and release cytokines, such as IFN-γ, that stimulate other anti-tumour responses. Pagès and colleagues illustrated how patients with colon tumours that are heavily infiltrated by CTLs and NK cells have a better prognosis than those who lack such an infiltration (Pagès *et al*., 2010). This is also the case in ovarian tumours (Nelson*,* 2008). In addition, there has been evidence that immunosuppressed organ transplant recipients can develop donor-derived cancer (Strauss and Thomas, 2010).

Cancer cells can develop ways to subvert the anti-tumour activities of the immune system. They can prevent infiltration by CTLs and NK cells by secreting immunosuppressive factors such as transforming growth factor beta (TGF-β) (Shields *et al*., 2010, Yang *et al*, 2010). TGF-β can suppress antigen-specific CD8$^+$ T cell function, and can change macrophage populations from classically activated M1 to pro-tumour M2 forms (Biere and Moses, 2006). In addition, lymphocytes that are actively immunosuppressive, such as T$_{REG}$ cells and myeloid-derived suppressor cells (MDSCs), are recruited to suppress the actions of CTLs (Mougiakakos *et al*., 2010, Ostrand-Rosenberg and Sinha, 2009).

Lung cancer is sometimes considered non-immunogenic and incapable of provoking an immune response. Smoking is thought to contribute to this unresponsiveness, but can exert pro-inflammatory effects on the immune system (Cramer and Finn, 2011). Smoking induces an increase in production of pro-inflammatory cytokines such as TNF-α and IL-6 by pulmonary epithelial cells, as well as a decrease in production of anti-inflammatory cytokines, e.g. IL-10. Asbestos has been reported to reduce anti-tumour immunity by reducing IFN-γ production in stimulated CD4$^+$ T cells, as well as the expression of the chemokine receptor CXCR3, which is

expressed by memory T cells (Kumagi-Takei *et al.*, 2011). CXCR3 expression and IFN-γ production are stimulated by T cell activation leading to increased anti-tumour immunity (Wenzel *et al.*, 2008 and Strieter *et al.*, 2006). IFN-γ also promotes the expression of CXCR3 ligands. One of these, CXCL10, has been demonstrated to inhibit NSCLC tumorigenesis and spontaneous metastasis in severe combined immunodeficiency (SCID) mice (Arenberg *et al.*, 2001).

II.II.III. Immune cells and their roles in anti- and pro-tumorigenesis

Immune cells originate from hematopoietic stem cells in the bone marrow. These hematopoietic stem cells give rise to two major lineages: lymphoid progenitor cells and myeloid progenitor cells. Lymphocytes are generated from the former and consist of T cells, B cells and NK cells. Myeloid cells are generated from the latter and consist of dendritic cells, neutrophils, eosinophils, basophils, macrophages and monocytes.

II.II.III.i. T cells and their conflicting roles in cancer

T cells have a central role in the activation and regulation of immune responses. These cells mature in the thymus (Miller and Osoba, 1967), located in the upper thorax, which is also a major source of antigen-presenting cells (APCs), dendritic cells and epithelial cells. Immature thymocyte precursors enter the thymus lacking CD4, CD8 and a functional T cell receptor (TCR). Here they acquire the expression of CD4, CD8 and a TCR. They leave the thymus as naïve cells that are either CD4$^+$ or CD8$^+$ with a functional TCR and circulate through the lymph nodes, where they can be activated following antigen presentation by dendritic cells. The interaction between T cells and dendritic cells is integral to establishing cell-mediated adaptive immunity. The T cell family is composed of helper T cells (T$_H$), memory T cells, regulatory T cells (T$_{reg}$), CTLs, NKT cells, mucosal-activated invariant T (MAIT) cells, and γδT cells.

II.II.III.i.i.   CD4$^+$ T cell heterogeneity and their role in tumour development

CD4$^+$ T cells are a highly heterogeneous population of cells that develop along distinct functional lineages due to cytokine signals during antigen activation (Zhou *et al*., 2009). These cells were classically divided into two subsets: T_H1 and T_H2 lineages. T_H1 cells are generated by exposure to pro-inflammatory cytokines such as IL-12, IL-18 and TNFα. T_H1 cells stimulate immune surveillance by promoting CD8$^+$ T cell and NK cell activity (Romagnani *et al*., 1997), as well as activating and recruiting macrophages. In comparison, T_H2 cells are primed in the presence of IL-4. These cells fight extracellular pathogens, such as parasitic worms, and are involved in allergies (Robinson *et al*., 1992). Their main functions include the induction of B cell proliferation and antibody production, eosinophil and mast cell degranulation (Mosmann and Coffman, 1989 and Paul and Sedar, 1994). Three additional lineages of CD4$^+$ T cells have now been identified: T_H17 cells, which are crucial in fighting extracellular bacteria and fungi (Ma *et al*., 2008); T_{REG} cells that are essential to maintain self-tolerance (Sakaguchi, 2005); and follicular T helper (T_{FH}) cells that assist B cells in antibody production (Crotty, 2011).

T_H1 cells contribute both directly and indirectly to suppress tumorigenesis by killing tumour cells through the release of high levels of IFN-γ, TNF-α and cytolytic granules, and through the stimulation of CD8$^+$ CTL responses (Romagnani *et al*., 1997). In comparison, T_H2 cells aid tumour progression through inhibition of T cell-mediated cytotoxicity, promoting T cell anergy, and encouraging humoral immune responses (Parker, 1993 and Pollard, 2004). T_H2 cells can inhibit apoptosis and promote proliferation of breast carcinoma cells, while *in vivo* the presence of the T_H2 cytokines, IL-4 or IL-13, encourages breast cancer growth (Aspord *et al*., 2007 and DeNardo *et al*., 2009). Supporting these results is the correlation of a high ratio of T_H2^+ to T_H1^+ cells with tumour size, grade and lymph node metastasis of breast cancers (Chin *et al*., 1992).

T_H17 cells have been implicated in the development of inflammation-related colonic tumours in response to a common human commensal bacteria (Wu *et al*., 2009). T_H17 infiltration has been

observed in several human cancers including ovarian and prostate, and is associated with poor prognosis (Miyahara *et al.*, 2008 and Sfanos *et al.*, 2008). IL-17 promotes tumour growth through angiogenesis in mouse models of NSCLC (Numasaki, *et al.*, 2003 and Numasaki *et al.*, 2005). Interestingly, in a B16 mouse melanoma model, reduction of IL-17 increased susceptibility to metastasis, and adoptive transfer of tumour-specific T_H17 cells encouraged immune surveillance by dendritic cells and CD8^{+} CTLs (Martin-Orozco *et al.*, 2009).

T_{REG} cells are believed to play an important role in the regulation of tumour immunity. Increased prevalence of CD4^{+} T_{REG} cells correlates with improved survival in many cancers including follicular lymphoma (Carreras *et al.*, 2006), however, the opposite has been reported for non-small cell lung cancer (Peterson *et al.*, 2006), breast carcinoma (Merlo *et al.*, 2009) and renal cell carcinoma (Siddiqui *et al.*, 2007). T_{REG} cells can mediate tumour development by suppressing the anti-cancer activities of CD8^{+} CTLs and NK cells (Trzonkowski *et al.*, 2004).

A retrospective study found that NSCLC tumours which had extensive infiltration by CD4^{+} T cells correlated with a favourable clinical outcome (Wakabayashi *et al.*, 2003). This study found that higher numbers of CD4^{+} T cells in the cancer stroma corresponded with increased survival times. In comparison, higher infiltrations of CD4^{+} T cells in renal cancers correlated with decreased overall survival (Siddiqui *et al.*, 2007).

II.II.III.i.ii.   CD8^{+} T cell dysfunction in cancer

CD8^{+} T cells are effector cells that are critical for immune defence against pathogens and tumours. When naïve CD8^{+} T cells interact with their cognate antigen, they quickly become activated, expand and differentiate into CTLs that provide immunity against pathogens using two major mechanisms (Kaech and Cui, 2012). Firstly CD8^{+} T cells have cytotoxic granules that contain perforin and granzymes. When CD8^{+} T cells come into contact with target cells expressing a specific antigen these are released leading to apoptosis of the target cells. Secondly, they secrete cytokines such as IFN-γ and TNF-α which have anti-tumour and anti-

microbial affects, through activation of NK cells and macrophages. CD8^{+} T cells can also induce apoptosis in target cells through the ligation of Fas orTNF-related apoptosis-inducing ligand (TRAIL), and also perforin and granzyme. While the majority of CTLs die by apoptosis following pathogen clearance, about 5 – 10% survive to mature into memory CD8^{+} T cells.

CD8^{+} T cells have the ability to specifically target host cells that have undergone oncogenic transformation. These protective characteristics that have been elucidated in both animal models and humans, have led to a focus on cancer vaccines on the induction of effector CD8^{+} T cells that kill cancer cells (Kirkwood *et al*., 2012 and Restifo *et al*., 2012).

The presence of an abundant TIL infiltration has been shown to correlate to a positive clinical outcome in a wide variety of human cancers. Studies by Pagès *et al*. and Galon *et al*. have demonstrated that high densities of localised CD8^{+} effector memory T cells correlate with a favourable outcome in colorectal cancer (Pagès *et al*., 2005 and Galon *et al*., 2006). Several studies have demonstrated that the presence of TILs with memory phenotype is a favourable prognostic indicator in lung cancer (Al-Shibli *et* al., 2008, Dieu-Nosjean, *et al*., 2008 and Kawai *et al*., 2008). The study by Dieu-Nosjean found that CD8^{+} T cell infiltrations were distributed predominantly around the tumour, as well as in tertiary lymphoid structures which are associated with long-term survival in lung cancer patients (Dieu-Nosjean *et al*., 2008). However, the study by Wakabayashi mentioned above, found that large infiltrations of CD4^{+} T cells, and not CD8^{+} T cells were indicative of favourable prognosis in lung cancer patients (Wakabayashi *et al*., 2003). CD4^{+} T are necessary to initiate and sustain anti-tumour responses, without which CD8^{+} T cells cannot differentiate into sustainable memory cells (Klebanoff *et al*., 2006). In spite of this, the study by Al-Shibli *et al.* demonstrated that high densities of CD8^{+} T cell infiltrations in the stroma were significantly associated with improved survival in NSCLC patients, independent of the presence of CD4^{+} T cell infiltrations (Al-Shibli *et* al., 2008). These studies

indicate that the absence of CD8^{+} T cell infiltrations is associated with a poor prognosis in lung cancer patients.

The formation of pleural effusions, the accumulation of fluid in the pleural cavity around the lung, are the result of tumour invasion of the pleura in late stage lung cancer, particularly in adenocarcinoma; this occurs in approximately 15 – 20% of primary lung cancer cases (Prado-Garcia *et al*., 2012). Pleural effusions are predictive of poor prognosis in lung cancer with a median survival of 4 months (Heffner, 2008). Most studies indicate that most effusions comprise of both neoplastic and lymphocytes, in large quantities (Prado-Garcia *et al*., 2005 and Atanakovic *et al*., 2004). CD8^{+} T cell numbers are reduced in pleural effusions compared to peripheral blood, while CD4^{+} T cells are increased (Lucivero, *et* al., 1988, Okamoto, *et* al., 2005 and Prado-Garcia, *et al*., 2005). The study by Prado-Garcia *et al*. found a higher percentage of CD8^{+} memory T cells and a lower percentage of CD8^{+} effector T cells in plural effusions compared with peripheral blood. They suggest that the terminal differentiation of CD8^{+} T cells is blocked and that naïve cells undergo apoptosis once they reach effector phenotype (Prado-Garcia *et al*., 2005).

Although memory CD8^{+} T cells can infiltrate lung tumours, they are functionally impaired and show little or no response to T cell-activating stimuli. Several studies have found that these cells have reduced proliferation, decreased production of T_H1 cytokines, and reduced cytotoxic potential (Prado-Garcia *et al*., 2005, Trojan *et al*., 2004 and Chen *et al*., 2000). These studies suggest that CD8^{+} T cell response is impaired in NSCLC.

II.II.III.iii.   B cells and cancer development

B cells constitute an essential component of humoral immunity, and provide defence against pathogens through antibody production, antigen presentation and cytokine secretion. These cells constitute approximately 5 - 15% of peripheral blood leukocytes and differentiate from lymphoid progenitor cells in the bone marrow. These are a population of cells that express

clonally diverse cell surface immunoglobulin (Ig) receptors that recognise specific antigenic epitopes (LeBien and Tedder, 2008).

The majority of mature B cells that reside in the secondary lymphoid system encounter and respond to T cell-dependent foreign antigens bound to dendritic cells or macrophages, and either differentiate into plasma cells or enter germinal centre (GC) reactions. This process requires Ig-mediated internalisation of antigen and its presentation on MHC class II molecules to follicular helper T cells (T_{FH}), which reciprocally induce B cell maturation, Ig isotype switching and affinity maturation. High affinity B cell clones are produced that form the memory sections of humoral immunity (Jacob *et al.*, 1991 and Kelsoe, 1996). GC-derived memory B cells generated in the wake of a primary antibody response can acquire point mutations in their BCRs that lead to enhanced affinities for antigens. These memory B cells persist and can rapidly expand during secondary responses to the same antigen, and have the ability to terminally differentiate into antibody-secreting plasma cells (McHeyzer-Williams and McHeyzer-Williams, 2005).

Several studies have highlighted the role of B cells in tumorigenesis. De Visser *et al.* demonstrated how depletion of B cells in a murine model of inflammation and *de novo* epithelial carcinogenesis impeded tumorigenesis (De Visser *et al.*, 2005). Two studies illustrated how B cell-deficient mice were resistant to several types of cancer, including MC38 colon carcinoma (Inoue *et al.*, 2006 and Shah *et al.*, 2005). Mice deficient in B cells showed robust development of anti-tumour CTLs and increased IFN-γ production by CD8$^+$ T cells and NK cells compared with the wild-type (Inoue *et al.*, 2006). Another study showed that TNF-receptor-associated factor 3 (TRAF3) causes B cells to become hyper reactive to antigens and toll-like receptor (TLR) agonists which resulted in autoimmunity, chronic inflammation and a significant increase in the incidence of squamous cell carcinomas (Zapata *et al.*, 2009). These studies indicate that B cells are capable of fostering tumorigenesis *in vivo*.

II.II.IV.iv.   NK cells in cancer

NK cells are a subset of innate lymphoid cells that were originally discovered by their ability to kill tumour cells (Oldham and Herberman, 1973, Herberman *et al.*, 1974 and Kiessling *et al.*, 1975). They express the transcription factor E4-promoter-binding protein 4 (E4BP4), and secrete cytokines such as IFN-γ that participate in the adaptive immune response (Vivier *et al.*, 2011). NK cells rapidly respond to virus infections (Lee *et al.*, 2007), and tumours (Smyth *et al.*, 2002). NK cells can directly induce death of virus-infected cells and tumour cells without specific immunization, by the release of perforin and granzyme or ligation of death receptors such as Fas and Trail that induce apoptosis in target cells. NK cells do not express Ag-specific receptors but their activities are controlled by a variety of stimulatory, co-stimulatory and inhibitory receptors. NK cells can both positively and negatively affect host B and T cell immunity (Andrews *et* al., 2010, Kreb *et* al.; 2009, Robins *et al.*, 2007), depending on the nature of antigen challenge.

NK cells have the ability to discern stressed cells, such as cancer cells and infected cells, from healthy cells. They can kill a diverse range of cancer cells both *in vivo* and *in vitro* (Vivier *et al.*, 2012). An epidemiologic study by Imai *et al.* looked at a large cohort of Japanese men and women to see if differences in peripheral blood cytotoxic activity could predict cancer risk. They found that individuals with low NK cell activity had an increased risk of cancer (Imai *et al.*, 2000). In addition, NK cell tumour infiltration is associated with improved prognosis in NSCLC carcinomas (Platonova *et* al., 2011 and Carrega *et al.*, 2008) and colorectal carcinomas (Halama *et al.*, 2011). A study by Ménard and colleagues demonstrated that NK cell anti-tumour effects are through direct cytolytic activity as well as cytokine production (Ménard *et al.*, 2009). The ability of NK cells to distinguish tumour cells from healthy cells have made them an exciting target of cancer immunotherapy (Vivier *et al.*, 2012).

II.II.III.v.   γδ T cells and cancer

γδ T cells are a subset of T cells that were discovered in 1986 by Brenner and colleagues; and express T cell antigen receptors (TCRs) using γ (Brenner *et al.*, 1986) and δ rearranging genes. γδ cells do not to respond to peptide antigens presented by the major histocompatibility complex (MHC) class I and II, instead they recognise and respond to self- and foreign nonpeptide antigens presented by various antigen-presenting molecules (Tanaka *et al.*, 1995 and Spada *et al.*, 2000). γδ T cells also express Toll-like and natural killer (NK) receptors which allow them to respond to other non-peptide microbial components or to changes in the expression of normal cell surface ligands (Morita *et al.*, 2007). Large expansions of γδ T cells in human have been observed doing microbial infections, suggesting a unique role in host immunity. The vast majority of human γδ cells use one of three major Vδ gene segments – Vδ1, Vδ2 and Vδ3. Despite the observation that at birth there is a collection of different γδ chain pairs; by the second decade of life the majority of γδ T cells are in fact Vγ9Vδ2 T cells (Parker *et al.*, 1990). γδ T cells are considered a part of innate immunity and have a key role in immunosurveillance and anti-tumour immunity (Hayday, 2000 and Thedrez *et al.*, 2007).

γδ T cells can infiltrate many human tumours, and can kill tumour cells *ex vivo* (Kabelitz *et al.*; 2007). These cells have considerable functional heterogeneity and functions that include cytotoxicity, cytokine secretion and antigen presentation. These cells comprise between 1 and 10% of peripheral blood cells, and are abundant in tissue, particularly in the epithelial layers (Hayday, 2009).

As mentioned previously, TILs are often associated with a positive prognosis. Several studies have demonstrated that γδ T cells are present as TILs in many different types of cancer (Lo Presti *et al.*, 2014). A study by Bialasiewicz and colleagues examined specimens of necrotising choroidal melanoma and found that 52% of samples had infiltrating γδ T cells, predominantly Vδ1, and their numbers correlated with improved survival (Bialasiewicz *et al.*, 1999). Another

study examined melanoma samples and found that γδ TILs comprised of equal percentages of Vδ1 and Vδ2 T cells, and that the presence of these cells indicated early stage melanoma. However, a study of colorectal cancer found that γδ T cells were the predominant source of IL-17, the majority of which were Vδ1. The authors found that the presence of infiltrating γδ T cells positively correlated with tumour staging, size, metastasis and invasion (Wu *et al.*, 2014). These results suggest that γδ T cells can act as both suppressors and promoters of tumorigenesis.

II.II.IV.v.i.   Vδ1 T cells

Vδ1 T cells are predominantly located at the mucosal surfaces. They frequently express CD8 and exhibit T$_H$1 phenotypes characterised by cytotoxicity and IFN-γ secretion (Deusch *et al.*, 1991) or T$_H$17 phenotypes characterised by IL-17 and IL-22 secretions (Maher *et al.*, 2015 and Fenoglio *et al.*, 2009). Vδ1 T cells have been shown to have a role in tumour surveillance, two groups have shown the ability of these cells to kill epithelial tumours (Groh *et al.*, 1999 and Maeurer *et al.*, 1996). This is believed to occur through recognition of MHC class I-related molecules MICA and MICB (Groh *et al.*, 1999 and Groh *et al.*, 1998). These molecules are expressed on epithelial cells following induced stress, such as heat shock or oxidative stress, as well as being constitutively expressed on many epithelial tumour cells (Groh *et al.*, 1998). Some Vδ1 T cells have also been shown to respond to phospholipids presented by CD1 (Russano *et al.*, 2007), sulfatides presented by CD1d (Bai *et al.*, 2012), and glycolipids presented by CD1c (Spada *et al.*, 2000)

II.II.III.v.ii.   Vδ2 T cells

Vδ2 T cells make up the majority of γδ T cells in the peripheral blood, and are only found in humans and higher primates. Vδ2 T cells recognise non-peptide phosphoantigens such as (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate (HMB-PP) and isopentenyl pyrophosphate (IPP), which are both intermediates in the synthesis of isoprenoids. Vδ2 T cells are also activated by bisphosphonates and alkylamines by virtue of the abiliityy of these drugs to inhibit isoprenoid

synthesis, allowing for the accumulation of IPP (Morita *et al*., 2007). In normal conditions, Vδ2 T cells make up approximately 2 − 5% of peripheral blood cells. However under adverse conditions, such as bacterial and protozoan infections, Vδ2 T cells have been found to expand to such high levels that these cells comprise the majority of circulating T cells (Morita *et al*., 2000).

Tumour cells have also been shown to stimulate Vδ2 T cells *in vitro* resulting in inflammatory cytokine secretion and cytotoxicity (Wroebel *et al*., 2007). A study by Wroebel *et al*. showed that many tumours were susceptible targets of Vδ2 T cell-dependent lysis. Recognition of tumour cells can occur by either the Vδ2 TCR (Morita *et* al., 2007 and Wroebel *et al*., 2007) or by NKG2D ligation (Wroebel *et al*., 2007). The finding that there are highly active 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductases, a limiting factor in the mevalonate pathway, in human lymphoma and leukaemia cells would suggest that recognition is from the high levels of IPP found within these tumour cells, (Harwood *et al*., 1991).

Aminobisphosphonates have been of interest in cancer therapeutics and at present there are trials involving the use of zoledronate to treat many cancers including B cell malignancies (Wilhelm *et* al., 2003) and prostate cancer (Dieli *et al*., 2007). While these trials have shown that zoledronate can stimulate Vδ2 T cells *in vivo*, they have shown low clinical efficacy as cancer therapeutics (June *et al*., 2009).

II.II.III.v.iii.   Vδ3 T cells

Vδ3 T cells make up approximately 0.2% of circulating T lymphocytes, and express both CD4 and CD8, although the majority tend to be double negative for these markers (Mangan *et al*., 2013). Vδ3 T cells were shown to recognise and kill CD1d-transfected HeLa cells in the absence of added glycolipid (Mangan *et al*., 2013). The same study also showed that CD1d was necessary for Vδ3 cytotoxicity as blocking CD1d abrogated cytolytic degranulation. These cells were also shown to

secrete cytokines that mediate T_H1, T_H2 and T_H17 responses such as IFN-γ, IL-17 and IL-4 (Mangan *et al.*, 2013).

II.II.III.vi.   iNKT cells and their role in tumour immunity

NKT cells are a heterogeneous population of T cells that recognise glycolipid antigens presented by the MHC class I-like molecule CD1d. In addition to a CD1d-restricted TCR, NKT cells express a number of NK cell stimulatory receptors, such as NKG2D, and are CD1d-restricted. CD1d is a non-polymorphic, MHC class I-like molecule that is largely conserved between species (Brigl and Brenner, 2004). It is the essential molecule involved in thymic selection, development and immune recognition by iNKT cells.

Invariant or type 1 natural killer T (iNKT) cells are characterized by their expression of an invariant T cell receptor (TCR), Vα24-Jα18/Vβ11 in humans, which recognises glycolipid antigens presented by CD1d. iNKT cells respond to α-galactosylceramide (αGalCer), marine sponge-derived glycolipid (Kawano *et al.*, 1997) with a high affinity for CD1d (Metelitsa *et al.*, 2001). iNKT cells account for less than 0.1% of human peripheral blood T cells but they are enriched in liver and adipose tissue. When stimulated these cells mediate direct cytotoxicity and rapidly produce a variety of cytokines including IL-4, IL-10, INF-γ and TNF-α. iNKT cells are some of the first responders during a wide variety of immune responses and mediate critical immunomodulatory functions against infections, immunologic and neoplastic diseases (Brigl and Brenner, 2004).

Type 2 NKT cells are a heterogeneous group of CD1d-restricted T cells that use a diverse αβ and γδ TCRs, and recognise a variety of glycolipids, but not αGalCer. Most human hepatic NKT cells are type 2 NKT cells (Berzins *et al.*, 2011 and Kenna *et al.*, 2003). Humans also have T cells that recognise glycolipids presented by the other CD1 isotypes – CD1a, CD1b and CD1c.

CD1d bears a striking similarity in its quaternary, tertiary and secondary structures to MHC class I molecules, where the α chain is folded into three domains that are non-convalently associated

with β2 microglobulin (Zeng *et al*., 1995). These molecules are similar in all key features but only the α chain contains the lipid-binding groove (Koch *et al*., 2005). Similar to the mouse CD1d structure, the human CD1d α chain has large, hydrophobic, antigen-binding groove in the core of the α1 and α2 domain (Rossjohn *et* al., 2012, Koch *et al*., 2005 and Zeng *et al*., 1995). The groove is composed of two channels that attach directly to the surface; these channels accommodate the two alkyl chains of αGalCer (Koch *et al*., 2005). CD1d α chains are folded and assembled with β2m in the endoplasmic reticulum; from there they pass through the Golgi and to the cell surface via the secretory pathway (Gumperz, 2006). Following cellular expression, CD1d molecules are re-internalised and trafficked through parts of the endoplasmic reticular system before returning to the cell surface (Gumperz *et al.*, 2006). CD1d allows NKT TCRs to recognise and bind to a variety of self (Facciotti *et* al., 2012, Rossjohn *et al*., 2012 and Brennan *et al*., 2011), microbial (Mattner *et al*., 2005) and glycosphingolipids (Kinjo *et al*. 2005) such as LPS, phospholipids, and glycosylceramide. CD1d is expressed in low levels on myeloid cells such as dendritic cells, macrophages and monocytes (Brigl and Brenner, 2004), but when loaded with αGalCer, these cells can stimulate iNKT proliferation and cytokine secretion (Spada *et al*., 2000). In comparison, many circulating and splenic B cells express CD1d at considerable levels (Exley *et al*., 2000). CD1d is not detectable on mature T cells, but after stimulation with PHA, low levels of cell-surface CD1d expression were observed (Exley *et al*., 2000) and it would appear that CD1d accumulates intracellularly (Salamone *et al*., 2001).

iNKT cells were first shown to be necessary for the elimination of tumours in a mouse murine model (Cui *et al*., 1997). Mice with iNKT cell deficiencies were unable to reject tumours in an IL-12-mediated fashion. While it was previously believed that the anti-tumour effect of IL-12 was mediated through NK cells and T cells, this study found that iNKT cells were an essential target of IL-12. αGalCer was identified as a ligand for iNKT cells (Kawano *et al*., 1997). It was shown that activated iNKT cells kill tumour cells following presentation of αGalCer by dendritic cells (Kawano *et al*., 1998). Cytotoxic activity against B cell melanoma was observed when mice with

iNKT cells were injected with αGalCer but not with the vehicle control. This study showed that αGalCer-activated iNKT cells kill tumour cells. Adoptive transfer of αGalCer-pulsed dendritic cells in a murine model showed that targeted delivery of αGalCer triggered optimal stimulation of iNKT cells, which was maintained after the challenge had dissipated (Macho-Fernandez *et al.*, 2014). Park and colleagues illustrated that iNKT cells directly respond to IL-12 in a B6 melanoma model to secrete IFN-γ, although they are unnecessary for effective anti-tumour immunity in this disease model (Park *et al.*, 2003).

iNKT cells have been shown to have a protective role in immunosurveillance in various murine tumour models (Vivier, *et al.*, 2012). Smyth and colleagues compared wild-type mice with Jα18-deficient mice, and found that the wild-type mice were protected from spontaneous tumours by a carcinogen (Smyth *et al.*, 2000). Adoptive transfer of iNKT cells into Jα18-deficient mice protected them from tumour growth (Crowe *et al.*, 2002). Tumour regression was mediated, in part by induction of IL-2 production and activation of CD8^{+} T cells and NK cells. Activation of iNKT cells and resulting IFN-γ production was instrumental in the anti-tumour activity of αGalCer. (Reilly *et* al., 2010, Berzofsky and Terabe, 2009, Dhopdapkar, 2009 and Swann *et al.*, 2007).

While there have been extensive studies in mice to validate the protective role of iNKTs against tumours, several phase I trials of the anti-tumour activity of αGalCer in humans have shown little therapeutic benefit (Exley and Nakayama, 2011). αGalCer was first tested in humans in a phase I trial in patients with refractory solid tumours(Giaccone *et al,.* 2002). αGalCer was administered intraveneously and the trial sought to identify the maximum tolerated dose and the optimum biologically active dose. There was minimal toxicity but the group found that immunological effects of αGalCer was confined to the patients with high pre-treatment numbers of iNKT cells (Giaccone *et al.*, 2002). Disease stabilisation occurred in 7 out of 24 patients but no optimum biologically active dose was identified. A phase I study of αGalCer-

pulsed dendritic cells in patients with advanced or recurrent NSCLC saw no serious adverse effects but no patient achieved a partial or complete response (Ishikawa *et al*., 2005). A phase I clinical trial of intra-arterial infusion of *in vitro* expanded iNKT cells with injection of αGalCer-pulsed APCs in 8 patients with recurrent head and neck carcinoma resulted in a transient partial response in 3 patients and disease stabilisation in 4 patients (Kunii *et al*., 2009). It is thought that such discrepancies between mouse and human trials are the result of dramatic differences in iNKT abundancies between the two species, and as such an inherent flaw with conventional mouse models.

iNKT cells can directly recognise and kill CD1d-bearing tumour cells. Some tumour types such as prostate cancers, myelomonocytic leukaemia and some neurologic tumours, express CD1d and as such can be targeted for NKT-mediated cell killing (Metelitsa *et al*., 2003). However, most human and mouse solid tumours are CD1d negative. Over the last number of years increasing evidence has suggested that cancer cells are capable of downregulating MHC class I molecules in an attempt to evade immunosurveillance (Algarra *et al*., 2004 and Jäger *et al*., 2001). It is possible that they similarly downregulate CD1d expression to evade the actions of iNKT cells.

Direct cell cytotoxicity is mediated through perforin and granzyme secretion, as well as ligation of Fas and TRAIL ligands. Several studies illustrate that iNKT cells can detect and kill CD1d-bearing tumour cells *in vitro* (Kenna *et al*., 2007, Dhodapkar *et al*., 2004 and Metelitsa *et al*., 2003). Hepatic and peripheral blood iNKT cells have been shown to secrete IFN-γ in response to C1R cells expressing transfected CD1d (Kenna *et al*., 2007). Dhodapkar and colleagues demonstrated how myeloma cells expressing CD1d were killed by autologous iNKT cells (Dhodapkar *et al*., 2004). iNKT cell-mediated killing of myelomonocytic leukaemia cells was found to occur primarily through the secretion of perforin and granzyme (Metelitsa *et al*., 2003). As mentioned previously, many tumour types do not express CD1d, possibly in an attempt at immune evasion. Even among the CD1d-positive tumours, there are those that become CD1d

negative in the advanced stages. Early and intermediate myeloma cells express CD1d, and therefore can be targeted for iNKT cell-mediated killing. However, this expression can be lost in advanced myeloma cells (Spanoudakis *et* al., 2009, Song *et* al., 2008 and Chang, *et al*., 2006).

Many cancer patients have shown depletions of iNKTs in affected tissues, and in some cases iNKT cells are replaced with type 2 NKT cells which are frequently pro-tumorigenic (Lynch *et* al., 2009, Kenna *et al*., 2007 and Kenna *et al*., 2003). There is increasing evidence that the presence of IFN-γ-producing T_H1-type iNKT cells strongly correlate to a positive prognosis in cancers such as haematological cancers and head and neck squamous cell carcinoma (de Lalla *et al*., 2011, Mölling *et al*., 2007 and Tahir, *et al*., 2001). These results suggest that iNKT cells play a key role in tumour immunosurveillance and that their absence predisposes to tumorigenesis.

II.II.V.   Immunotherapies for NSCLC

Lung cancer is the most common cause of cancer-related death in the world. Approximately 85% of cases are NSCLC. Most lung cancer patients are diagnosed in the advanced stages of the disease which has a five year survival of 3.5%. For the 15% of patients who are eligible for surgery at diagnosis, more than 40% will have local or systematic reoccurrences and eventually succumb to the disease (Jemal *et al*., 2011). Treatments for advanced disease are mostly palliative, and the benefit of platinum-based chemotherapy regimens have plateaued (Crombet Ramos *et al*., 2015). As such there is an urgent need for novel efficacious therapies against NSCLC.

Immunotherapy has seen a resurgence of interest in the last two decades, including an increasing interest in developing immunotherapies for the treatment of lung cancer. While immunotherapy is rarely curative, it has the distinct advantage of being a targeted therapy, potentially prolonging survival for lung cancer patients with fewer side effects. Despite a strong preclinical rationale there had been a limited success to date for immunotherapies for solid tumours. In recent years, improvements in overall survival rates in phase 3 clinical trials and the

approvals of monoclonal antibodies for the treatment of melanoma have led to a resurgence of interest in immunotherapy of solid tumours (Roberts *et al.*, 2011). There has also been success for NSCLC when, in February of this year, the anti-PD1 drug Opdivo by Bristol Myers Squibb was granted accelerated approval by the FDA after interim analysis showed superiority of Opdivo over docetaxol in patients with squamous cell carcinoma.

Immunotherapy has become an important modality in the treatment of malignant tumours after surgery, radiotherapy and chemotherapy (Dougan and Dranoff, 2009). There are three categories of immunotherapies that are currently used in the treatment of NSCLC: supportive immunotherapy, active immunotherapy and passive immunotherapy (Decoster *et al.*, 2011). Bevacizumab and cetuximab are both examples of supportive immunotherapy. Bevacizumab is an anti-VEGF monoclonal antibody that is indicated for metastatic cancers including some colon carcinomas, lung and ovarian cancers. It works by blocking angiogenesis and was the first clinical angiogenesis inhibitor available in the US (Shih and Lindley, 2006). Cetuximab is an EGFR-inhibitor that is indicated for metastatic colon cancer, metastatic NSCLC, and head and neck tumours. Both of these monoclonal antibodies have been included in the National Comprehensive Cancer Network guidelines for NSCLC as first line therapies (Zheng *et al.*, 2013).

Vaccinations are examples of active immunotherapies, and aim to promote T cell or humoral responses against tumour-specific or associated antigens (Liu, 2014). Vaccination therapy for NSCLC has not been met with great success. Trials examining different vaccines such as L-BLP-25 and MAGE-A3 had disappointing results (Crombet Ramos *et al.*, 2015). L-BLP-25 is a mucin 1-based vaccine that was evaluated in stage III NSCLC patients. Mucin 1 is a glycoprotein that is over-espressed and abnormally glycosylated in certain tumour types including NSCLC (Crombet Ramos *et al.*, 2015). The double-blind, randomised Phase III trial found that there were no significant improvements in overall survival between the control and experimental groups (Crombet Ramos *et al.*, 2015). MAGE-A3 was evaluated in a large, double-blind, randomised,

placebo-controlled Phase III clinical trial in patients with NSCLC that had undergone surgical resection. No experimental group saw an improvement following treatment with MAGE-A3. This was the largest vaccination trial ever undertaken in lung cancer (Domingues *et al.*, 2014). CIMAvax-EGF is a vaccine for cancer immunotherapy that is currently under review. EGFR is over-expressed in around 50% of lung cancers (Sharma *et al.*, 2007), and CIMAvax-EGR aims to prevent EGFR activation. Thus far it has been well tolerated and significant improvement in survival was observed in advanced NSCLC patients (Crombet Ramos *et al.*, 2015).

Passive immunotherapy is concerned with the adoptive transfer of immune cells. It was initially used in leukaemia in patients who relapsed following allogeneic bone marrow transplantation, and is now finding application for solid cancers (Zheng *et al.*, 2013). Current adoptive immunotherapies include a variety of cell types such as TILs, γδT cells, lymphokine-activated killer (LAK) cells, cytokine-induced killer (CIK) cells. TILs have high affinity TCRs against tumour antigens which they recognise in the context of MHC molecules (Wu *et al.*, 2012). These cells can be expanded efficiently *ex vivo* and their tumour reactivity can be tested *in vitro* (Nguyen *et al.*, 2010), however, this type of adoptive therapy is limited as TILs can only be obtained through surgery or biopsy. γδT cells can recognise tumours in an MHC-unrestricted manner, and these cells can also directly influence adaptive immunity by acting as APCs (Yoshida *et al.*, 2011 and Hayday, 2009). γδT cells have cytotoxic activity against a diverse range of cancers including renal tumours, head and neck squamous carcinoma and colon cancer (Alexander *et al.*, 2008, Viey *et al.*, 2008, Corvaisier *et al.*, 2008 and Viey *et al.*, 2005). They are able to rapidly release IFN-γ and TNF-α. LAK cells are generated from T cells exposed to IL-2 and can lyse NK-resistant tumour cells (Sinkovics and Horvath, 2005). CIK cells are heterogeneous lymphocytes that possess MHC-unrestricted anti-tumour immunity (Mesiano *et al.*, 2012 and Lin and Hui, 2010). These cells are generated *in vitro* by incubating PBMCs with anti-CD3 mAb, INF-γ, IL-1α, and IL-2 (Hontscha *et al.*, 2011). Clinical trials have shown that CIK cells can prevent recurrence and can improve

quality of life and progression free survival in several solid tumours (Hui, 2012, Ma *et al*., 2012, Zhang *et al*., 2012 and Hontscha *et al*., 2011).

Dendritic cells are a heterogeneous group of cells that link the innate and adaptive immune systems. Mature dendritic cells enhance anti-tumour immunity in lung cancer by stimulating CD8^{+} T cells via antigen presentation and IL-12 secretion. However, the maturation of dendritic cells in NSCLC is suppressed by IL-10, VEGF and TGF-β (Shen *et al*., 2010 and Bergeron *et al*., 2006). Immature dendritic cells can play a role in tumour progression through facilitating tumour migration, invasion and epithelial-to-mesenchymal transition (Schneider *et al*., 2011). Dendritic cells are currently being investigated as *in vitro* vehicles to develop vaccines against lung cancer. Studies have demonstrated that tumour antigen-pulsed dendritic cells can increase the numbers of CD4^{+} T cells, CD8^{+} T cells and NK cells (Kato *et al*., 2011, Zhong *et al*., 2011, Bergeron *et al*., 2006 and Chen *et al*., 2001). In addition, αGalCer-pulsed dendritic cells has been demonstrated to result in sustained activation of iNKT cells *in vivo* (Macho-Fernandez *et al*., 2014). Clinical studies suggest that adoptive immunotherapy is a powerful treatment for NSCLC, resulting in improved clinical outcome with little toxicity (Zheng *et al*., 2013).

II.II.V.i. iNKT cells and tumour therapy

αGalCer can induce potent iNKT cell activation, and anti-tumour activity in mice (Cui *et al*., 1997 and Kawano *et al*., 1997), sparking global interest in its use as an immunotherapy. αGalCer is a potent ligand for iNKT cells that is presented by CD1d. It induces iNKT cell activation and proliferation, as well as production of cytokines such as IFN-γ to activate other immune cells, resulting in a T_H1 immune response. Early preclinical models demonstrated that αGalCer could induce anti-tumour activity against multiple types of tumour, including colon cancer and lymphoma (Schneiders *et al*., 2011). The models were free from side effects except for microgranuloma formation in mice, rats and monkeys. The first phase I study evaluated IV administration of αGalCer in patients with refractory solid tumours, and looked at identifying

the maximum tolerated dose as well as the optimum biologically active dose. The study found minimal toxicity in patients, but no anti-tumour immune responses were observed. Disease stabilisation was detected in 7 out of 24 patients but neither the maximum tolerated dose nor the optimum biologically active dose were identified (Giaccone *et al.*, 2002).

Macho-Fernandez and colleagues demonstrated that targeted delivery of αGalCer by dendritic cells triggered optimal stimulation of iNKT cells, which was maintained after the challenge had dissipated (Macho-Fernandez *et al.*, 2014). This and other studies showing the increased anti-tumour activity using αGalCer-pulsed DCs led to new clinical trials evaluating this method of delivery in patients. These trials indicated that administration of αGalCer leads to a sharp decline of circulating iNKTs, but this was followed by sustained expansion of iNKT cells (Schneiders *et al.*, 2011). The immunological effects of αGalCer were best in patients with relatively high numbers of iNKT cells pre-administration. While αGalCer-based immunotherapies were well-tolerated in patients, more work is necessary to ensure that these anti-tumour effects are consistently observed.

II.II.V.ii.　　　　γδ T Cell Immunotherapy

Vδ2 T cells can recognise phosphoantigens through by TCRs as well as MICA, MICB and through NKG2D. Vδ2 T cells are attractive candidates for immunotherapy because of their cytotoxic activity against a variety of cancer including renal tumours, head and neck squamous carcinoma and colon cancer (Alexander *et al.*, 2008, Viey *et al.*, 2008, Corvaisier *et al.*, 2005 and Viey *et al.*, 2005), and their ability to rapidly release IFN-γ and TNF-α. A phase I trial on adoptive transfer of Vδ2 T cells in NSCLC patients with recurrent or advanced disease found that the treatment with well-tolerated with half the patients achieving stable disease (Kakimi *et al.*, 2014). A phase I/II trial of adoptive transfer of Vδ2 T cells with zoledronic acid and IL-2 in 11 patients with advanced renal cell carcinoma was well tolerated with one patient achieving a complete response and 5

patients achieving stable disease (Kobayashi *et al.*, 2011). This and other trials suggest that there is potential for effective γδ T cell therapies for NSCLC.

II.III.   Epigenetics

Epigenetics is defined as the sum of all stable and heritable changes to gene expression that does not occur as a result of changes to DNA sequences (Probst *et al.*, 2009). Chromatin structure defines the condition in which DNA is organized and allows for the transcriptional repression or activation of genes (Sharma *et al.*, 2010). Chromatin modification is an essential focus of epigenetic therapies as it is the organisation of chromatin which provides the functionality of epigenetic changes rather than the underlying DNA sequence. The two forms of epigenetic mechanisms which are explored in this report are DNA CpG methylation and histone post-translational modifications (HPTMs). The combined mechanism of both of these modifications is known as gene bookmarking (Zaidi *et al.*, 2010). These modifications also interact with one another to establish the chromatin landscape and gene expression. Failure to retain the proper epigenetic marks can lead to the activation or inhibition of signalling pathways which has been implicated in the aetiology of many diseases including cancer, (Sharma *et al.*, 2010).

Cancer is the result of widespread epigenetic mutations and genetic alterations, which have an important role in disease initiation and progression. Such mutations are selected for in a cancer cell population because of the advantages they confer. Epigenetic mutations contribute to the criteria for Knudson's Two-Hit Hypothesis, the theory by which tumorigenesis is caused by two neoplastic events, such as silencing of tumour suppressor genes, and activating oncogenes.

II.III.I.   DNA CpG methylation

DNA methylation is the most well-established epigenetic mechanism in mammals, and works as a gene silencing mechanism that has an integral role in chromatin structure and regulation of gene expression. In mammals, it principally occurs by the covalent modification of CpG dinucleotides, which are clustered in small areas known as CpG islands and regions of repetitive sequences (Bird 2002). These islands occupy up to 60% of gene promoters and methylation at these sites can lead to transcriptional repression, a feature found in cancer. Hypermethylation of these regions is well established; it is present in almost all types of human cancers and is associated with transcriptional silencing of important genes (Jones and Baylin, 2002), such as tumour suppressor genes. Aberrant hypomethylation causes activation of genes such as proto-oncogenes in cancer. It has been shown that the enzymes responsible for DNA methylation; DNA methyltransferases (DNMTs) DNMT1, DNMT3A, DNMT3B; are up-regulated and associated with poor prognosis in lung cancer (Lin *et al*., 2007). Indeed, aberrant gene silencing in NSCLC by DNA CpG methylation has been linked to platinum-based resistance.

Abnormal DNA methylation was the first epigenetic mutation discovered in cancer (Feinberg and Vogelstein, 1983). Cancer is characterised by "genome-wide hypomethylation and site-specific CpG promoter hypermethylation", (Sharma *et al*., 2010). It has been suggested that such changes occur at the initiation of cancer and aid in its progression, (Feinberg *et al*., 2006). Genome-wide hypomethylation has an important role in tumorigenesis by creating genomic instability (Jones *et al*., 2002), and activating growth-promoting genes and oncogenes, and to cause a loss of imprinting. Genomic imprinting is a phenomenon whereby certain genes are expressed in a "parent-of-origin" manner which is different to classical Mendelian inheritance. Promoter hyper-methylation on the other hand serves to aid cancer initiation and progression by silencing important tumour suppressor genes (Figure 2.4). Many of these genes are integral to several processes including DNA repair, apoptosis and angiogenesis.
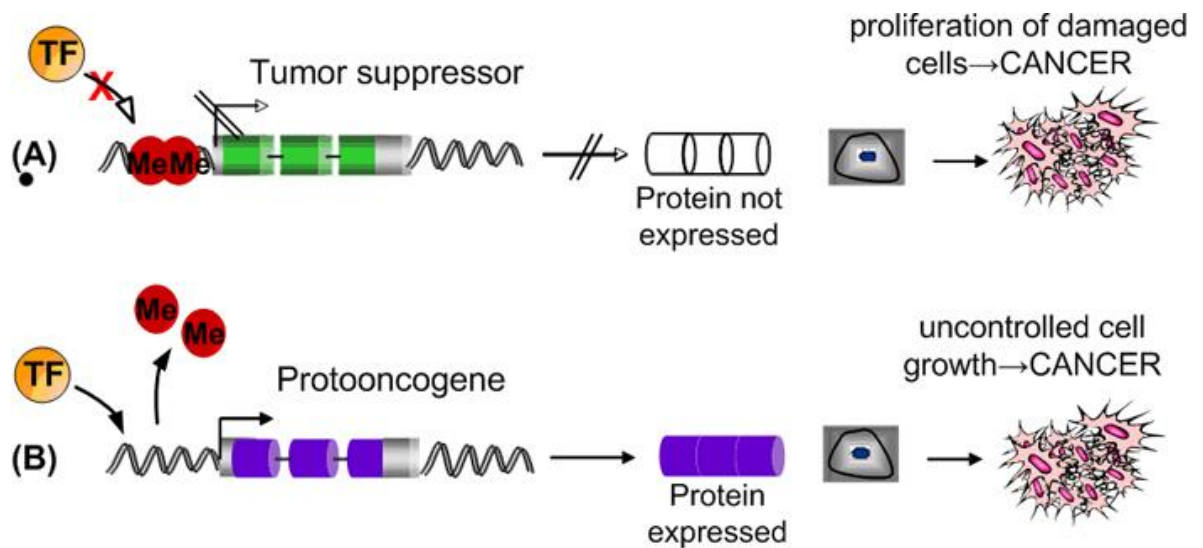
Figure 2.4: Schematic of aberrant DNA methylation marks that can lead to cancer. A) The promoter region of a tumour suppressor gene is methylated so the transcription factor cannot bind. The protein cannot be expressed and as a result there is a proliferation of damaged cells leading to cancer. **B)** The promoter region of a protooncogene is demethylated, facilitating the binding of a transcription factor. This leads to the expression of the protein, allowing uncontrolled cell growth leading to cancer. Adapted from Nelson, 2008.

Evidence exists which suggests the potential of DNA methylation as a biomarker for the detection of disease and its prognosis, for example methylation of the ASC/TMS1 gene has been shown to indicate late stage lung cancer (Machida *et al.*, 2006). Although methylation analysis is relatively easy in homogeneous samples, the heterogenetic nature of clinical samples poses a significant hurdle. Each component of the sample displays its own methylation pattern, which can vary over time. In addition the variability of samples and differential degrees of neoplastic transformation adds complexity. Cell-free circulating DNA (cfcDNA) are small pieces of DNA that are released by dying tumour cells. This DNA has been shown to carry tumour-specific mutations which have been used to characterize tumours, however the delay in its appearance does not allow for early diagnosis (Levenson, 2010). Aberrant methylation can also be detected in cfcDNA, and has shown potential for the early detection, diagnosis and prediction of therapeutic response (Belinsky, 2004).

II.III.II. Histone modifications

HPTMs are also important epigenetic mechanisms that are involved in gene regulation and cellular function. Histone proteins are composed of a globular C-terminal domain, as well as an unstructured N-terminal tail, and make up the nucleosome core (Luger *et* al., 1997). The N-terminal tails can undergo a variety of modifications including acetylation, phosphorylation, ubiquitination, methylation and sumoylation on specific residues (Kouzarides 2007). HPTMs can cause either gene silencing or induction by changing the accessibility of transcription factors to the chromatin (Sharma *et al.*, 2010). Aberrant modifications have been implicated in many different types of cancer, including NSCLC, where it has been seen to have both a predictive and prognostic value (Barlési *et al.*, 2007).

The proteins of interest in this study are histone deacetylases (HDACs) which are associated with abnormal expression in NSCLC. HDACs are enzymes which are involved in gene regulation by the removal of acetyl groups from ε-N-acetyl lysine amino acids on histones. Lysine acetylation

usually correlates to gene activation (Sharma *et al*., 2010). Conversely, a loss of histone acetylation causes the repression of gene expression and as such an overexpression of HDACs has been observed in a variety of cancers, (Halkidou *et al*., 2004). In addition, alterations in methylation patterns in histones, *e.g.* H3K9, have been linked to aberrant gene silencing in many cancer types (Nguyen *et al.,* 2002).

II.III.III.   Epigenetic alterations in NSCLC

Hypermethylation and silencing of tumour suppressor genes is a frequent occurrence in NSCLC. Tumour suppressor gene silencing is involved in nearly all of the hallmarks of cancer (Hanahan and Weinberg, 2011). Studies have identified methylation patterns that are associated with repression of tumour suppressor gene expression in the reoccurrence of NSCLC following surgical resection. Sterlacci and colleagues showed that demethylation of p16, a tumour suppressor protein, and the subsequent loss of expression occurs in just under two-thirds of all NSCLC cases. Loss of p16 is more frequent in squamous cell carcinoma (84%) than adenocarcinoma (50%) and is associated with shorter survival time following resection (Sterlacci *et al*., 2011).

Another study found that methylation of four genes, including p16, is associated with relapse following surgical resection in patients with stage I NSCLC (Brock *et al*., 2008). Genome-wide association studies have identified a more extensive observation of methylation patterns in NSCLC. One study identified 766 common differentially methylated genes between matched adenocarcinoma and normal samples. They identified two different subgroups and found that one was associated with increased hypermethylation and an increased chance of concurrent KRAS mutation. Approximately three-quarters of these genes were frequently hypermethylated and silenced in NSCLC (Selamat *et al*., 2012).

Three DNMTs are involved in the methylation of CpG dinucleotides in mammalian cells, DNMT1, DNMT3a and DNMT3b. DNMT1 is primarily involved in maintenance of methylation, while

DNMTs 3A and 3B are involved in *de novo* methylation. In cancer DNMT1 is believed to be involved in CpG *de novo* hypermethylation in cancer (Jair *et al.*, 2006), while both DNMT1 and DNMT3B appear to play an important role in methylation and silencing in cancer cells (Rhee *et al.*, 2002 and Rhee *et al.*, 2000). All three DNMTs have been shown to be overexpressed in NSCLC, and DNMT1 over-expression in particular correlates with poor prognosis (Tang *et al.*, 2012, Lin *et al.*, 2007 and Kim *et al.*, 2006). The study by Tang and colleagues showed that overexpression of DNMT3A was associated with hypermethylation of tumour suppressor genes (Tang *et al.*, 2012).

HDAC expression is a common alteration in a variety of human cancers (Vendetti and Rudin, 2013). Over-expression of HDAC1 has been documented in NSCLC and correlates with poor prognosis (Minamiya *et al.*, 2011 and Sasaki *et al.*, 2004). One study by Bartling and colleagues found that HDAC3 expression was elevated in more than 90% of squamous cell carcinoma samples (Bartling *et al.*, 2005). In addition, HDAC5 and HDAC10 are commonly downregulated in NSCLC, and reduced expression of either has been shown to be a negative prognostic indicator (Osada *et al.*, 2004). These studies demonstrate the role of epigenetic dysregulation in NSCLC and give targets for epigenetic therapy to combat this disease.

II.III.IV.   Epigenetic targeted therapies

In recent years there has been a drive to discover novel epigenetic therapies because of the reversible nature of these marks; indeed here has been some progress with the advent of epigenetic drugs that work to irreversibly restore the normal epigenome, (Yoo and Jones, 2006). DNA methylation inhibitors were among the first to be discovered, and some such as gemcitabine (GEM) have become the standard of care for some cancers. These cytotoxic drugs work by inhibiting the function of DNMTs (Egger *et al.*, 2004). Although there are some concerns over the use of DNMT inhibitor drugs because of their incorporation into DNA, studies have demonstrated that they are relatively safe in long-term treatment (Sharma *al.*, 2010). HDACs

are another therapeutic target because of the possibility of restoring normal histone acetylation patterns. There has been some success, with inhibitors showing successful inhibition of tumour progression by apoptosis and differentiation. An example of this is suberoylanilide hydroxamic acid (SAHA), which has been approved in the treatment of T cell cutaneous lymphoma.

II.III.V.   Epigenetics and immune evasion in cancer

Epigenetic modifications and their role in cancer development and progression have been studied extensively. Increasing evidence suggests that epigenetics plays a key role in downregulating the expression of molecules that are critical in immunogenicity and tumour recognition, leading to immune evasion. While immunotherapies have been shown to be a successful therapeutic option for cancer patients, only a handful successfully achieve long-term clinical benefit (Sigalotti *et al*., 2014). Elucidation of the mechanisms responsible for the ineffectiveness of immunotherapies in the majority of cancer patients is of critical importance. Epigenetic modifications leading to tumorigenesis are potentially reversible, and the efficacy of epigenetic drugs has been proven making them an attractive option in overcoming the shortcomings of immunotherapies.

CTLs recognise neoplastic cells because of antigen presentation in the form of antigenic peptides. These peptides are associated with human leukocyte antigen (HLA) class I molecules on the surface of cancer cells. Tumour cells have the capacity to alter expression of HLA genes so that the cells are not recognised by CTLs (Seliger, 2008). Several studies suggest that epigenetic dysregulation is responsible for the downregulation of HLA class I molecules in cancer (Sigalotti *et al*., 2014). Aberrant hypermethylation of HLA genes has been demonstrated in gastric carcinoma and human oesophageal squamous cell carcinoma (Sigalotti *et al*., 2014). This hypermethylation correlated with downregulation of the respective protein and/or mRNA (Qifeng *et* al., 2011, Ye *et al*., 2010 and Nie *et al*., 2001). Nie and colleagues demonstrated that HLA antigens were absent in 89% of oesophageal squamous cell carcinoma samples, and that

treatment with 5-aza-2'-decytidine was able to reactivate HLA B expression in a oesophageal squamous cell carcinoma cell line (Nie *et al.*, 2001). The study by Ye and colleagues illustrated how gastric cancer cells had between a 3- and 8-fold decrease in HLA molecule expression compared with their adjacent non-cancer tissue. Treatment with 5'aza-2'-decytidine was sufficient to re-establish HLA-A expression in a gastric cancer cell line (Ye *et al.*, 2010).

Epigenetic targeting drugs can also increase the anti-tumour capabilities of cells of the innate immune system. HDAC inhibitors can prevent the suppression of NKG2D ligand expression by tumour cells, as shown in human hepatocellular carcinoma cells treated with HDAC inhibitor sodium valproate (Armeanu *et al.*, 2005). NK cell lysis was improved through increase in NKG2D ligand recognition in many other cancer types such as cervical, glioblastoma and prostate carcinoma using a variety of HDAC inhibitors (Sigalotti *et al.*, 2014). This increase in NKG2D ligand expression was also possible using DNMT inhibitors alone or in combination with HDAC inhibitors (Yamanegi *et al.*, 2012, Chávez-Blanco *et al.*, 2011 and Tang *et al.*, 2008).

These studies show that immune evasion by tumour cells can be achieved through epigenetic dysregulation. The regulation of CD1d expression in solid tumours is still largely unknown, but a study by Yang and colleagues found that CD1d expression could be induced in mouse and human tumour cell lines using HDACi (Yang *et al.*, 2012). By targeting these epigenetic aberrations using DNMT and HDAC inhibitors, alone or in conjugation, immune cells can prevent tumour escape.

II.III.VI. Potential of epigenetics and cancer therapies

While the identification of genetic mutations has led to the development of novel therapeutics, such as tyrosine kinase inhibitors, these have only had favourable responses in some patients (Sun *et al.*, 2007). At present, around 80% of NSCLC patients have no currently identifiable genetic mutations. Advances in the field of epigenetics have demonstrated that human cancer cells conceal various genetic mutations, as well as global epigenetic abnormalities (Jones and Baylin, 2007), which interact at each stage of cancer development to promote tumour

progression (Jones and Laird, 1999). While the genetic origin of cancer is well-established, recent studies suggest that epigenetic alterations play key roles in the initiating events of some cancers (Feinberg *et al.*, 2006). Since epigenetic abnormalities have the potential to be reversed, unlike genetic mutations, they make attractive candidates for novel therapies.

II.IV.   Hypothesis

The World Health Organisation has predicted that cancer mortality will rise to 11 million a year by 2030. In light of this, novel therapeutics which specifically target cancer are required. At present the standard of care for NSCLC remains a platinum-based therapy which has shown limited capabilities. In order to combat the disease, mechanisms which aid the proliferation of cancer must be pursued as a target for therapeutics. Immunotherapies are an attractive target for use as an adjunct therapy against NSCLC, given that these are targeted therapies that can improve overall survival while causing fewer toxic effects. This study focuses on the MHC class I-like molecule, CD1d, which is downregulated in most human solid tumours, thereby contributing to immune evasion. It seeks to target CD1d expression using DNMT and HDAC inhibitors, and in doing so aid in CD1d-restricted immune cell cytotoxicity by iNKT cells.

II.IV.I.   Objectives

The main objectives of this this study are:

1. To enumerate immune cells from blood and BAL samples from patients with NSCLC and as controls, non-cancer patients. Cells will be phenotypically characterized using multicolour flow cytometry.

2. To treat a panel of NSCLC cell lines with various epigenetic targeting agents and to characterise the changes in CD1d expression using qPCR and flow cytometry. To confirm

that the changes in expression observed are a direct consequence of epigenetic targeting by chromatin immunoprecipitation analysis at the promoter level.

3. To generate lines of iNKT cells from blood and to functionally characterize them with regard to antigen specificity, CD1d recognition, cytolytic activity against lung cancer cell lines.

4. To determine if epigenetic up-regulation of CD1d expression by NSCLC cells can sensitise them for cytolysis by iNKT cells.

III. Materials and Methods

III.I. Materials

Table 3.1.1: Equipment and software used in study

Equipment/Software	Model	Company	Location
Balance	Scout® Pro	Ohaus Corporation	New Jersey, USA
Centrifuge	Eppendorf 5415D	Eppendorf	Hamburg, Germany
Centrifuge	Eppendorf 5810	Eppendorf	Hamburg, Germany
Centrifuge	Mikro 200	Hettrich Zentrifguen	
Class II laminar air flow cabinet	MSC 1.2 Advantage	Thermo-Scientific Nunc	Roskilde, Denmark
Class II Microbiological Safety Cabinet	BioMAT^2	Medical Air Technology	Manchester, UK
CO_2 Incubator	Galaxy S	RS Biotech	Ayrshire, UK
CO_2 Incubator	HERAcell 150i	Thermo-Scientific Nunc	Roskilde, Denmark
Fluorescence microscope	Eclipse E200	Nikon	NY, USA
Flow Cytometer	FACSCanto™ II	Beckton Dickenson	New Jersey, USA
Flow Cytometry software	FACSDiva	Beckton Dickenson	New Jersey, USA
FlowJo	v10.0.8	Treestar Incorporated	New Jersey, USA
Gel Electrophoresis Apparatus (Horizontal)	Gibco BRL Horizon® 58	Life Technologies™	Carlsbad, CA, USA

GraphPad Prism	v5.0	GraphPad Software Incorporated	La Jolla, CA, USA
Inverted microscope	CK30	Olympus	Tokyo, Japan
MACS magnet	LS magnet	Miltenyi Biotech	Surrey, UK
MACSmix™ tube rotator		Miltenyi Biotech	Surrey, UK
MACS stand	MACS Multi Stand	Miltenyi Biotech	Surrey, UK
MoFlo™ cell sorter	XDP	Beckman Coulter	High Wycombe, UK
Nanodrop	ND-1000 Spectrophotometer	Labtech International	East Sussex, UK
Haemocytometer	Neubauer	Marienfield Superior	Germany
Haemocytometer (disposable)	Kova slides	Una Health Ltd	UK
Plate reader	Sunrise	TECAN Group Ltd.	Mannedorf, Switzerland
Power Supply	EC600-90	E-C Apparatus Co.	Swarthmore, PA, USA
Rotary Shaker		Grant Bio	UK
Stirer	HB501	Bibby Sterilin	Staffordshire, UK
SuperCycle Thermal Cycler	SC360	Kyratec	Queensland, Australia
Tube-Strip Picofuge™	PMC-860	Stratagene California	La Jolla, CA, USA
Vacuum Adaptor		Promega	Madison, WI, USA
Vacuum Manifold	Vac Man®	Promega	Madison, WI, USA

| Vortex | Vortex Genie 2 | Scientific Industries | NY, USA |
| Water bath | YCW-010E | GEMMY | Taiwan |

Table 3.1.2: Plastic ware used in study

Material	Company	Location
6 well plates	Corning Life Sciences	MA, USA
10 cm plates (Single use)	Thermo Scientific	MA, USA
10 cm plates	Corning Life Sciences	MA, USA
24 well plates	Corning Life Sciences	MA, USA
96 well plates, round bottomed	Corning Life Sciences	MA, USA
96 well plates, flat bottomed	Thermo Scientific	MA, USA
BD Falcon™ tube (5 ml)	BD Biosciences	Oxford, UK
Cell scrapper(disposable)	Fisher Scientific	Pittsburgh, PA, USA
Eppendorf tubes (1.5 ml)	Eppendorf	Hamburg, Germany
FACS tubes	BD Biosciences	Oxford, UK
Falcon tubes (15 ml)	Sarstedt	Nümbrecht, Germany
Falcon tubes (50 ml)	Sarstedt	Nümbrecht, Germany
Filter (0.2 µM) Minisart Plus	Fisher Scientific	Pittsburgh, PA, USA
Fisherbrand® Nitrile Gloves (S)	Fisher Scientific	Pittsburgh, PA, USA
LS separation columns	Miltenyi Biotech	Bergisch-Gladbach, Germany
Maxisorp ELISA plates	Thermo-Scientific Nunc	Roskilde, Denmark
Micro tube (0.5 ml)	Sarstedt	Nümbrecht, Germany
Micro tube (2 ml)	Sarstedt	Nümbrecht, Germany
Parafilm "M" Laboratory Film	Bemis®	USA
Pastette (3.5 ml)	Sarstedt	Nümbrecht, Germany

PCR plate (96 well)	Fisher Scientific	Pittsburgh, PA, USA
PCR tubes (0.2 ml)	Thermo Fisher Scientific	USA
Pipettes (25 ml)	Sarstedt	Nümbrecht, Germany
Pipettes (10 ml)	Sarstedt	Nümbrecht, Germany
Pipettes (10 ml)	Sterilin®	Newport, UK
Pipette tips (1 ml)	Biopointe Scientific	San Diego, CA, USA
Pipette tips (1 ml)	Fisher Scientific	Pittsburgh, PA, USA
Pipette tips (200 µl)	Biopointe Scientific	San Diego, CA, USA
Pipette tips (200 µl)	Fisher Scientific	Pittsburgh, PA, USA
Pipette tips (20 µl)	Biopointe Scientific	San Diego, CA, USA
Pipette tips (10 µl)	Biopointe Scientific	San Diego, CA, USA
Pipette tips (10 µl)	Sarstedt	Nümbrecht, Germany
Pre-separation filters	Miltenyi Biotech	Bergisch-Gladbach, Germany
Optically Clear Adhesive Seal Sheets	Thermo-Scientific Nunc	Roskilde, Denmark
Semperguard® Latex Gloves (Medium 7 – 8)	Semperit Group	Vienna, Austria
Sterlin tubes (30 ml)	Fisher Scientific	Pittsburgh, PA, USA
Syringe (50 ml)	Terumo Europe N.V.	Leuven, Belgium
T25 tissue culture flasks	Thermo-Scientific Nunc	Roskilde, Denmark
T75 tissue culture flasks	Thermo-Scientific Nunc	Roskilde, Denmark

Table 3.1.3: Reagents used for Cell Culture

Reagent	Concentration	Company	Location
0.05% Trypsin-EDTA	1X	Gibco Invitrogen	Paisley, UK

2-Mercaptoethanol	50 mM	Gibco Invitrogen	Paisley, UK
Cellbanker2®		AMS Biotech	Oxfordshire, UK
Dulbecco's Minimum Essential Medium (DMEM)		Gibco Invitrogen	Paisley, UK
Dulbecco's Phosphate Buffered Saline (DPBS)	1X	Gibco Invitrogen	Paisley, UK
Eagle's Minimum Essential Medium (EMEM)		Lonza	Basel, Switzerland
F (Ham's)-12 media		Lonza	Basel, Switzerland
Fungizone® (Amphotericin B)	250 μg/ml	Gibco Invitrogen	Paisley, UK
HEPES Buffer Solution	1M	Gibco Invitrogen	Paisley, UK
Hyclone Fetal Bovine Serum		Thermo-Scientific	Logan, UT, USA
L-glutamine	2 mM	Gibco Invitrogen	Paisley, UK
Lymphoprep		Gibco Invitrogen	Paisley, UK
Minimum Essential Medium (MEM) Amino Acids	50X	Gibco Invitrogen	Paisley, UK
Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA)	100X	Gibco Invitrogen	Paisley, UK
Penicillin/-streptomycin (PS)	500 U/ml	Gibco Invitrogen	Paisley, UK
RPMI Medium 1640 (1X) + GlutaMAX™-I		Gibco Invitrogen	Paisley, UK
Sodium Pyruvate	100 mM (100X)	Gibco Invitrogen	Paisley, UK

Table 3.1.4: Reagents used in cell enumeration and viability assessments

Material	Concentration	Company	Location

Acridine Orange	15 mg	Sigma-Aldrich	Poole, UK
Ethidium Bromide	50 mg	Sigma-Aldrich	Poole, UK
95% EtOH	1 ml	Sigma-Aldrich	Poole, UK

Table 3.1.5: Kits and reagents for magnetic bead expansion of iNKT cells

Material	Concentration	Company	Location
Anti-iNKT MicroBeads		Miltenyi Biotech	Bergisch-Gladbach, Germany
DPBS	1X	Gibco Invitrogen	Paisley, UK
Bovine Serum Albumin (BSA)	30%	Sigma-Aldrich	Poole, UK
Ethylenediaminetetraacetic acid (EDTA)	500 mM	Sigma-Aldrich	Poole, UK

Table 3.1.6: List of antibodies used for flow cytometry

Antibody	Clone	Company	Location
TCR Vδ3 (unconjugated)		Beckman Coulter	Pasadena, CA, USA
APC anti-human TCR Vα24-Jα18	6B11	Biolegend	San Diego, CA, USA
FITC anti-human CD107a (LAMP-1)	H4A3	Biolegend	San Diego, CA, USA
FITC anti-human CD56 (NCAM)	HCD56	Biolegend	San Diego, CA, USA
FITC anti-human TCR Vδ2	B6	Biolegend	San Diego, CA, USA
FITC anti-human TCR Vδ1	TS8.2	Thermo Scientific	MA, USA
Fixable Viability Dye eFluor® 506		eBioscience	San Diego, CA, USA
Pacific Blue™ anti-human CD3	HIT3a	Biolegend	San Diego, CA, USA

PE anti-human CD1d	51.1	Biolegend	San Diego, CA, USA
PE mouse anti-human CD1d		Beckton Dickenson	New Jersey, USA
PE anti-human CD8a	HIT8a	Biolegend	San Diego, CA, USA
PE anti-human CD56(NCAM)	MEM-188	Biolegend	San Diego, CA, USA
PE anti-human TCR Vα24-Jα18	6B11	Biolegend	San Diego, CA, USA
PE anti-human TCR Vδ2	B6	Biolegend	San Diego, CA, USA
PE/Cy7 anti-human CD4	SK3	Biolegend	San Diego, CA, USA
PE/Cy7 anti-human CD19	HIB19	Biolegend	San Diego, CA, USA
PerCP anti-human CD8	SK1	Biolegend	San Diego, CA, USA
PerCP anti-human CD8a	HIT8a	Biolegend	San Diego, CA, USA
PerCPCy5.5 anti-human CD8a	HIT8a	Biolegend	San Diego, CA, USA

Table 3.1.7: List of reagents used for flow cytometry

Material	Concentration	Company	Location
BD Compensation Beads		BD Biosciences	Oxford, UK
BSA	30%	Sigma-Aldrich	Poole, UK
DPBS	1X	Gibco Invitrogen	Paisley, UK
Sodium Azide (NaN$_2$)	10%	Sigma-Aldrich	Poole, UK
Paraformaldehyde (PFA)	1%	Santa Cruz	Santa Cruz, CA, USA

Table 3.1.8: List of antibodies used for FACS

Antibody	Clone	Company	Location
PE anti-human CD1d	51.1	Biolegend	San Diego, CA, USA

PE anti-human TCR Vα24-Jα18	6B11	Biolegend	San Diego, CA, USA
PE TCR Vβ11	C21	Beckman Coulter	High Wycombe, UK
Pacific Blue™ anti-human CD3	HIT3a	Biolegend	San Diego, CA, USA
FITC TCR Vα24	C15	Beckman Coulter	High Wycombe, UK

Table 3.1.9: List of reagents used for FACS

Material	Concentration	Company	Location
2-Mercaptoethanol	50 mM	Gibco Invitrogen	Paisley, UK
BSA	2%	Sigma-Aldrich	Poole, UK
DPBS	1X	Gibco Invitrogen	Paisley, UK
Fungizone® (Amphotericin B)	250 µg/ml	Gibco Invitrogen	Paisley, UK
HEPES Buffer Solution	1M	Gibco Invitrogen	Paisley, UK
L-glutamine	2 mM	Gibco Invitrogen	Paisley, UK
Minimum Essential Medium (MEM) Amino Acids	50X	Gibco Invitrogen	Paisley, UK
Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA)	100X	Gibco Invitrogen	Paisley, UK
Penicillin/-streptomycin (PS)	500 U/ml	Gibco Invitrogen	Paisley, UK
RPMI Medium 1640 (1X) + GlutaMAX™-I		Gibco Invitrogen	Paisley, UK
Sodium Pyruvate	100 mM (100X)	Gibco Invitrogen	Paisley, UK

Table 3.1.10: List of reagents used for RNA analysis

Material	Concentration	Company	Location
1-bromo-3-chloropropane (BCP)		Sigma-Aldrich	Poole, UK
AccuGENE™ Molecular Biology Water		Lonza	Basel, Switzerland
Acetic acid		Sigma-Aldrich	Poole, UK
Agarose (Molecular Grade)		Bioline	UK
dATP	10 mM	Thermo Scientific	Logan, UT, USA
dCTP	10 mM	Thermo Scientific	Logan, UT, USA
dGTP	10 mM	Thermo Scientific	Logan, UT, USA
dTTP	10 mM	Thermo Scientific	Logan, UT, USA
Ethanol (EtOH)	96%	Sigma-Aldrich	Poole, UK
Ethidium bromide (Electrophoresis Grade)		Fisher Scientific	Pittsburgh, PA, USA
EDTA		Sigma-Aldrich	Poole, UK
GeneRuler (100 bp) Plus DNA ladder	50 µg	Thermo Scientific	Logan, UT, USA
GoTaq® Green Master Mix	2X	Promega	Madison, WI, USA
Loading Dye	6X	Thermo Scientific	Logan, UT, USA
Oligo dT$_{12-18}$ Primer		Eurofins	Ebersberg, Germany
Propan-2-ol		Sigma-Aldrich	Poole, UK
Random Hexamers			
Reaction Buffer	5X	Thermo Scientific	Logan, UT, USA
RevertAid Reverse Transcriptase		Thermo Scientific	Logan, UT, USA
Ribonuclease RNase Inhibitor		Thermo Scientific	Logan, UT, USA

Trizma® base		Sigma-Aldrich	Poole, UK
TRI Reagent®		Sigma-Aldrich	Poole, UK

Table 3.1.11: Kits and reagents used for ChIP assay

Material	Concentration	Company	Location
Dimethyl sulfoxide (DMSO)		Lennox	Ireland
DPBS	1 X	Gibco Invitrogen	Paisley, UK
Formaldehyde	1%	Sigma-Aldrich	Poole, UK
SDS Buffer		Merck Millipore	MA, USA
OneDay ChIP Kit		Diagenode	Liege, Belgium

Table 3.1.12: List of kits and reagents used for transfecting NSCLC cells

Material	Concentration	Company	Location
Buffer 2 + BSA		New England Biolabs	MA, USA
Buffer A		Roche Life Science	Germany
Buffer B		Roche Life Science	Germany
Buffer H		Roche Life Science	Germany
EcoRI		Invitrogen (Thermo Fisher Scientic)	MA, USA
EcoRI/ApaI		Invitrogen (Thermo Fisher Scientic)	MA, USA
FuGENE® Transfection Reagent		Promega	Madison, WI, USA
Eagle's Minimum Essential Medium (EMEM)		Lonza	Basel, Switzerland

F (Ham's)-12 media		Lonza	Basel, Switzerland
L-glutamine	2 mM	Gibco Invitrogen	Paisley, UK
Luria Broth (LB) with Ampicillin	50 µg/ml		
Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA)	100X	Gibco Invitrogen	Paisley, UK
One Shot® DH5α^{TM}-T1^R		Life Technologies^{TM}	Carlsbad, CA, USA
Penicillin/-streptomycin (PS)	500 U/ml	Gibco Invitrogen	Paisley, UK
Pure Yield^{TM} Plasmid Midiprep System		Promega	Madison, WI, USA
PvuI		Invitrogen (Thermo Fisher Scientic)	MA, USA
T4 DNA ligation kit		New England Biolabs	MA, USA
TOPO® TA Cloning® Kit		Life Technologies^{TM}	Carlsbad, CA, USA
Wizard® SV Gel and PCR Clean-Up System		Promega	Madison, WI, USA

Table 3.1.13: List of kits used for ELISA

Material	Company	Location
Interleukin-4 (IL-4)	eBioscience	San Diego, CA, USA
Interlekin-10 (IL-10)	R&D Systems	Abingdon, UK
Interleukin-13 (IL-13)	eBioscience	San Diego, CA, USA
Interferon gamma (IFN-γ)	eBioscience	San Diego, CA, USA
Tumour Necrosis Factor alpha (TNF-α)	eBioscience	San Diego, CA, USA

Table 3.1.14: List of reagents used for ELISA

Material	Concentration	Company	Location
BSA	1%	Fisher Scientific	Pittsburgh, PA, USA
NaCl		Fisher Scientific	Pittsburgh, PA, USA
Sodium dihydrogenphosphate ($Na_2H_2PO_4$)		BDM Laboratory Supplies	Poole, UK
Disodium anhydrous hydrogen orthophosphate (NNa_2HPO_4)		Fisher Scientific	Pittsburgh, PA, USA
3, 3', 5, 5'-Tetramethylbenzidine (TMB)		Sigma-Aldrich	Poole, UK

Table 3.1.15: General reagents used

Reagent	Concentration	Company	Location
Ethanol (EtOH)	96%	Sigma-Aldrich	Poole, UK
Industrial Methylated Spirits (IMS)		Hazardous Materials Trinity College Dublin	Dublin, Ireland
Methanol (MeOH)		Sigma-Aldrich	Poole, UK
Virkon		Fisher Scientific	Pittsburgh, PA, USA

Table 3.1.16: List of cell stimulators for co-culture assays

Material	Concentration	Company	Location
Phorbol 12-Myristate 13-Acetate (PMA)	50 ng/ml	Sigma-Aldrich	Poole, UK
Ionomycin	1 μg/ml	Sigma-Aldrich	Poole, UK

Table 3.1.17: List of cytokines used

Material	Concentration	Company	Location
Interleukin-2 (IL-2)	100 ml	Peprotech	New Jersey, USA

Table 3.1.18: List of antibiotics used

Material	Concentration	Company	Location
Monensin	2mM in ethanol	Biolegend	San Diego, CA, USA
G418 Sulfate	100 mg/ml	InvivoGen	San Diego, CA, USA

Table 3.1.19: List of glycolipids for co-culture assays

Material	Concentration	Source	Location
α-galactosylceramide (αGalCer)	100 ng/ml	Funokoshi & Co. Ltd.	Tokyo, Japan
7Dw8.5	100 ng/ml		

Table 3.1.20: List of epigenetic drugs used

Material	Concentration (used)	Treatment Time	Company	Location
5-Aza-2'Deoxycytidine (DAC)	50 nM 100 nM 200 nM 500 nM 1 µM 5 µM	24 h 72 h	Merck	Darmstadt, Germany
Gemcitabine hydrochloride (2'-Deoxy-2', 2'diflurocytidine hydrochloride)	200 nM 1 µM	24 h	Eli Lilly	Cork, Ireland
Trichostatin A (TSA)	250 ng/ml	24 h	Calbiochem	San Diego, CA, USA
Suberanilohydroxamic acid (SAHA) or Vorinostat	5 µM	24 h	Caymen Chemicals	Cambridge, UK

Table 3.1.21: Cell lines and associated media

Cell Line	Media	Source	Location
Adenocarcinoma (A549)	F-12 supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and PS (500 U/ml)	AATC (LGC Promochem)	Teddington, UK
HeLa-mock	DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 0.1 M	Prof. Steven Porcelli Albert Einstein College of Medicine	NY, USA

	MEM NEAA, and PS (500 U/ml)		
HeLa-CD1d	DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 0.1 M MEM NEAA, and PS (500 U/ml)	Prof. Steven Porcelli Albert Einstein College of Medicine	NY, USA
Invariant Natural Killer T (iNKT) cells	RPMI supplemented with 10%(v/v) FBS, 1 M HEPES, PS (500 U/ml), 2 mM L-glutamine, 0.1M MEM NEAA, 50 mM 2-Mercaptoethanol, 100 mM Sodium Pyruvate, 50X MEM amino acids and Fungizone® (Amphotericin B) (250 µg/ml)	Enriched from PBMCs from the Irish Blood Transfusion Service (IBTS)	Dublin, Ireland
Irradiated Peripheral Blood Mononucleocytes (PBMCs)	RPMI supplemented with 10% (v/v) FBS, 1 M HEPES and PS (500 U/ml)	Irish Blood Transfusion Service (IBTS)	Dublin, Ireland
PBMCs	RPMI supplemented with 10% (v/v) FBS, 1 M HEPES and PS (500 U/ml)	Irish Blood Transfusion Service (IBTS)	Dublin, Ireland

Squamous cell carcinoma (SK-MES-1)	EMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 0.1 M MEM NEAA, and PS (500 U/ml)	AATC (LGC Promochem)	Teddington, UK

III.II. Methods

All equipment and plastics that were utilised during this project are listed in tables 3.1.1 and 3.1.2 above.

III.II.I.　　Cell culture

III.II.I.i.　　　　Maintenance of cancer cell lines

A549, mock-transfected and CD1d-transfected HeLa-CD1a cells (HeLa-mock and HeLa CD1d) and SK-MES-1 cells were maintained at a constant temperature of 37°C in a humidified atmosphere of 5% CO_2. A549 cells are an adenocarcinomic human alveolar basal epithelial cell line and were cultured in F-12 (Ham) medium supplemented with 10% (v/v) FBS (fetal bovine serum) in addition to 2 mM L-glutamine and penicillin streptomycin (500 U/ml). HeLa cells are an adherent cervical cancer cell line that was maintained in DMEM (Dulbecco's minimum essential medium) supplemented with 10% (v/v) FBS and 2 mM L-glutamine and penicillin streptomycin (500 U/ml). SKMES-1 cells are a human lung squamous cell carcinoma cell line and was maintained in EMEM (Eagle's minimum essential medium) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 0.1M non-essential amino acids and penicillin/-streptomycin (500 U/ml). All media were pre-warmed in a water bath (37°C) for thirty minutes prior to culturing cells.

All cell culture was performed in a laminar air flow cabinet which was sanitised before use with industrial methylated spirits (IMS), which was previously diluted to 70% using distilled water,

and using sterile or autoclaved materials and reagents. Cells were split when they reached 80% confluence. The cell supernatant was removed from each tissue culture flask and disposed of in Virkon solution. Cells were was washed with sterile DPBS (Dulbecco's Phosphate Buffered Saline) (5 ml) which was then discarded. Trypsin (2 ml) was added to each flask and the samples were incubated for 5 min at 37°C to allow for the cells to detach from the flasks. The appropriate cell line-specific medium (4 ml) was added to each flask, and the cell suspension was removed to sterile 50 ml falcon tubes. Cells were pelleted by centrifugation at 1,300 X g for 3 min. The supernatant was discarded and the pellet was diluted in the appropriate medium (5 ml). This cell suspension was then used to seed fresh sterile T75 flasks at a density of 500,000 cells per ml which were placed in the CO_2 incubator.

III.II.I.ii. Cell enumeration and viability

A portion of cells (10 µl) was removed for cell enumeration and assessment of viability. A haemocytometer was used for cell counting. Ethidium bromide (50 mg dissolved in 95% EtOH (1 ml) was added to acridine orange (15 mg) with H_2O (49 ml) and stored at -20°C. Before use aliquots (1 ml) were added to PBS (100 ml) to make up EBAO. EBAO was added to the cells at a 1:2 to 1:20 ratio depending on an estimation of cell numbers and cell density. Acridine orange stains viable cells green and dead cells are stained orange with ethidium bromide, on the basis of membrane integrity, and so can be excluded from the count. The haemocytometer chamber was filled with the cell suspension by capillary action. The number of viable cells in the four larger corner squares (1 mm x 1 mm) comprising nine smaller squares were counted, and the results were averaged. The number of cells per ml was calculated using the equation below:

(Average Number of Viable Cells) x 10 x 1000 x Diliution factor = No. of cells/ml

Where 1000 = µl volume in the chamber

III.II.I.ii. Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were prepared from buffy packs obtained from the Irish Blood Transfusion Service (IBTS) at St. James's Hospital, Dublin 8, Ireland. PBMCs were diluted with DPBS at a 1:1 ratio. Lymphoprep (10 ml) was added to a sterile 50 ml Falcon tube, and the PBMC:DPBS mixture (40 ml) was carefully layered over before being centrifuged at 400 X g for 25 min without acceleration or brakes. The buffy coat layer was carefully extracted using a sterile 3 ml pastette to a clean Falcon tube and DPBS was added to 50 ml, before centrifugation at 800 X g for 5 min. The supernatant was removed and the cells vortexed; DPS was added to 50 ml before a final centrifugation at 400 X g for 10 min. Supernatant was removed and cells were vortexed and re-suspended in complete RPMI (RPMI media supplemented with 10% (v/v) FBS, 1 M HEPES and PS (500 U/ml)).

III.II.I.iii. Long-term cell storage and recovery of cells

Adherent cells were washed with DPBS and incubated with Trypsin at 37°C for 5 min as outlined above before centrifugation at 1,300 X g for 3 min. Non-adherent cells were transferred to a 50 ml Falcon tube and centrifuged at 800 X g for 5 min. Supernatants were removed and cells were vortexed. Cells were enumerated using the method outlined above. Cellbanker2 (1 ml) was added and the cell suspension was removed to a sterile 2 ml Eppendorf tube for long-term storage at -80°C.

Cells were removed from incubation at -80°C and allowed to come to room temperature. Cells were transferred to a sterile 50 ml falcon tube and cell-appropriate media (5 ml) was added. Cells were centrifuged at 800 X g for 5 min and supernatants were discarded. Cells were briefly vortexed. Adherent cells were re-suspended in cell-appropriate media (Table 3.1.21) and added to a T-75 flask. Non-adherent cells were re-suspended in cell-appropriate media (20 ml) (Table 3.1.21) and plated out in a 96-well sterile plate. All cells were incubated at 37°C.

III.II.II.   Flow cytometry

III.II.II.i.   Principles of flow cytometry

Flow cytometry is a research and clinical diagnostic tool that determines the physical and/or chemical attributes of single cells. Heterogenic suspension of cells are passed through an interrogation point generally consisting of a laser. The laser hits the cells and a disruption of laser light occurs which is scattered in all direction. This allows single cells that pass through the interrogation to achieve a uniform maximum light scatter.

Photodetectors detect both transmitted and scattered light. Light scatter is measured as both forward light angle scatter (Forward Scatter or FSC) and as right angle light scatter (Side Scatter or SSC). FSC gives information on the relative size of the cell, while SSC analyzes the internal complexity of the cell. In this way smaller non-granular cells such as lymphocytes can be easily distinguished from other larger and more granular cells such as monocytes.

Photodetectors can also detect fluorescent light so that is emitted when cells bound with fluorochrome-labelled monoclonal antibodies (mAbs) are excited by the laser. A wide array of fluorochromes exist that emit at different wavelengths allowing for cell distinction by fluorescent detectors within the flow cytometer. By conjugating flurochromes to mAbs specific to molecules of interest, cell characteristics can be examined in cell populations by the presence or absence of these flurochromes. The fluorescence emitted by these fluorochrome-labelled mAbs is interpreted by flow cytometry software and is given by mean fluorescence intensity (MFI), which correlates to extent of surface marker expression by the cells of interest ().

III.II.II.ii.   Fluorescence activated cell sorting (FACS)

FACS analysis uses the principles of flow cytometry to sort individual cells by disrupting the stream of cells following their passage through the interrogation point. Cells are given either positive or negative charge based on their fluorescent emission. Cells of interest are drawn away

from the stream by opposite electrical charge and collected while the other cells are allowed to flow through as waste.

III.II.II.iii.   Cell-surface staining for flow cytometry

Cell surface staining of both immune and cancer cells was assessed by flow cytometry using 1.0 x 10^5 – 1.0 x 10^6 cells per tube. Adherent cells were rinsed with DPBS and trypsinised before centrifugation at 800 X g for 5 min, and the supernatant was removed. Non-adherent cells were washed with DPBS at 800 X g for 5 min and the supernatant was removed. To assess cell viability cells were stained with (50 µl) Fixable Viability Dye eFluor® 506. This stain was previously diluted at a 1:1000 ratio with DPBS. Cells were incubated in the dark at room temperature for 15 min. Cells were stained for characteristics of interest using the antibodies listed in Table 4.6 and PBA buffer, (DPBS with 10% sodium azide (NaN_2) and 30% BSA), and incubated in the dark at room temperature for 15 min. Cells were washed with PBA (1 ml) at 800 X g for 5 min. The supernatant was discarded and cells were re-suspended in PBS (0.5 ml). Cells were analysed immediately using a flow cytometer. Cells that were not immediately analysed were fixed with 1% PFA (paraformaldehyde) and incubated in the dark at 4°C. Each experiment required some essential controls. Unstained cells were used to adjust some parameters in the flow cytometer such as voltages, while BD Compensation Beads were individually stained with each flurochrome and used as single stains to carry out compensation controls. FMOs ("Fluorescence minus one" controls where samples contained all but one fluorchrome) were used for establish gates when required. Doublets and dead cells were excluded from the analysis (Figure 3.1).

III.II.II.v.   Flow cytometry analysis

Cells were examined on a Beckton Dickenson FACSCanto™ II using BD FACSDiva software. Gain and voltage parameters were set on unstained control cells for FSC and SSC respectively to distinguish cells by size and complexity. Using the compensation control channel, unstained control and single stain control beads were used to compensate accordingly. Positive
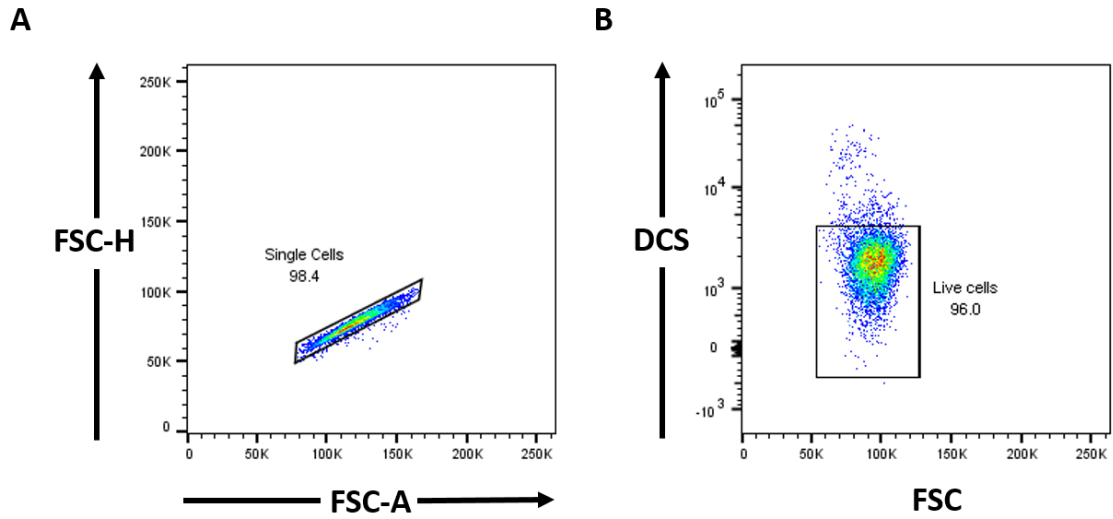
Figure 3.1: Doublets and dead cells were excluded from flow cytometric analysis. A) Flow cytometric dot plot showing iNKT cells which were gated on to included only single cells. **B)** iNKT cells were stained with a dead cell stain (DCS), eFluor-506. Cells expressing eFluor-506 were excluded from the analysis.

populations were gated on so as to compensate for any spectral overlap of flurochromes. Spectral overlap occurs when photons from an excited fluorochrome spill into a second detector causing single stained cells to appear double positive, because of similar light emission wavelengths. A mathematical correction is applied to address this spillover. On BD FACSCanto™ II machines, compensation can be calculated by the software. Where needed, FMO controls were used to gate on positive and negative populations. Post-acquisition analysis was carried out using FlowJo software v10.0.8 and Graphpad Prism software v5.0.

III.II.II.v.   Cell-surface staining for cell sorting

As many as 1.0×10^8 cells per sample were stained in sterile FACS tubes. Adherent cells were rinsed with DPBS and trypsinised before centrifugation at 1,300 X g for 3 min, and then washed with DPBS at 400 X g for 10 min. Non-adherent cells were washed using DPBS and centrifuged at 400 X g for 10 min. The supernatant was removed and cells were stained with antibodies (Table 3.1.8) and DPBS with 2% BSA in the dark at 4°C for 30 min. Stained cells were topped up to 10 ml with DPBS with 2% BSA and centrifuged at 400 X g for 10 min. The supernatant was removed completely and cells were re-suspended in cell-appropriate media (1 ml) (Table 3.1.21) and kept on ice until they were sorted using a Beckman Coulter MoFlo™ XDP. Required controls were used as outlined above (Section IV.II.II.iv.) After sorting cells were centrifuged at 400 X g for 10 min and re-suspended in cell-appropriate media (Table 4.21). Non-adherent cells were plated out in 96-well plates and adherent cells were seeded in T-75 flasks.

III.II.III.   Epigenetic treatment of cancer cells for mRNA analysis

III.II.III.i.   Treatment with epigenetic targeting therapies

A549 and SK-MES-1 cells were treated with a panel of epigenetic targeting therapies to assess whether CD1d expression could be up-regulated at the mRNA level. Cells were split at 80%

confluency as described above (Section IV.II.I.). Cells were incubated overnight at 37°C to allow time for cells to adhere.

Cells were treated with the HDACi (Histone deacetylase inhibitor) SAHA (Suberanilohydroxamic acid) dissolved in DMSO (dimethyl sulfoxide) at 5 µM for 24 hours, or treated for 24 hours with a recovery period of 24 hours, in cell-appropriate media (Table 3.1.21). Cells were also treated with theDNMTi (DNA methyltransferase inhibitor) DAC (5-Aza-2'Deoxycytidine) dissolved in MeOH (methanol) at 200 nM or 1 µM and or Gemcitabine (Gemcitabine hydrochloride; 2'-Deoxy-2',2'-difluorocytidine hydrochloride) dissolved in DPBS at 200 nM or 1 µM for 48 hours. Both the media and the drugs were replaced every 24 hours.

III.II.III.ii.　　　Total RNA isolation and cDNA synthesis

Following treatment with the DNMT and HDAC inhibitors the supernatant was removed from each flask. TRI reagent (1 mL) was added to each flask and spread evenly over the cells several times using a cell scraper. The TRI reagent was removed from the flask and transferred to a 2 ml micro tube. BCP (1-bromo-3-chloro-propane) (100µl) was added to each sample. The samples were shaken for 15 s, and then stored for 10 min at room temperature before being centrifuged at 13,500 rpm for 15 min at 4°C. The colourless phase was removed to separate tubes, and propan-2-ol (500 µl) was added; following mixing the samples were stored at room temperature for 10 min. Each sample was centrifuged at 13,500 rpm for 15 min at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol (1 mL) prior to storage at room temperature for 5 min. Each sample was centrifuged for a third time at 13,500 rpm for 5 min at 4°C. The supernatant was removed and the pellets were allowed to air dry for 5 min. Each sample was then re-suspended in molecular grade water (50 µl).

RNA was quantified using a Nanodrop ND-1000 Spectrophotometer (Table 4.1) and 1 µg was taken for cDNA synthesis. cDNA was generated using RevertAid and oligo-dT(20) primers. The template DNA (1 µg) was added to oligo-dT(20) (1 µl) or random hexamers (1 µl) and the

reaction volume was made up to 12.5 µl with molecular grade water. The samples were centrifuged briefly and then incubated at 65°C for 5 min. The following master mix was prepared (volumes are given per sample): 5X Reaction Buffer (4 µl); Ribonuclease RNase Inhibitor (0.5 µl); 10 mM dNTP mix (2 µl); RevertAid Reverse Transcriptase (1 µl). The final solution (7.5 µl) was added to each tube and mixed by pipetting. The samples were incubated at 42°C for 1 h, followed by a further incubation of 10 min at 70°C.

III.II.III.iii. Real time polymerase chain reaction (RT-PCR) amplification

Cell lines were examined for the expression of CD1d and β-actin by RT-PCR using the primers illustrated below (Table 3.2.1). Primers were purchased through Sigma-Aldrich. The template RNA (1 µl) was added to a master mix containing the following (per reaction): GoTaq 2x (10 µl); forward primer (2 µl); reverse primer (2 µl) and water (6 µl). Cycling conditions consisted of 95°C for 5 min followed by repeat cycles of 1 min at 94°C, 1 min at the appropriate annealing temperature (Table 3.2.1) and 1 min at 72°C with a final extension of 72°C for 10 min.

Experiments were carried out in triplicate and PCR products were electrophoresed in 1% agarose gels (agarose (1 g) in 1X Tris Acetate (TAE, 100 ml) and quantified using Tina 2.09c densitometry software. Target mRNA expression was standardised against *β-actin* controls, and expressed as a ratio of *CD1d* mRNA expression: *β-actin* expression.

Table 3.2.1: Primers used for PCR

Primer	Sequence	Annealing Temp	No. of cycles
β-actin (510 bp)	Forward 5'-AGCACTGTGTTGGCGTACAG-3' Reverse 5'-TGTTTGAGACCTTCAACACCC-3'	56°C	35
CD1d	Forward 5'-AAGCCTGTATGGGTGAAG-3'	56°C	40

(234 bp)	Reverse 5'-GACTGCCAAGGCAATCAAGC-3'		
pCDNA CD1d (cloning) (1008 bp)	Forward (HindIII) 5'-CG**AAGCTT**GCGATGGGGTGCCTGCTGTTTCTGCTGCTCTGG-3' Reverse (XhoI) 5'-TAGC**CTCGAG**TCACAGGACGCCCTGATAGGAAGTTTGCCT-3'	58°C	40

III.II.III.iv. Chromatin immunoprecipitation (ChIP) assay

ChIP assays are immunoprecipitation techniques that investigate the interaction between protein and DNA. It is used to determine whether specific proteins are associated with specific genomic regions. It is also used to determine the precise region in the genome that various histone modifications are associated with, thereby indicating the target of the histone modifiers. A549 cells were seeded into flasks with complete media until approximately 70-80% confluency was reached. The cells were treated with either DMSO (10 µl), for the control set, or TSA (Trichostatin A) (250 ng/mL), for 24 hours. Formaldehyde was added to a final concentration of 1% to allow the DNA to cross-link to histones. The samples were incubated at 37°C for 5 min. The supernatant was removed and the cells were washed twice using cold DPBS. Following this the cells were scraped into 1.5 ml Eppendorf tubes and centrifuged at 14,000 X g for 4 min at 4°C. The supernatant was removed and RT SDS lysis buffer (200 µl) was added in order to re-suspend the cells. The cells were then incubated for a period of 30 min. The cells were sonicated thrice, for 10 s followed by a rest period of 30 s, on ice. Samples were then centrifuged for 10 min at 13,000 X g at 4°C. The supernatant was collected for use in the ChIP assay, and the pellet was discarded.

A OneDay ChIP Kit was used to perform the ChIP assay. PI-ChIP buffer (1452µl) was added to the samples (396µl) and vortexed. 280µl of the sample:buffer mixture was added to separate tubes kept on ice. The desired antibodies (listed in Table 3.2.2 below) were added to the tubes which were then vortexed and incubated overnight in a rotary shaker at 4°C. During this

incubation the binding beads were washed twice in ChIP buffer and pelleted by centrifugation (3 min at 2,000 X g); the supernatant was discarded. ChIP buffer (10.5 mL) was used to re-suspend the beads and 500 µl was added to appropriately labelled tubes on ice. The beads were pelleted as above, the supernatant discarded, and the beads were stored on ice. Following incubation with specific antibodies (Table 4.23), the tubes were centrifuged at 4°C for 10 min at 12,000 X g.

Table 3.2.2: Selection of antibodies required for ChIP assay

Antibodies	Abbreviation
Pan-acetylated histone H3	α-H3Ac (pan)
Pan-acetylated histone H4	α-H4Ac (pan)
Histone H3 acetylated at lysine 9	α-H3K9Ac
Histone H3 acetylated at lysine 9 and 14	α-H3K9/K14Ac
Histone H3 methylation marker at lysine 4	α-H3K4 me
Histone H3 dimethylation marker at lysine 4	α-H3K4 me2
Histone H3 dimethylation marker at lysine 9	α-H3K9 me2
Histone H3 acetylated at lysine 9 and phosphorylated at serine 10	α-H3K9Ac/pS10
Histone H4 trimethylation marker at lysine 20	α-H4K20 me3
Histone H3 trimethylation marker at lysine 27	α-H3K27 me3

PCR was performed on the supernatant obtained from the ChIP assay. A 2.5 µl aliquot of supernatant was added to a PCR master mix (volumes are per sample): GoTaq 2x (Green) (10µl); forward primer (2µl); reverse primer (2µl) and water (6µl). The cycling conditions were as follows: 95°C for 5 min; 95°C for 1 min, 60°C for 1 min, 72°C for 1 min for a total of 35 cycles; a final 10 min at 72°C. The primer information is listed below in Table 3.2.3. Primers were purchased through Sigma-Aldrich.

Table 3.2.3: Primer information for ChIP sequencing

Primer name	Sequence	Annealing Temp	No. of cycles
CD1d ChIP (bp)	Forward 5'-CCACCTAGAGACATGTACTGC-3' Reverse 5'-CGCTCACTTCAGTAGGTTTC-3'	56°C	40

III.II.IV.   Establishing cancer cell lines over-expressing *CD1d*

III.II.IV.i.   Digestion and ligation of expression vectors

Expression vectors are used to introduce a specific gene into a target cell. This vector is generally a virus or plasmid designed for protein expression in cells. The vector inserts the gene of interest and can utilise the cell's mechanisms for protein synthesis to produce the corresponding protein. Vectors are engineered so that they have regulatory sequences that act as enhancer and promoter regions to allow for the successful transcription of the gene of interest. In this case an expression vector was used to attempt to insert the *CD1d* gene into A549 and SK-MES-1 cell lines to establish cultured cell lines over-expressing *CD1d.*

Plasmid A (Figure 3.2) contained the *CD1d* gene sequence and was purchased through AMS Biotech. Plasmid B was the expression vector and carried the CMV (cytomegalovirus) promoter and was also purchased with AMS Biotech. This promoter is popular for transfection as it provides the highest level of expression activity in the largest variety of cell types. Both plasmids (25 µl) were digested using HindIII and XhoI primers (1 µl each) along with Buffer B (5 µl) or Buffer 2 (5 µl) + BSA (0.5 µl) and H_2O (up to 50 µl) (Table 3.1.12) and heat shocked at 65°C for 5 min. The plasmids were incubated at 37°C overnight before running them out on a 1% agarose gel to assess whether complete digestion had occurred.

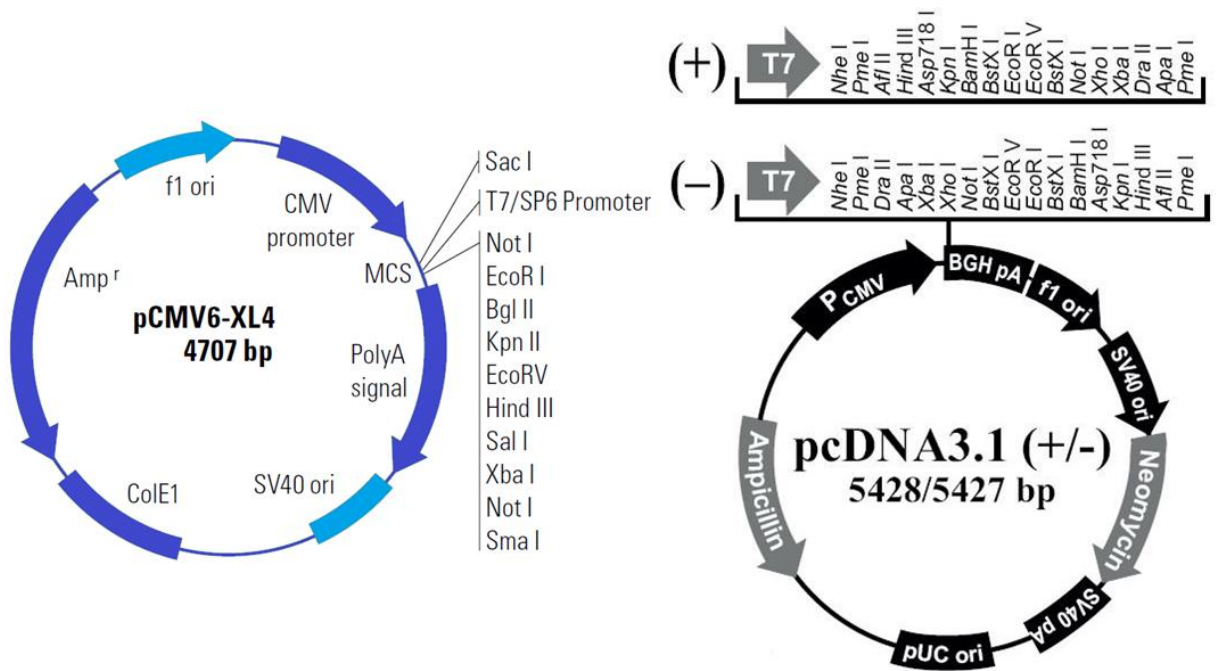**Figure 3.2: pCMV6-XL4 insertion vector and pcDNA3.1 expression vector used to transfect A549 and SK-MES-1 cells.** Plasmid A or pCMV6-XL4 (left) is an untagged insertion vector that contains a neomycin mammalian selection vector. CD1d cDNA was shuttled into this vector for insertion into Plasmid B or pcDNA3.1 (+/-) (right). Plasmid B allows for cloning and expression of *CD1d*. A list of the restriction sites is also depicted.

The Wizard® SV Gel and PCR Clean-Up System was designed to extract and purify DNA fragments >100 bp from standard agarose gels in TAE. PCR products were purified to remove excess nucleotides and primers. This is a membrane-based system that allows recovery of isolated DNA fragment that can then be used for a number of purposes including cloning and restriction enzyme digestion. DNA from the gel was excised and transferred to a microtube (1.5 ml) with Membrane Binding Solution (10 µl). The mixture was vortexed and then incubated at 65°C for 10 min. The Vacuum Adaptor was added to the manifold port and the SV minicolumn was placed into the Adaptor. The dissolved gel mixture was applied and incubated for 1 min at room temperature. The vacuum was applied and was not released until all of the liquid had passed through the minicolumn. Membrane Wash Solution (700 µl) was added and the vacuum was applied. Membrane Wash Solution (500 µl) was added and the vacuum was applied. The minicolumn was removed to a collection tube and centrifuged at 16,000 X g for 5 min. The collection tube was emptied and the minicolumn and collection tube were centrifuged again for 1 min. The minicolumn was transferred to a micro tube (1.5 ml) and Nuclease-Free Water (50 µl) was added. Tubes were incubated at room temperature for 1 min and then centrifuged at 16,000 X g for 1 min. The concentration of the CD1d DNA insert as well as an empty vector was measured using a Nanodrop ND-1000 Spectrophotometer (Table 3.1.1). For ligation 10X T4 DNA Ligase Buffer (2 µl) was added to the vector DNA (50 ng) and insert DNA (37.5 ng), T4 DNA Ligase (1 µl) and Nuclease-Free Water (to 20 µl). The mixture was kept on ice, and gently mixed by pipetting up and down and centrifuged briefly. The mixture was incubated at room temperature for 2 hours and then heat inactivated at 65°C for 10 min.

III.II.IV.ii.   Transformation of DH5α cells and colony PCR

DHα5 cells are the most commonly used *E. coli* (*Escherichia coli*) strain used for routine cloning applications. These cells can support blue/white cloning as well as increasing insert ability and quality of plasmid DNA prepared from minipreps. These cells were used for the insertion of the

pcDNA3.1 plasmid to establish transformed bacteria. The kit used was the One Shot® DH5α™-T1^R Competent Cells (Table 3.1.12).

TOPO® Cloning reaction was set up as follows: Fresh PCR product (4 µl) with NaCl (Sodium chloride) solution (1 µl) and TOPO® vector (1 µl). The reaction was mixed gently and incubated at room temperature for 5 min and then placed on ice. TOPO® Cloning reaction (2 µl) was added into a vial of One Shot® Chemically Competent *E. coli* cells and then mixed gently. The mixture was incubated on ice for 5 min and then the cells were heat-shocked for 30 seconds at 42°C. The tubes were transferred to ice and S.O.C medium (250 µl) from the kit was added. The tubes were shaken horizontally at 37°C for 60 min. 50 µl of each transformation was spread onto a pre-warmed ampicillin-selective LB (luria broth) plate from the kit, and incubated at 37°C overnight.

Twenty colonies were selected at random and streaked out on LB ampicillin-selective plates. The plate was incubated at 37°C overnight. The following day a pipette tip was used to select a small portion of a colony which were then suspended in H$_2$O (10 µl). This was repeated for each of the twenty colonies. The samples were heated at 95°C for 5 min and centrifuged at 8,000 X g for 5 min. 2 µl of each sample was added to GoTaq 2x Green (10 µl), CD1d cloning primers HindIII Forward Primer (2 µl) and XhoI Reverse Primer (2 µl) (Table 3.2.3) and H$_2$O (6 µl). Cycling conditions consisted of 95°C for 5 min followed by 40 repeat cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C with a final extension of 72°C for 10 min. PCR products were run out on a 1% agarose gel to assess which colonies carried the insert (Figure 3.3).

III.II.IV.iii.　　　Large-scale plasmid preparation

A large-scale plasmid preparation was carried out on cultures that were found to express CD1d mRNA using the Pure Yield™ Plasmid Midiprep System (Table 3.1.12) to isolate high quality plasmid DNA for transfection. DNA purification was carried out by vacuum to remove any
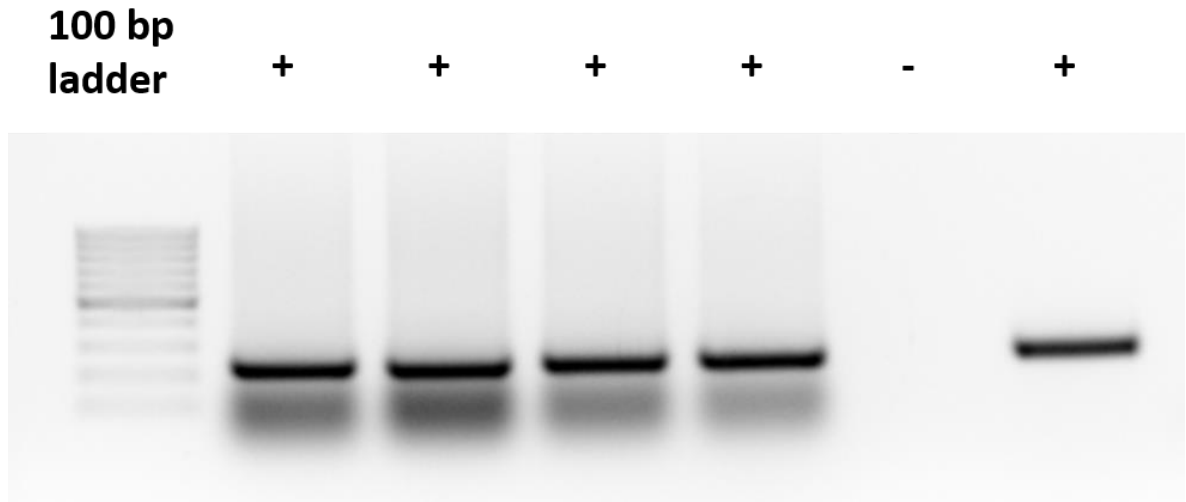
Figure 3.3: Agarose gel of colony PCR showing colonies that are positive and negative for the _CD1d_ insert. pcDNA3.1 (Plasmid B) was inserted into _E. coli_ cells to establish transformed bacteria. Following transformation cells were spread on a LB ampicillin-selective plate and incubated at 37°C overnight. Twenty colonies were selected at random and streaked out on LB ampicillin-selective plates and incubated at 37°C overnight. A portion of each colony was analysed by PCR analysis to determine which colonies carried the insert.

contaminants such as RNA, protein and endotoxin contaminants that could affect the robustness of any subsequent transfection.

Transformed cells were grown in culture overnight at 37°C. Cells were pelleted at 5000 X g for 10 minutes and the supernatant was aspirated completely. Cells were re-suspended in Cell Resuspension Solution (3 ml). Cell Lysis Solution (3 ml) was added and mixed gently followed by a 3 min incubation at room temperature. Neutralisation Solution (5 ml) was added and mixed gently. Lysed cells were incubated at room temperature until a white precipitate formed. Pure Yield^{TM} Clearing Columns were placed on top of Pure Yield^{TM} Binding Columns and then into a vacuum manifold (Table 3.1.1). The lysate was poured into the Clearing Column and incubated at room temperature for 3 min. The vacuum was applied until all the liquid had passed through, and the Clearing Column was discarded.

Endotoxin Removal Wash (5 ml) was added to the column and the vacuum was applied. Column Wash Solution (20 ml) was added and the vacuum was applied. The vacuum was reapplied to dry the membrane for 30 s. The Binding Column was removed and any excess EtOH was removed; the column was placed in a Falcon tube (50 ml). Nuclease-Free Water (600 μl) was added and the column was centrifuged at 2,000 X g for 5 min to elute the DNA. The DNA of the CD1d insert as well as an empty vector was measured using a Nanodrop ND-1000 Spectrophotometer (Table 3.1.1).

III.II.IV.iv. Transient transfection of NSCLC cell lines

The plasmid was digested with EcoRI with Buffer H and EcoRI/ApaI with Buffer A to confirm that the insert was present. FuGENE® HD Transfection Reagent was incubated at room temperature for several minutes and then vortexed briefly. A549 and SK-MES-1 cells were plated at a density of 3.0 x 10^5 cells/well of a 6 well plate with the appropriate media (3 ml) (Table 3.2.1). CD1d plasmid or an empty vector (3.3 μg) was added to FuGENE® HD Transfection Reagent (9.9 μl) with sterile deionised water (up to 155 μl). The complex was mixed gently by pipetting and then
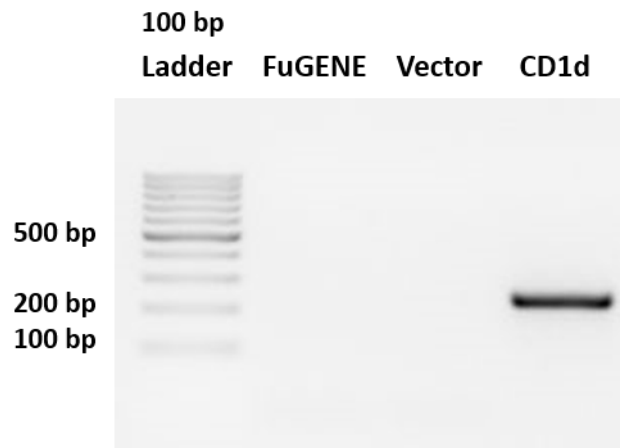
Figure 3.4: A549 cells transiently transfected with CD1d. A549 cells were transfected with an expression vector containing either the *CD1d* gene or empty vector using the FuGENE protocol. Cells were solubilised for analysis of CD1d mRNA by RT-PCR.

incubated for 5 min at room temperature. The complex was added to the appropriate wells and thoroughly mixed before incubation at 37°C for 48 hours. Presence of CD1d over-expression was assessed by PCR (Table 3.2.2) (Figure 3.4).

III.II.IV.v. Stable transfection of NSCLC cell lines

A double digestion was performed using PvuI to linearise the plasmid. Linear DNA is more effective for stable transfections because of its optimal integration into the host genome. The free ends means that it is more likely to be recombinant and therefore more likely to be taken up although the caveat is that it is taken up less efficiently by the cell. The FuGENE protocol was followed as before (Section III.II.IV.iv) except with 6.6 µg of DNA and 20 µl of FuGENE® HD Transfection Reagent. Cells with stable integration were chosen for enrichment. Cells were maintained in cell-appropriate media with G418 Sulfate, a selective antibiotic that is used for the selection and maintenance of eukaryotic cells carrying the *neo* gene. The media and antibiotic were replaced every 3 – 4 days. After two weeks remaining cells carried a copy of either the empty vector or the CD1d insert. Cells were assessed by PCR to see whether the empty vector and CD1d insert expressed CD1d. Following confirmation of the presence or absence of CD1d over-expression, cells were sorted by FACS to ensure pure transfectant cell lines of >98%. Transfectant cells were maintained in G418-media to ensure stable transfection.

III.II.V. Induction of CD1d in NSCLC

III.II.V.i. Single high dose DAC treatment

CD1d has been shown to be epigenetically regulated at the histone level in lung adenocarcinoma cells (Yang *et al.*, 2012). Treating A549 cells with HDACi TSA and SAHA resulted in an induction of CD1d through the inhibition of HDAC1 or HDAC2 (Yang *et al.*, 2012). We decided to investigate whether treatment with a DNMTi, DAC, could result in a similar induction of CD1d expression. CD1d expression could be up-regulated at the protein level. Cells were split at 80% confluency A549 and SK-MES-1 cells were treated with DAC at 100 nM, 1 µM and 5 µM to assess whether
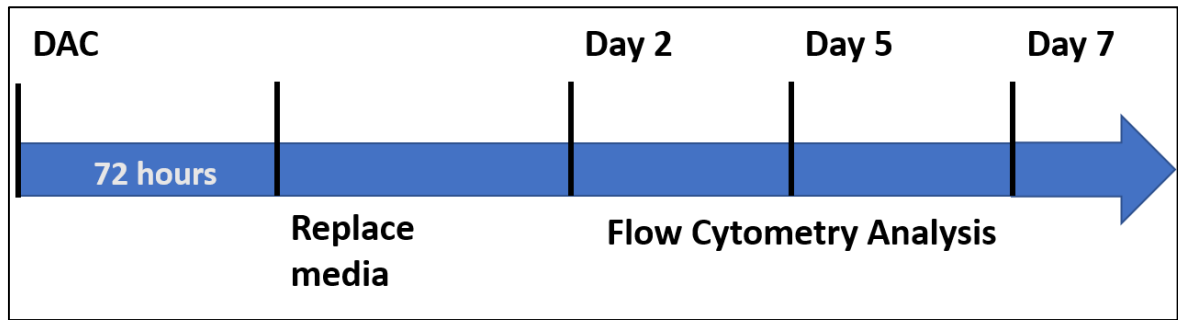
Figure 3.5 High Dose DAC Treatment to Induce CD1d Expression in NSCLC. Cells were treated with a once-off treatment of DAC at 100nM, 1 µM and 5µM. After 72 hours the medium was replaced and cells were cultured in the absence of DAC for 2, 5 or 7 days and analysed by flow cytometry.

as described above (Section III.II.I.). Cells were incubated in 10 cm plates overnight at 37°C to allow time for cells to adhere. DAC was diluted in MeOH to either 100 nM, 1 µM or 5 µM. Cells were treated one of the three concentrations of DAC, or PBS. MeOH was used as a vehicle control. The medium was replaced after 72 hours and cells were given a recovery period of either 2, 5 or 7 days (Figure 3.5). Following this recovery period cells were assessed for expression of CD1d by flow cytometry.

III.II.V.ii.   Transient DAC treatment

Past trials with high doses of DAC have been plagued by extreme toxicities (Abele *et al.*; 1987). Transient low doses of DAC have been shown to sustain long-term anti-tumour effects and is at least temporarily associated with its ability to maintain it's targeting of DNA methylation processes and alterations of gene expression (Tsai *et al.*, 2012). A549 and SK-MES-1 cells were treated with transient low doses of DAC at 50 nM, 100 nM or 1 µM for 72 hours. DAC was diluted in MeOH as before. Cells were treated with one of the three concentrations of DAC, or PBS. MeOH was used as a vehicle control. Both the media and the treatments were replaced every 24 hours. Cells were assessed for CD1d expression by flow cytometry immediately after treatment and again following a recovery period of 4 days (Figure 3.6).

II.II.V.iii.   Combination DAC + SAHA treatment

Heavily methylated DNA correlates with chromatin that are in a transcriptionally repressive state due to hypomethylated histones (Eden *et al.*, 1998). These epigenetic processes have been linked; studies have shown that the methyl-CpG-binding protein MeCP2 appears to be present in a complex with histone deacetylase activity (Nan *et al.*, 1998 and Jones *et al.*, 1998). MeCP2 interacts specifically with DNA methylation to facilitate transcriptional repression (Nan *et al.*, 1998) and this repression can be reversed by inhibiting histone deacetylase (Jones *et al.*, 1998).
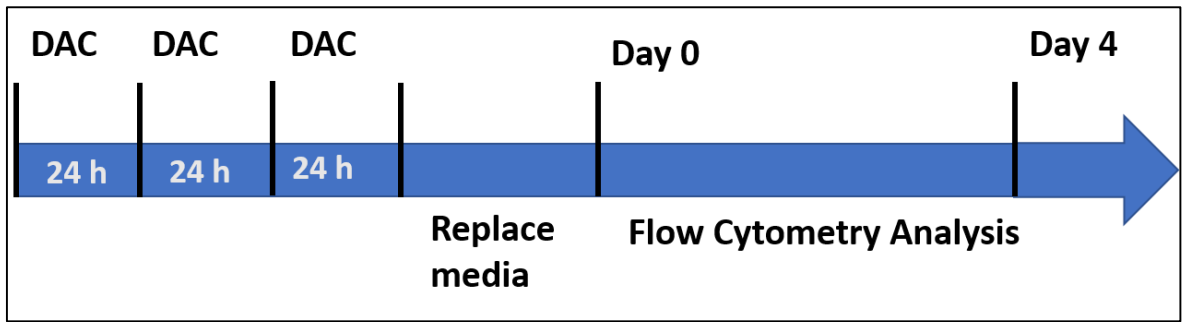
Figure 3.6: Transient Low Dose DAC Treatment to Induce CD1d Expression in NSCLC. Cells were treated with of DAC at 50nM, 100 nM and 1 μM. Both the medium and the treatment was replaced every 24 hours. After 72 hours of treatment the medium was replaced and cells were immediately analysed by flow cytometry, or cultured in the absence of DAC for 4 days and then analysed by flow cytometry.

Figure 3.7: Combinatorial DAC + SAHA Treatment to Induce CD1d Expression in NSCLC. Cells were treated with of DAC at 100nM, 500 nM and 1 μM for 72 hours. Both the medium and the treatment was replaced every 24 hours. After 48 hours 5 μM SAHA was added. After 72 hours of treatment the medium was replaced and cells were immediately analysed by flow cytometry, or cultured in the absence of DAC and SAHA for 4 days and then analysed by flow cytometry.
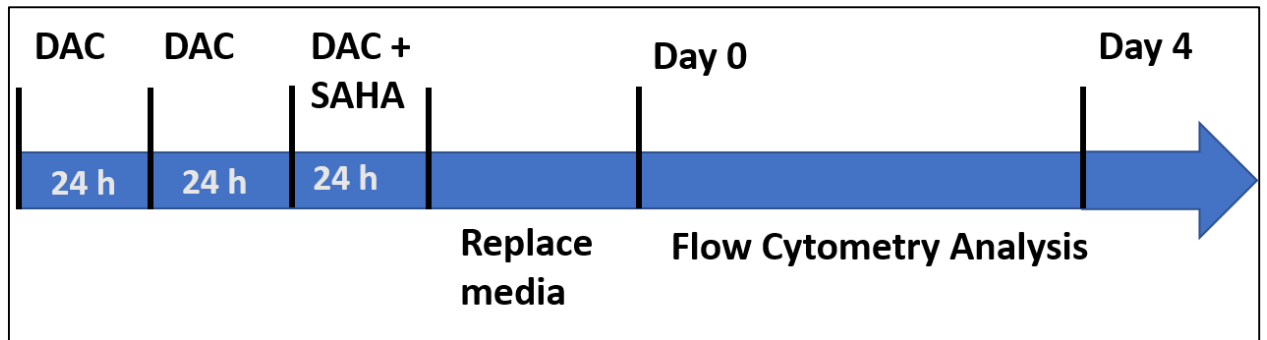
Cameron and colleagues showed that they could reactivate several hypermethylated genes by TSA and DAC in combination (Cameron *et al.*, 1999).

In order to examine whether a combined therapy of DAC and SAHA could up-regulate CD1d expression, A549 and SK-MES-1 cells were treated with both drugs for a period of 72 hours as depicted in Figure 3.7 below. DAC was diluted in MeOH to 100 nM, 500 nM and 1 µM. SAHA was diluted to 5 µM in DMSO. Cells were treated with one of the three concentrations of DAC with SAHA or PBS. MeOH was used as a vehicle control. Both the drugs and media were replaced every 24 hours. Cells were assessed for presence of CD1d by flow cytometry directly following the treatment period, and again following a four day recovery period.

III.II.VI.   Invariant natural killer T (iNKT) cells

III.II.VI.i.   iNKT cell isolation using magnetic bead enrichment

iNKT (6B11^{+}) cells were enriched from PBMCs following isolation of PBMCs as outlined above, using Miltenyi MACS MicroBeads. MACS MicroBeads are 50 nM nanoparticles that are non-toxic and do not activate cells. They are conjugated to highly specific antibodies specific for a particular antigen. Anti-iNKT MicroBeads were developed for the selection of cells expressing the TCR α-chain Vα24-Jα18.

Cells were centrifuged at 300 X g for 10 min and the supernatant was carefully removed. Cells were vortexed briefly and re-suspended in Miltenyi Buffer (1 X DPBS supplemented with 500 mM ethylenediamenetetraacetic acid (EDTA) and 30% BSA (Bovine Serum Albumen)). Miltenyi Buffer was made up fresh each time and kept on ice. Anti-iNKT MicroBeads (100 µl) was added and cells were vortexed gently and incubated on ice for 15 min. Cells were washed with Miltenyi Buffer (2 ml) and centrifuged at 300 X g for 10 min. The supernatant was removed completely and cells were re-supended in Miltenyi Buffer (0.5 ml).

Cells were applied to an LS column set up in the magnetic apparatus that had previously been rinsed with Miltenyi Buffer (3 ml). The flow-through containing unlabelled cells were collected. The column was rinsed with Miltenyi Buffer (3 ml) thrice to remove any unlabelled cells. iNKT⁺ cells were eluted by removing the column to a sterile collection tube and flushing the cells through using Miltenyi Buffer (5 ml) and the accompanying plunger. The collected positively-labelled cells were enriched over a second column using the same method to increase purity. Cell were enumerated and cell purity was assessed by flow cytometry.

III.II.VI.ii.   Purification of iNKT cells by MoFlo cell sorting

As many as 1.0×10^8 cells per sample were stained in sterile FACS tubes. iNKT cells were washed using DPBS and centrifuged at 400 X g for 10 min. The supernatant was removed and cells were stained with antibodies specific for Vα24 and Vα11 (Table 3.1.8) and DPBS with 2% BSA in the dark at 4°C for 30 min. Stained iNKT cells were topped up to 10 ml with DPBS with 2% BSA and centrifuged at 400 X g for 10 min. The supernatant was removed completely and cells were re-suspended in cRPMI with IL-2 (1 ml) (Table 3.1.21) and kept on ice until they were sorted using a Beckman Coulter MoFlo™ XDP. Required controls were used as outlined in Section III.II.II.iv.

III.II.VI.iii.   iNKT cell expansion and maintenance

After sorting cells were centrifuged at 400 X g for 10 min and re-suspended in cRPMI (Table 3.1.21) with IL-2 (250U/ml). αGalCer, a marine sponge-derived glycolipid that activates iNKT cells (Kawano *et al.*, 1997) was brought to room temperature. αGalCer was heated to 80°C for 2 minutes and then sonicated for 10 mins and added to the iNKT cell suspension at 1 mg/ml. iNKT cells were cultured in the presence of irradiated PMBCs and incubated at a constant temperature of 37°C in a humidified atmosphere of 5% CO_2. After 4 weeks cells were ≥95% purity as assessed by flow cytometry. cRPMI media with IL-2 was replaced every 2 – 3 days. Cells were monitored closely for changes in colour and appearance of smooth, round cell pellets

which indicated expansion. iNKT cells were kept at high cell densities and split 1 in 2 in fresh 96 well plates when multiple cell layers were observed using an inverted microscope.

III.II.VII.   iNKT CD107a degranulation assay

Cancer cell lines were co-cultured with iNKT cells to assess their susceptibility to iNKT cell-mediated cytolytic degranulation by measuring the expression of cell-surface CD107a. Lysosomal-associated membrane protein -1 (LAMP-1 or CD107a) is a marker of degranulation following T or NK cell stimulation (Betts *et al*., 2003). Under normal conditions CD107a resides in vesicles within cells. When these cells come into contact with target cells CD107a is up-regulated on the cell surface (Figure 3.5). This up-regulation correlates with both cytokine secretion, as well as NK-mediated lysis of target cells (Alter *et al*., 2004 and Hogan *et al*.; 2011). iNKT cells were co-cultured with NSCLC cell lines or CD1d-transfected HeLa and SK-MES-1 cell lines or mock-transfected HeLa cell lines to assess whether degranulation had occurred. This was examined by measuring the amount of cell surface CD107a using flow cytometry.

Cancer cell lines were seeded in 96 well plates at 1.0×10^5 cells per well and pulsed with either αGalCer or 7Dw8.5 overnight. 7Dw8.5 is an analogue of αGalCer that was designed by Li and colleagues and has shown superior activity over αGalCer using enzyme-linked immunosorbent (ELISA) and enzyme-linked immunospot (ELISPOT) assays in mouse studies on HIV and malarial vaccines (Li *et al*., 2010). iNKT cells were added the following day at 1.0×10^5 cells per well; PMA and ionomycin were set up as positive controls. iNKT cells cultured in media alone were used as negative controls. Cells were stained with CD107a FITC (Table 3.1.6) and incubated for 1 hour at 37°C. 1000X Monensin was diluted in 1X in iNKT media (Table 4.20) and added to the each well and the plate was incubated for 3 hours at 37°C. Monensin is a protein transport inhibitor that is used to increase intracellular cytokine signals by inhibiting transport process during cell activation.
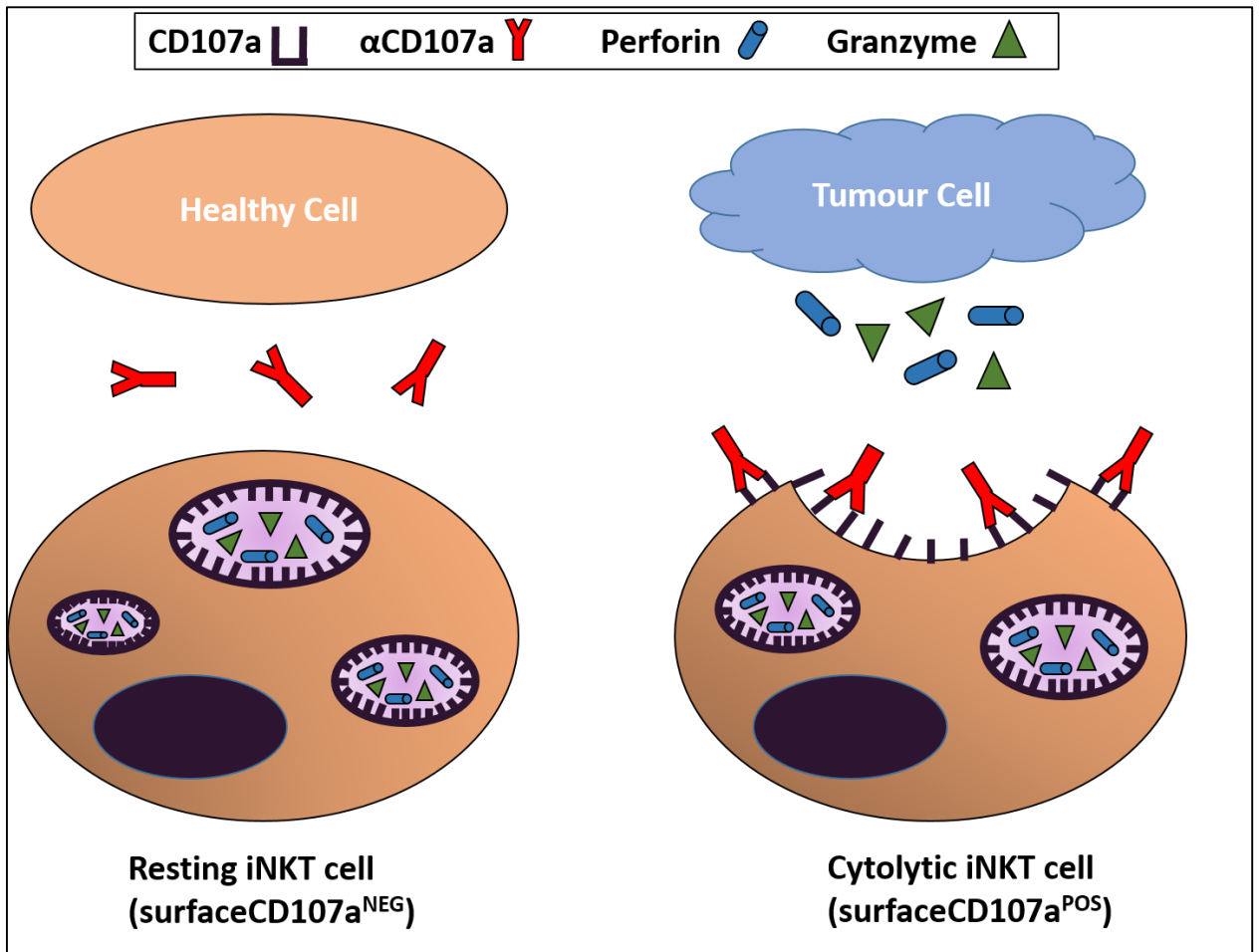
Figure 3.8: Depiction of CD107a degranulation assay. CD107a is contained in vesicles within the cell. When the cell comes into contact with a tumour cell, these vesicles migrate towards the surface of the cell. De-granulation is assessed by measuring the quantity of surface CD107a expression as measured by the intensity of anti-CD107a staining.

Following the 3 hour incubation the co-culture assay was centrifuged at 800 X g for 5 min and the supernatant was discarded. Cells were stained with Fixable Viability Dye eFluor® 506 (Table 4.6) for 15 min in the dark at room temperature. Cells were then stained with an antibody cocktail of 6B11 PE, CD3 PB, CD4 PeCy7 and CD8a PerCP (Table 4.6) in PBA for 15 min in the dark at room temperature. Cells were washed in PBA at 800 X g for 5 min. The supernatants were discarded and the cells were re-suspended in PBA and analysed by flow cytometry.

III.II.VIII. Phenotyping Patient Samples and Healthy Controls

III.II.VIII.i. Bronchial lavage samples

Bronchial lavage samples were retrieved at bronchoscopy after informed consent and as approved by the Research Ethics Committee of St. James's Hospital. Samples were retrieved from both NSCLC patients (Table 3.2.4) and non-cancer controls (Table 3.2.5) according to strict exclusion criteria as follows: under 18 years of age, absence of written informed consent, current respiratory infection, pulmonary fibrosis, sarcoidosis, HIV (Human Immunodeficiency Virus) or Hepatitis C. Following removal of alveolar macrophages by adherence purification, non-adherent cells were washed thrice with DPBS. Cells were enumerated and stained with Fixable Viability Dye eFluor® 506 (Table 3.1.6) for 15 min in the dark at room temperature. Following this each sample was split into two FACS tubes labelled Tube 1 and Tube 2 and stained with an antibody cocktail (Table 3.2.6) in PBA for 15 min in the dark at room temperature. Cells were washed in PBA at 800 X g for 5 min. The supernatants were discarded and the cells were re-suspended in PBA and analysed by flow cytometry.

Table 3.2.4: Table of characteristics of NSCLC patients enrolled in BAL study

Identifier	Gender	Age
B029	Male	88

B030	Male	57
B034	Male	70
B036	Female	67
B037	Male	91
B041	Male	65
B044	Male	62
B045	Female	71
B048	Male	81
B049	Male	59
B053	Female	59
B055	Male	69

Table 3.2.5: Table of characteristics of non-cancer patients enrolled in the BAL study

Identifier	Gender	Age	Smoking Status
B001	Male	73	Ex-Smoker
B002	Male	85	Ex-Smoker
B003		57	Smoker
B004	Male	28	Ex-Smoker
B005	Female	67	Smoker
B006	Female	75	Never Smoker
B007	Male	41	Smoker
B008	Female	42	Never Smoker
B009	Female	79	Smoker
B010	Female	44	Smoker
B011	Male	45	Smoker

B012	Female	38	Smoker
B013	Female	65	Smoker
B014	Female	53	Never Smoker
B015	Male	54	Ex-Smoker
B016	Male	60	Never Smoker
B017	Female	60	Never Smoker
B018	Female	60	Ex-Smoker
B019	Male	70	Ex-Smoker
B020	Male	67	Ex-Smoker
B021	Female	32	Smoker
B022	Male	64	Smoker
B023	Female	63	Ex-Smoker
B024	Female	75	Never Smoker
B025	Female	56	Never Smoker
B026	Female	56	Smoker
B027	Female	65	Smoker
B028	Female	62	Never Smoker
B031	Male	45	Never Smoker
B032	Male	64	Smoker
B033	Female	56	Smoker
B035	Female	54	Ex Smoker
B038	Male	76	Ex Smoker
B039	Female	80	Never Smoker
B040	Male	44	Never Smoker
B042	Male	78	Ex-Smoker
B043	Female	76	Never Smoker

B046	Female	74	Smoker
B047	Male	69	Ex-Smoker
B050	Male	69	Ex-Smoker
B054	Female	56	Ex-Smoker

Table 3.2.6: List of antibodies used in patient enumeration and phenotyping studies

Tube	Antibodies
1	6B11 PE, CD3 PB, CD4 PeCy7, CD8a PerCP, Vδ1 FITC
2	CD3 PB, CD19 PeCy7, CD56 FITC, Vδ2 PE, Vδ3 APC

III.II.VIII.ii. Blood samples

Blood samples were retrieved following informed consent and as approved by the Research Ethics Committee of St. James's Hospital. Samples were retrieved from lung cancer patients (Table 3.2.7) and age-matched healthy controls (Table 3.2.8). Samples were prepared by PBMC prep as described above (Section IV.II.I.ii.) and the samples were assessed by flow cytometry using the panel of antibodies described in Table 3.2.6.

Table 3.2.7: Table of characteristics of NSCLC patients enrolled in blood study

Identifier	Gender	Age
BL001	Female	60
BL002	Male	57
BL003	Female	65
BL004	Male	62
BL005	Male	65
BL006	Female	75
BL007	Female	75
BL008	Male	55

BL009	Female	53

Table 3.2.8. Table of characteristics of healthy controls enrolled in blood study

Identifier	Gender	Age
BC001	Male	54
BC002	Female	50
BC003	Male	53
BC004	Female	55
BC005	Female	60
BC006	Female	59
BC007	Female	58
BC008	Female	62
BC009	Male	64
BC010	Male	63
BC011	Male	61
BC012	Female	62
BC013	Female	59

III.II.IX.   Statistical analysis

Statistical analysis for all data was performed using GraphPad Prism v5.0. Data is expressed as mean ± standard error of the mean and analyzed using one- or two-way ANOVA with either Bonferroni or Dunnett's post-hoc test or a t test with Welch's correction. The Bonferroni Test was used for multiple comparison analysis while Dunnett's Post Test was used to compare each sample mean against a control mean. Welch's correction is used for unequal sample sizes and does not assume that the variances of the two populations are equal. The data was considered significant if $p < 0.05$.

IV. Phenotyping Lymphocyte Populations in NSCLC

IV.I. Introduction

Most chronic diseases, including cancer, stimulate MHC-unrestricted inflammatory responses in the host, and both innate and adaptive immune systems play tumour-promoting and tumour suppressing roles. Several studies have shown that lymphocytes play roles both in anti-tumour immunity, and in tumour-promoting inflammation.

Lymphocytes can infiltrate tumours where they kill tumour cells and release cytokines, such as IFNγ and TNFα, which promote anti-tumour responses of other cells. Increased tumour infiltration by CTLs and NK cells is associated with an improved prognosis in many cancers, including colon cancer (Pagès *et al*., 2010). CTLs recognise tumour-associated antigens presented by MHC class I molecules, whereas NK cells detect changes in the levels of expression of MHC class I molecules and other stress-inducible ligands that signify tumour transformation. Upon recognition of tumour cells, CTLs and NK cells can kill tumour cells by perforin or granzyme release, or by Fas ligand or TRAIL ligation. A prospective cohort study looking at a population in Japan found that individuals with medium or high cytotoxic activity of peripheral T lymphocytes had a lower risk of cancer that individuals with low cytotoxic activity (Imai *et al*., 2000).

TILs can promote tumorigenesis through a variety of mechanisms, such as preventing CTL recruitment and activation through the release of cytokines such as TGFβ and/or IL-10. Tumours can also actively recruit immunosuppressive T_{REG} cells. A study by Woo and colleagues demonstrated that T_{REG} cells from NSCLC patients were capable of suppressing the expansion of allogeneic T cells (Woo *et al*., 2002). Another study by Meloni and colleagues showed that immunosuppressive T_{REG} cells with increased functionality and cytokines were present in the

peripheral blood of NSCLC patients (Meloni *et al.*, 2006). These studies suggest that NSCLC has developed mechanisms to evade immune surveillance by activating tolerogenic immune cells.

The balance of pro- and anti-tumour immune responses is controlled, in part, by innate T cells. iNKT cells are required for tumour recognition in several murine models and are numerically and/or functionally impaired in many human cancers (Dhodapkar *et al.*, 2003, Crowe *et al*, 2002, Yanagisawa *et al.*, 2002, Tahir *et al.*, 2001, Cui *et al.*, 1997 and Kawano *et al.*, 1997). Various subsets of γδT cells have been implicated in promoting or inhibiting tumorigenesis in mice and humans (Wu *et* al, 2014, Cordova *et al.*, 2012, Wakita *et al.*, 2010, Casetti *et al.*, 2009, Corvaisier *et al.*, 2005 and Biaslasiewicx *et al.*, 1999). Clinical trials that target iNKT cells and Vδ2 T cells for cancer immunotherapies are ongoing for several cancers in humans.

Phenotypic analysis of circulating lymphocytes or TILs have revealed expansions and depletions of lymphocyte subsets that have provided clues to the pathogenesis of cancer and provided prognostic biomarkers. A retrospective study on NSCLC tumours found that extensive infiltration of tumours by CD4$^+$ T cells correlated with a favourable outcome, with higher numbers of CD4$^+$ T cells corresponding to increased survival times (Wakabayashi *et al.*, 2003). In comparison, a study of CD4$^+$ T cells in renal cancers found that higher infiltrations of these cells correlated with decreased overall survival (Siddiqui *et al.*, 2007). A study of CD8$^+$ T cells in NSCLC patients found that high density infiltrations in the lung stroma significantly correlated with improved survival (Al-Shibli *et al.*, 2008). Several other studies looking at CD8$^+$ T cell infiltration in lung tumours found that these cells show reduced proliferation, decreased production of T_H1 cytokines, and reduced cytotoxic potential (Prado-Garcia *et al.*, 2005, Trojan *et al.*, 2004 and Chen *et al.*, 2000).

NK cell tumour infiltration is associated with improved prognosis in colorectal carcinomas (Halama *et al.*, 2011) and NSCLC carcinomas (Platonova *et al.*, 2011 and Carrega *et al.*, 2008). A epidemiological study found that individuals with low NK cell activity had an increased risk of

cancer (Imai *et al*., 2000). In addition, solid tumours have significantly lower frequencies of iNKT cells and in some cases these cells are replaced by type 2 NKT cells which are frequently pro-tumorigenic (Kenna *et al*., 2007 and Kenna *et al*., 2003).

Research has demonstrated how inflammation and immune cells, namely those of the innate immune system, can promote tumorigenesis (Colotta *et al*., 2009, DeNardo *et al*., 2010, Grivennikov *et al*., 2010, Qian and Pollard, 2010). Tumour-promoting lymphocytes have been shown to release actively mutagenic chemicals to nearby cancer cells that aid in their evolution (Grivennikov *et al*., 2010). NF-KB has been shown to be involved in tumour initiation and progression in tissues prone to chronic inflammation (Karin, 2006, Greten *et al*., 2004). Inhibition of the NF-KB pathway in epithelial and myeloid cells prevented inflammation-associated tumour formation in a mouse model of colitis (Greten *et al*., 2004). Another mouse model showed that inhibition of NF-KB activity in the later stages of tumour formation led to the apoptosis of transformed cells and failure of tumour progression (Pikarsky *et al*., 2004). Tumour-associated macrophages (TAMs) are the primary leukocyte subset that amplify the inflammatory response in the tumour microenvironment. A variety of studies have demonstrated that an increase of TAMs is associated with poor prognosis and angiogenesis (Mantovani *et al*., 2008, Pollard, 2006, Balkwill *et al*., 2005 and Bingle *et al*., 2002). TAMs also contribute to tumorigenesis through secretion of cytokines, matrix-degrading enzymes and growth factors (Yang *et al*., 2008, Wyckoff *et al*., 2007, Condeelis and Pollard, 2006 and Mantovani *et al*., 2006).

These studies indicate that immune deficiencies are present in many cancers. We hypothesized that immune cell populations differ in NSCLC patients compared with controls, and that identifying these changes could lead to novel targets for immunotherapies against NSCLC.

IV.II. Objectives

The aim of the present study is to enumerate and phenotypically characterise lymphocyte subsets in blood and BAL of NSCLC patients, and to compare them with those in control subjects. Analysis of lymphocytes in BAL samples would allow us to characterise the cellular nature of the immune system in the tumour microenvironment, whereas analysis of blood samples may identify changes that can be used as potential prognostic biomarkers in NSCLC.

The specific aims of this chapter are as follows:

1) To enumerate T cells, including CD4$^+$ T cells and CD8$^+$ T cells, B cells, NK cells, γδT cells and iNKT cells in BAL samples from patients with NSCLC and non-cancer controls to identify lymphocyte subsets that contribute to tumorigenesis or anti-tumour immunity.

2) To enumerate T cells, including CD4$^+$ T cells and CD8$^+$ T cells, B cells, NK cells, γδT cells and iNKT cells in blood samples from patients with NSCLC and healthy controls to identify potential biomarkers that predict disease.

3) To determine whether peripheral blood analysis is comparable to BAL analysis in determining variations in immune populations between cancer patients and non-cancer or healthy controls

4) To analyse the effects of smoking status, gender, and age on immune cell populations.

IV.III. Methods

IV.III.I. NSCLC patients and control groups

BAL samples were taken from NSCLC patients (Table 4.3.1) and non-cancer controls (Table 4.3.2) after informed consent according to strict exclusion criteria: patients undergoing clinically indicated bronchoscopy under 18 years of age, a known (or ensuing) diagnosis of malignancy, sarcoidosis, HIV or Hepatitis C who did not give informed written consent prior to the procedure.

Only samples that were included in the analysis are included in the table below. Other samples could not be used because of a lack cells and difficulty in optimising the method and are listed in Table 3.2.4 and 3.2.5. Blood samples were taken from chemotherapy-naïve NSCLC patients (Table 4.3.3) and healthy controls (Table 4.3.4) after informed consent. Table 4.3.5 summarizes the characteristics of the NSCLC patients, non-cancer patients and healthy controls enrolled in the studies

Table 4.3.1 Table of characteristics of NSCLC patients enrolled in BAL study

Identifier	Gender	Age
B030	Male	57
B034	Male	70
B036	Female	67
B037	Male	91
B041	Male	65
B044	Male	62
B045	Female	71
B048	Male	81
B049	Male	59
B053	Female	59

Table 4.3.2 Table of characteristics of non-cancer patients enrolled in BAL study

Identifier	Gender	Age	Smoking Status
B014	Female	53	Never Smoker
B015	Male	54	Ex-Smoker
B017	Female	60	Never Smoker
B018	Female	60	Ex-Smoker

B019	Male	70	Ex-Smoker
B020	Male	67	Ex-Smoker
B021	Female	32	Smoker
B022	Male	64	Smoker
B023	Female	63	Ex-Smoker
B024	Female	75	Never Smoker
B025	Female	56	Never Smoker
B028	Female	62	Never Smoker
B031	Male	45	Never Smoker
B032	Male	64	Smoker
B033	Female	56	Smoker
B039	Female	80	Never Smoker
B040	Male	44	Never Smoker
B042	Male	78	Ex-Smoker
B043	Female	76	Never Smoker
B046	Female	74	Smoker
B047	Male	69	Ex-Smoker
B050	Male	69	Ex-Smoker
B054	Female	56	Ex-Smoker

Table 4.3.3. Table of characteristics of NSCLC patients enrolled in blood study

Identifier	Gender	Age
BL001	Female	60
BL002	Male	57
BL003	Female	65
BL004	Male	62

BL005	Male	65
BL006	Female	75
BL007	Female	75
BL008	Male	55
BL009	Female	53

Table 4.3.3. Table of characteristics of healthy controls enrolled in blood study

Identifier	Gender	Age
BC001	Male	54
BC002	Female	50
BC003	Male	53
BC004	Female	55
BC005	Female	60
BC006	Female	59
BC007	Female	58
BC008	Female	62
BC009	Male	64
BC010	Male	63
BC011	Male	61
BC012	Female	62
BC013	Female	59

Table: 4.3.5: Summary of characteristics of subjects enrolled in BAL and blood analysis studies

Population	% Female	% Male	Mean age (years)	% Smokers	% Ex Smokers	% Never Smokers

NSCLC patients (BAL)	30	70	62.8	50	50	0
NSCLC patients (blood)	55.56	44.44	63	NA	NA	NA
Non-cancer controls	56.52	43.48	62	21.74	39.13	39.13
Healthy controls	61.54	38.46	58.46	NA	NA	NA

IV.III.II.   Preparation of BAL samples

BAL samples were retrieved at bronchoscopy after informed consent and as approved by the Research Ethics Committee of Sr. James's Hospital, in partnership with Professor Joe Keane and Dr. Seónadh O'Leary of Trinity College Dublin. Samples were plated overnight to remove macrophages by adherence purification. Samples were washed thrice with PBS to remove any PBS and counted. Cells were stained with a panel of antibodies and analysed by flow cytometry (Section III.II.VIII.i.).

IV.III.III.   Preparation of blood samples

Blood samples were layered over Lymphoprep and centrifuged at 400 x g for 25 min without acceleration or breaks. The buffy coat layer containing PBMCs was removed and washed with PBS twice. PBMCs were counted and then stained with a panel of antibodies (Table 4.3.5) and analysed by flow cytometry (Section III.II.VIII.ii.).

IV.III.IV.   Antibody staining and flow cytometry

Samples were split into two tubes and cells were stained with a panel of antibodies (Table 4.3.5) in PBA in the dark for 15 min at room temperature. Cells were then washed with PBA and then re-suspended in PBS (0.5 ml). Cells were analysed immediately using a flow cytometer. Unstained cells were used to adjust some parameters in the flow cytometer such as voltages,

while BD Compensation Beads were individually stained with each flurochrome and used as single stains to carry out compensation controls. FMOs were used to establish gates when required. Doublets and dead cells were excluded from the analysis by gating on single cells and live cells respectively (Section III.II.II.iii.).

Table 4.3.5 Panel of antibodies used to phenotype lymphocyte populations in NSCLC patients and control groups

Tube	FITC	PE	PE-Cy7	PerCP	PB	APC	efluor 506
1	Vδ1	6B11	CD4	CD8	CD3	-	Dead cells
2	CD56	Vδ2	CD19	-	CD3	Vδ3	Dead cells

IV.III.V.   Analysis

Cells were examined on a Beckton Dickenson FACSCanto™ II using BD FACSDiva software. Post-acquisition analysis was carried out using FlowJo software v10.0.8. Statistical analysis was performed using Graphpad Prism software v5. A two-tailed or one-tailed t test with Welch's correction was used to analyse differences between the means of two groups. Welch's correction ensures that the test does not assume equal variance. Variance measures the spread of results in a given population. A one-way ANOVA was used to analyse differences between the means of three or more groups. Bonferroni's Test was used to for multiple comparison analysis. The data was considered significant if $p < 0.05$.

IV.IV. Results

IV.IV.I. T cell frequencies are similar in BAL samples from NSCLC patients and non-cancer patients

BAL samples were taken from NSCLC patients and non-cancer controls as described in section III.II.VIII.I. Following overnight adherence purification, cells were washed with PBS thrice, stained with a panel of antibodies (Table 4.3.5) and analysed by flow cytometry (Figure 4.1). After gating on lymphocytes (Figure 4.1A) cells were assessed for presence of CD3 (Figure 4.1B and C). Doublets and dead cells were excluded from the analysis. A t test was used with Welch's correction to assess whether there was a significant variation between the means. There was no significant variation between percentages of T cells in NSCLC patients compared with non-cancer controls (Figure 4.1D).

IV.IV.II. CD4$^+$, CD8$^+$ and CD4$^-$ CD8$^-$ T cell frequencies are similar in BAL samples from NSCLC patients and non-cancer patients

T cells were further examined on the basis of CD4 and CD8 positivity to assess whether there were any variations in populations between NSCLC patients and non-cancer controls. BAL cells were stained with mAbs specific for CD3, CD4 and CD8. After gating on CD3$^+$ cells (Figure 4.1). Cells were assessed for expression of CD4 and CD8 (Figure 4.2). Frequencies of these cells are presented as percentages of T cells. There were no differences in frequencies of CD4$^+$, CD8$^+$ or CD4$^-$ CD8$^-$ T cells between NSCLC patients and non-cancer controls. A two-tailed t test with Welch's correction found that there was a significant difference in the variance of CD4$^-$ CD8$^-$ T cells between the two populations (Figure 4.4F).
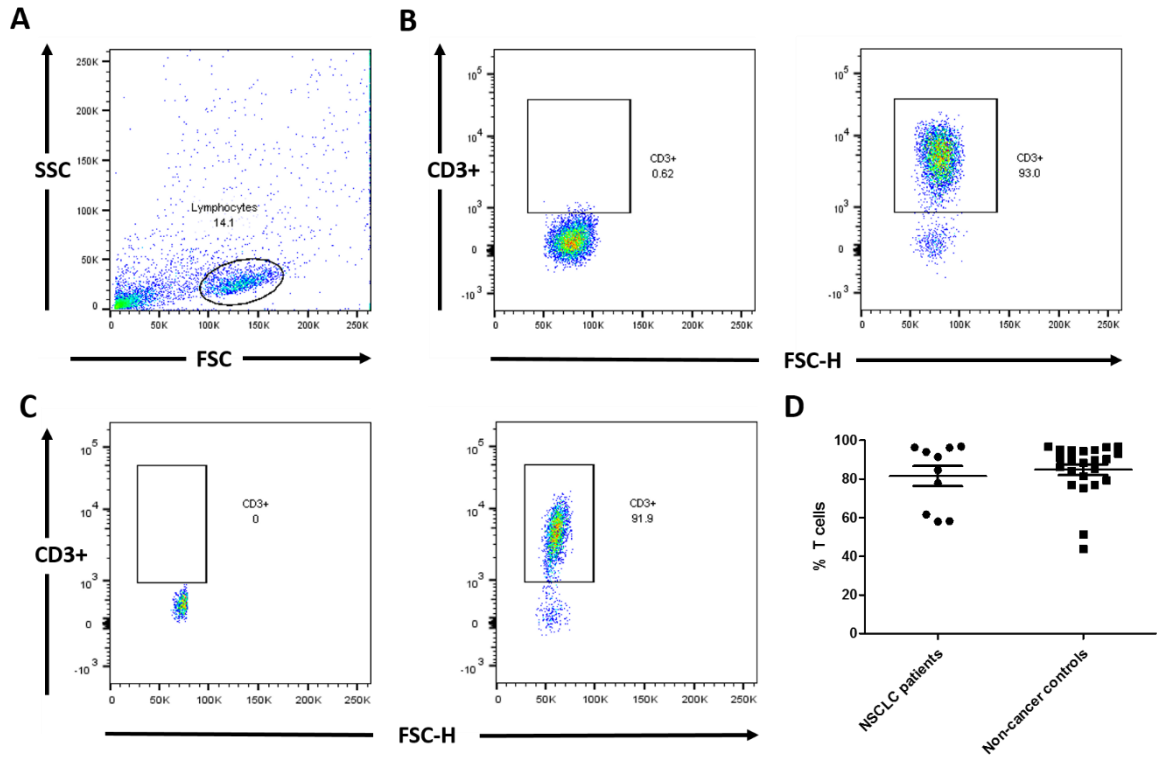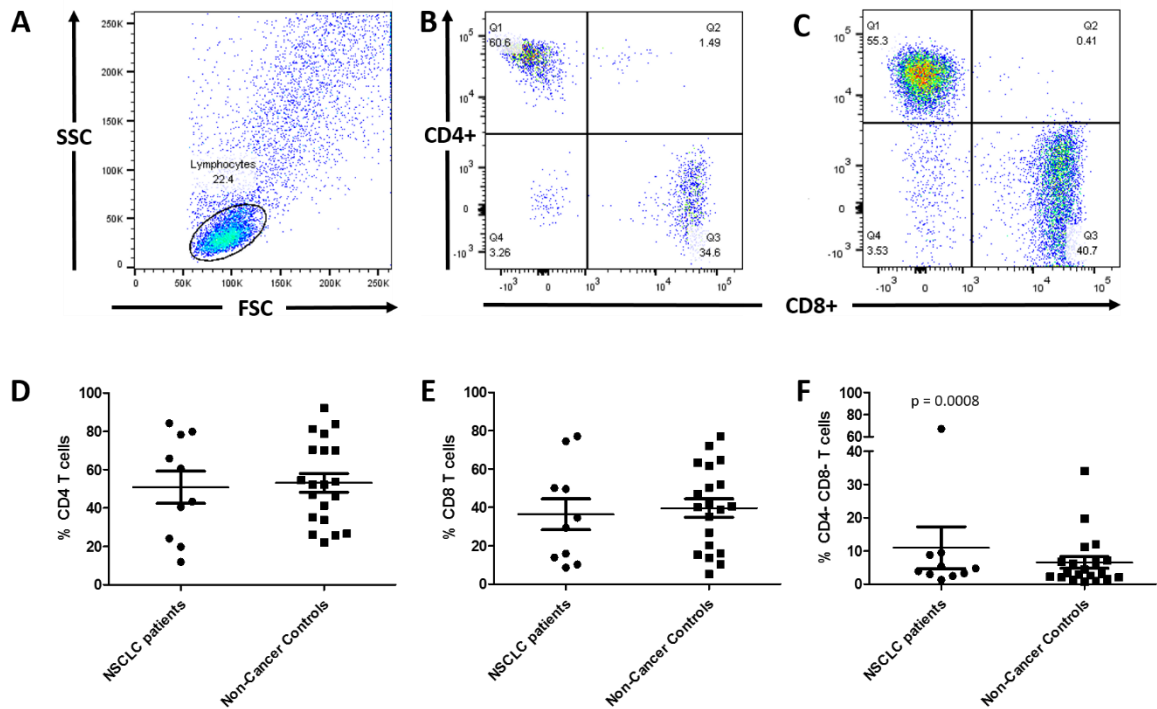
Figure 4.1: Flow cytometric analysis of CD3 expression by BAL cells from patients with NSCLC and non-cancer control subjects. BAL samples were obtained from 10 NSCLC patients and 23 non-cancer control subjects. Following removal of macrophages by adherence purification, cells were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. **A**) Flow cytometric dot plot showing FSC and SSC properties of BAL cells showing gated lymphocytes. **B)** and **C)** Flow cytometric dot plots showing CD3 expression by gated BAL lymphocytes from a patient with NSCLC (B) and a non-cancer control patient (C). Left panels shows unstained cells and right panels show anti-CD3 mAb-stained cells. **D)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and control subjects that expressed CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction.

Figure 4.2: Flow cytometric analysis of CD4⁺ and CD8⁺ expression by BAL cells from patients with NSCLC and non-cancer control subjects. BAL samples were obtained from 10 NSCLC patients and 20 non-cancer controls. Following removal of macrophages by adherence purification, cells were stained with a panel of antibodies (Table 4.3.5.) and analysed using flow cytometry. **A)** Flow cytometric dot plot showing FSC and SSC properties of BAL cells showing gated lymphocyte. **B)** and **C)** Flow cytometry dot plots showing CD4 and CD8 expression by T cells in a BAL sample from a NSCLC patient (B) and a non-cancer control subject (C). **D) – F)** Mean (± SEM) percentages of gated CD3⁺ cells from BAL samples from NSCLC patients and non-cancer control subjects that expressed CD4 (D), CD8 (E) and neither (F). Statistical analysis was performed using a two-tailed t test with Welch's correction. p value (p = 0.0008) represents significant difference in the variance of CD4⁻CD8⁻ T cell frequencies in NSCLC patients compared with non-cancer controls.

IV.IV.III.   B cell frequencies are similar in BAL samples from NSCLC patients and non-cancer patients

BAL samples from NSCLC patients and non-cancer controls were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. After gating on lymphocytes, doublets and dead cells were excluded. B cell frequencies were determined from the percentages of lymphocytes that expressed CD19 (Figure 4.3). A t test with Welch's correction was performed to assess whether there was a significant difference in mean B cell percentages between cancer patients and non-cancer controls. No significant difference was seen in the means between both groups. However, a statistically significant difference was observed in the variance of B cell frequencies in the two patient populations (Figure 4.3D).

IV.IV.IV.   NK cells frequencies are similar in BAL samples from NSCLC patients and non-cancer patients

BAL samples from NSCLC patients and non-cancer controls were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. After gating on lymphocytes (Figure 4.4A), doublets and dead cells were excluded. NK cell frequencies were determined from the percentages of live lymphocytes that expressed CD56 in the absence of CD3 (Figure 4.4B and C). Unstained controls were used for each sample. A t test with Welch's correction did not find any significant difference between percentages of NK cells in NSCLC patients and non-cancer controls (Figure 4.4D).
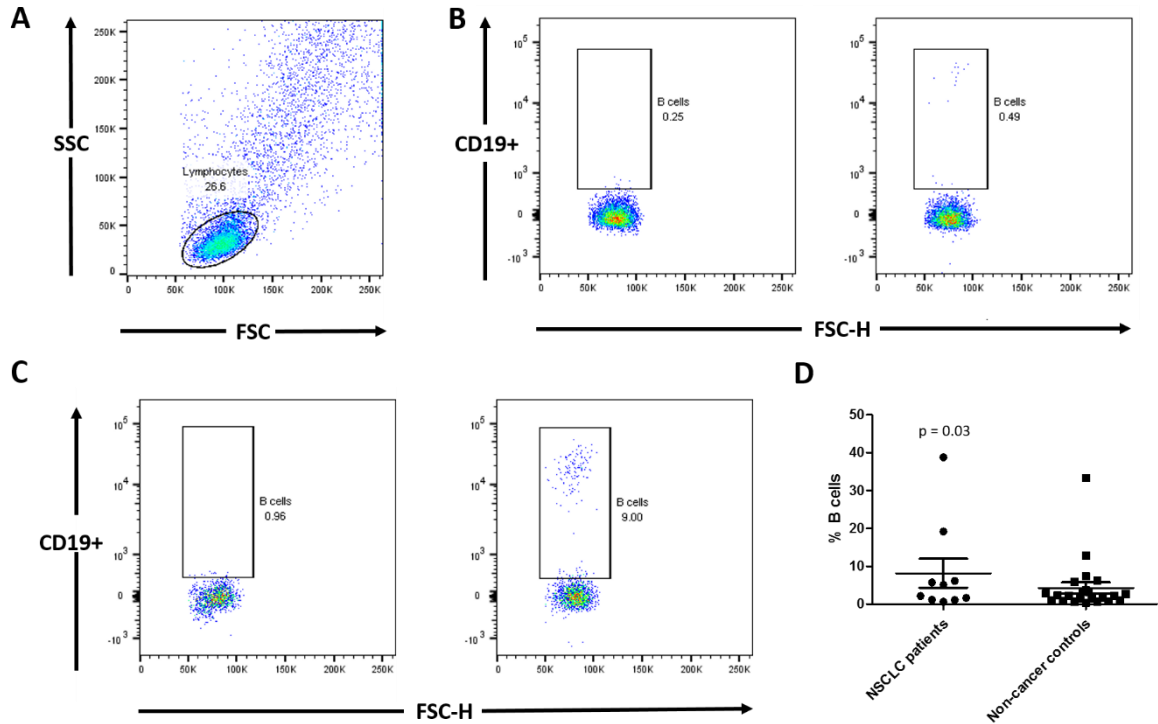
Figure 4.3: Flow cytometric analysis of CD19 expression by BAL cells from patients with NSCLC and non-cancer control subjects. BAL samples were obtained from 10 NSCLC patients and 23 non-cancer control subjects. Following removal of macrophages by adherence purification, cells were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. **A**) Flow cytometric dot plot showing FSC and SSC properties of BAL cells showing gated lymphocytes. **B)** and **C)** Flow cytometric dot plots showing CD19 expression by gated BAL lymphocytes from a patient with NSCLC (B) and a non-cancer control patient (C). Left panels shows unstained cells and right panels show anti-CD19 mAb-stained cells. **D)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and control subjects that expressed CD19. Statistical analysis was performed using a two-tailed t test with Welch's correction. p value compares the variance of B cell frequencies in NSCLC patients compared with non-cancer controls.
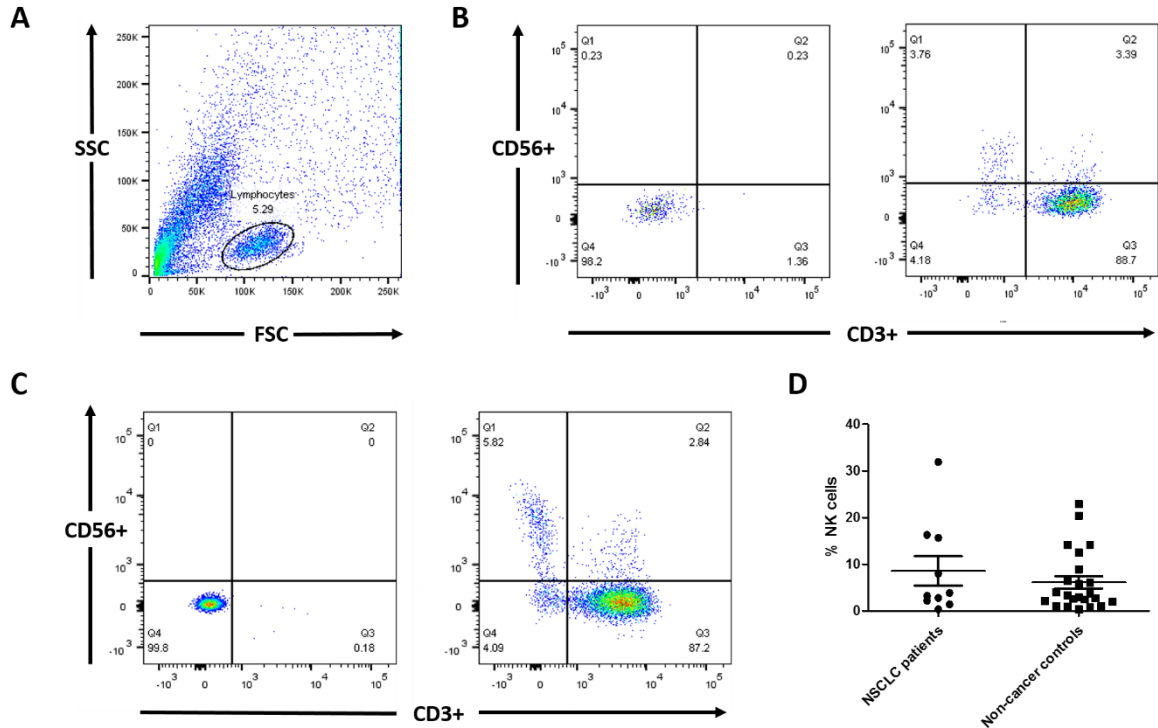
Figure 4.4: Flow cytometric analysis of CD56 expression by BAL cells from patients with NSCLC and non-cancer control subjects. BAL samples were obtained from 10 NSCLC patients and 23 non-cancer control subjects. Following removal of macrophages by adherence purification, cells were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. **A**) Flow cytometric dot plot showing FSC and SSC properties of BAL cells showing gated lymphocytes. **B**) and **C**) Flow cytometric dot plots showing CD56 expression by gated BAL lymphocytes from a patient with NSCLC (B) and a non-cancer control patient (C). Left panels shows unstained cells and right panels show anti-CD56 and anti-CD3 mAb-stained cells. **D**) Mean (± SEM) percentages of lymphocytes from NSCLC patients and control subjects that expressed CD56. Statistical analysis was performed using a two-tailed t test with Welch's correction.

IV.IV.V. Vδ1 T cell frequencies are similar in BAL samples from NSCLC patients and non-cancer patients

BAL samples from NSCLC patients and non-cancer controls were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. After gating on lymphocytes (Figure 4.5A), doublets and dead cells were excluded. Vδ1 T cell frequencies were determined from the percentages of T cells that expressed Vδ1 in the presence of CD3 (Figure 4.5B and C). Unstained controls were used for each sample. A t test with Welch's correction did not find any significant difference between percentages of Vδ1 T cells in NSCLC patients and non-cancer controls (Figure 4.5D).

IV.IV.VI. Vδ2 T cell frequencies are similar in BAL samples from NSCLC patients and non-cancer patients

BAL samples from NSCLC patients and non-cancer controls were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. After gating on lymphocytes (Figure 4.6A), doublets and dead cells were excluded. Vδ2 T cell frequencies were determined from the percentages of lymphocytes that expressed Vδ2 in the presence of CD3 (Figure 4.6B and C). Unstained controls were used for each sample. A t test with Welch's correction did not find any significant difference between percentages of Vδ2 T cells in NSCLC patients and non-cancer controls (Figure 4.6D).

IV.IV.VII. Vδ3 T cell frequencies are similar in BAL samples from NSCLC patients and non-cancer patients

BAL samples from NSCLC patients and non-cancer controls were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. After gating on lymphocytes (Figure 4.7A), doublets and dead cells were excluded. Vδ3 T cell frequencies were determined from the
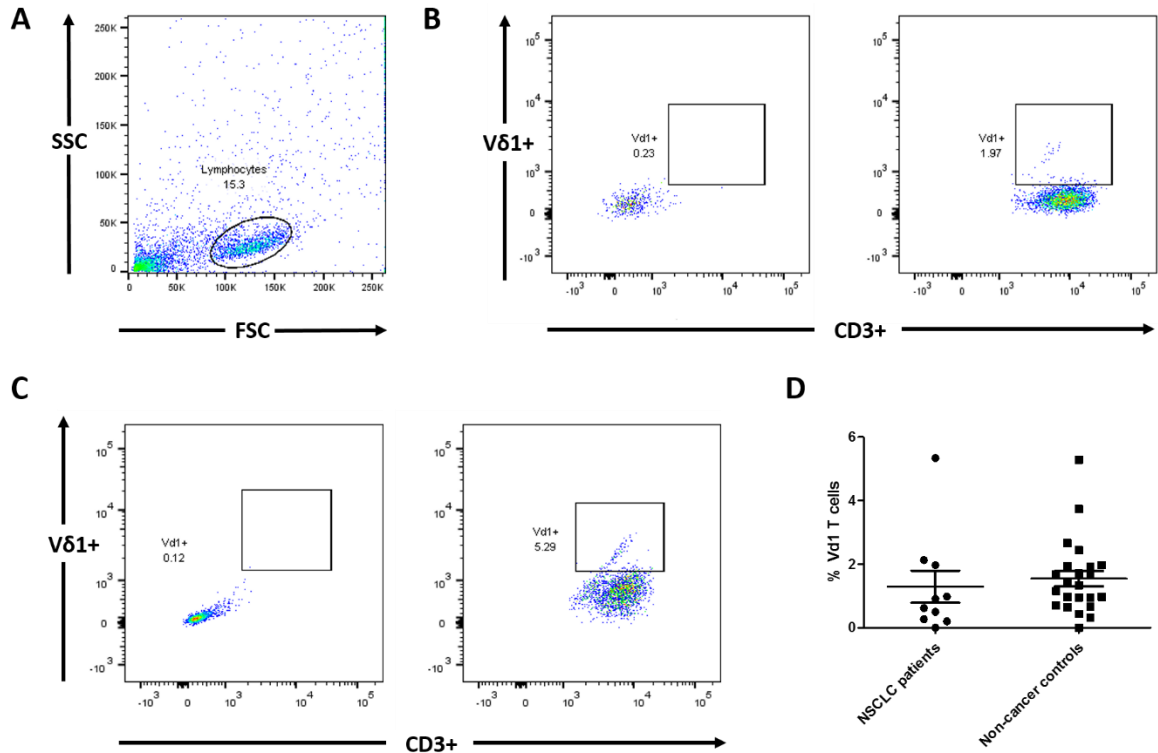
Figure 4.5: Flow cytometric analysis of Vδ1 expression by BAL cells from patients with NSCLC and non-cancer control subjects. BAL samples were obtained from 10 NSCLC patients and 23 non-cancer control subjects. Following removal of macrophages by adherence purification, cells were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. **A)** Flow cytometric dot plot showing FSC and SSC properties of BAL cells showing gated lymphocytes. **B)** and **C)** Flow cytometric dot plots showing Vδ1 and CD3 expression by gated BAL lymphocytes from a patient with NSCLC (B) and a non-cancer control patient (C). Left panels shows unstained cells and right panels show anti-Vδ1 and anti-CD3 mAb-stained cells. **D)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and control subjects that expressed Vδ1 and CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction.
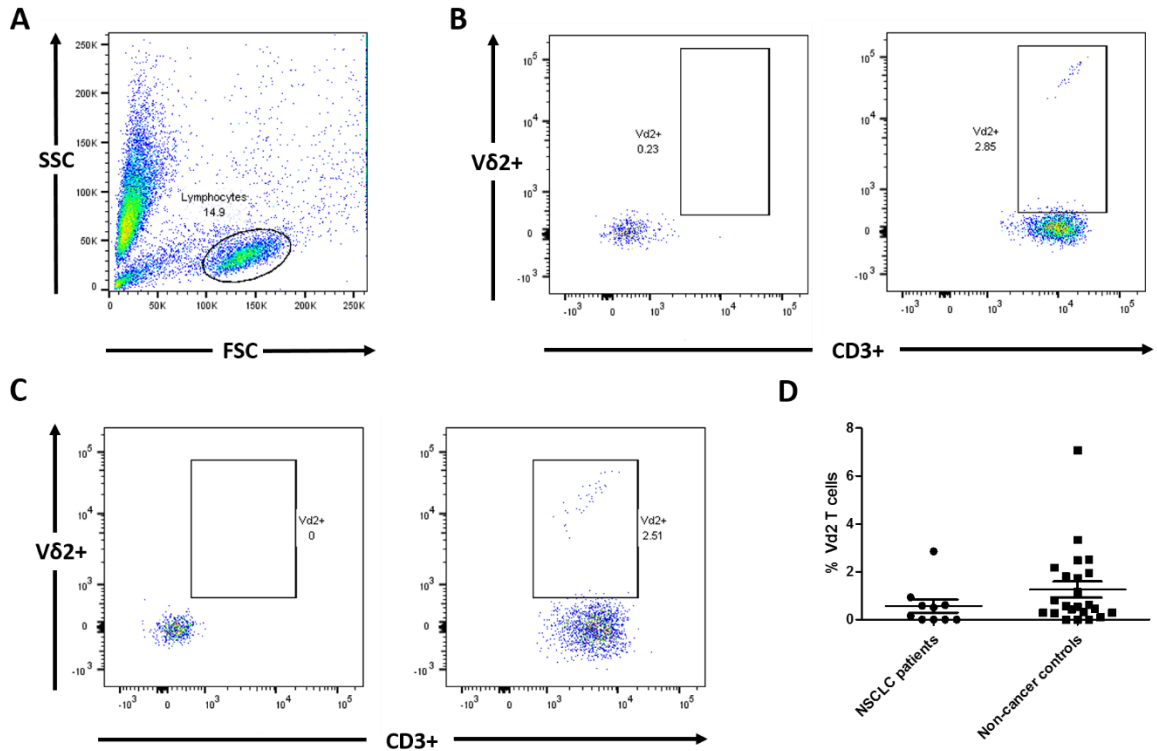
Figure 4.6: Flow cytometric analysis of Vδ2 expression by BAL cells from patients with NSCLC and non-cancer control subjects. BAL samples were obtained from 10 NSCLC patients and 23 non-cancer control subjects. Following removal of macrophages by adherence purification, cells were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. **A**) Flow cytometric dot plot showing FSC and SSC properties of BAL cells showing gated lymphocytes. **B)** and **C)** Flow cytometric dot plots showing Vδ2 and CD3 expression by gated BAL lymphocytes from a patient with NSCLC (B) and a non-cancer control patient (C). Left panels shows unstained cells and right panels show anti-Vδ2 and anti-CD3 mAb-stained cells. **D)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and control subjects that expressed Vδ2 and CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction.
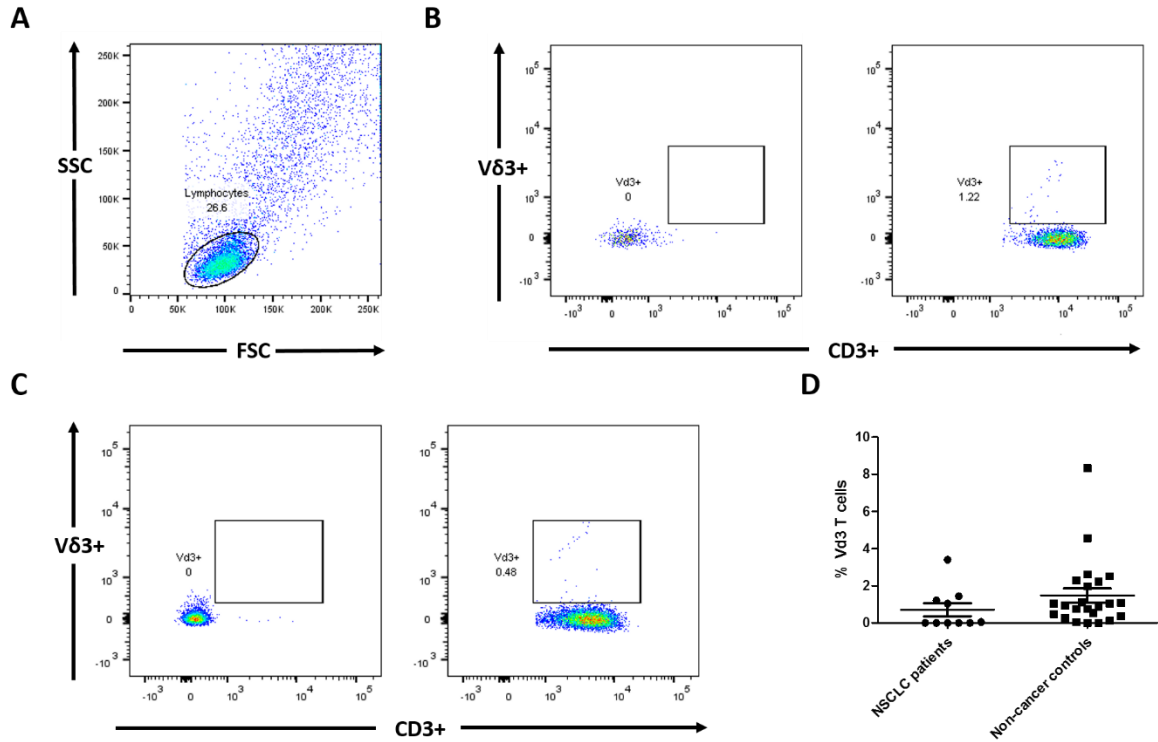
Figure 4.7: Flow cytometric analysis of Vδ3 expression by BAL cells from patients with NSCLC and non-cancer control subjects. BAL samples were obtained from 10 NSCLC patients and 23 non-cancer control subjects. Following removal of macrophages by adherence purification, cells were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. **A**) Flow cytometric dot plot showing FSC and SSC properties of BAL cells showing gated lymphocytes. **B)** and **C)** Flow cytometric dot plots showing Vδ3 and CD3 expression by gated BAL lymphocytes from a patient with NSCLC (B) and a non-cancer control patient (C). Left panels shows unstained cells and right panels show anti-Vδ3 and anti-CD3 mAb-stained cells. **D)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and control subjects that expressed Vδ3 and CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction.

percentages of lymphocytes that expressed Vδ3 in the presence of CD3 (Figure 4.7B and C). Unstained controls were used for each sample. A t test with Welch's correction did not find any significant difference between percentages of Vδ3 T cells in NSCLC patients and non-cancer controls (Figure 4.7D).

IV.IV.VIII. iNKT cells are depleted in BAL samples from lung cancer patients

The frequency of iNKT cells in BAL samples of NSCLC patients and non-cancer controls were determined after removal of macrophages by adherence purification. Cells were washed with PBS and stained with anti-CD3 and anti-Vα24Jα28 (6B11) mAbs and analysed by flow cytometry. Lymphocyte were gated on (Figure 4.8A) and any doublets or dead cells were excluded from the analysis. The frequency of iNKT cells (T cells that express 6B11 and CD3) was expressed as a percentage of total CD3$^+$ cells (Figure 4.8B and C). Unstained controls were used for each sample. iNKT cells were found to be significantly depleted in NSCLC patients compared to non-cancer controls (p = 0.04) (Figure 4.8B). The range of frequencies of iNKT cells in NSCLC patients was also significantly different to the range found in non-cancer controls (p = 0.0004). These results show that iNKT cell frequencies are significantly lower in lung cancer.

IV.IV.IX. Women have higher frequencies of iNKT cells

To assess whether smoking status, gender or age could affect iNKT cell frequencies in the lung, BAL samples from non-cancer controls were analysed by flow cytometry and sorted accorded to smoking status, gender and age range (Figure 4.9). Smokers were classed as current smokers or ex-smokers who have been off cigarettes for less than 1 year. Ex-smokers were classed as those who were off cigarettes for more than one year. Never Smokers are classed as those who have smoked less than 100 cigarettes in their lifetime. Statistical analysis was performed using a one-way ANOVA with Bonferroni's Multiple Comparison test. Smoking status did not affect iNKT cell frequencies.
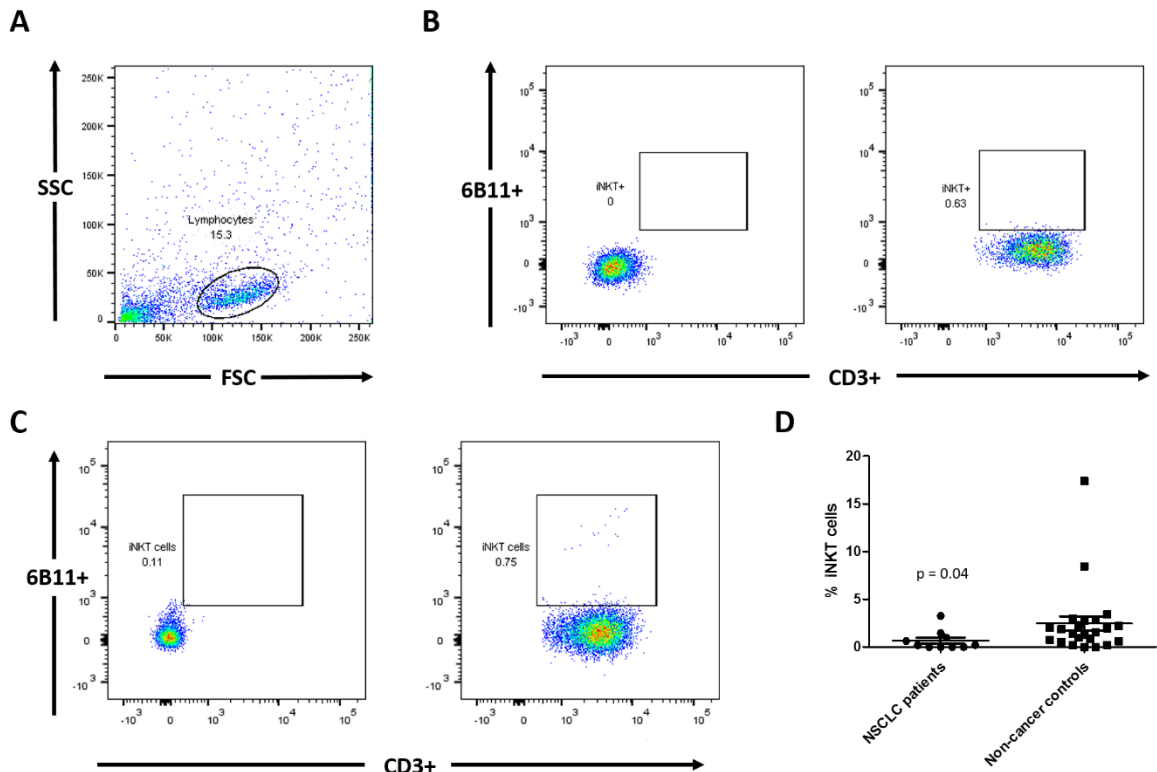
Figure 4.8: Flow cytometric analysis of 6B11 expression by BAL cells from patients with NSCLC and non-cancer control subjects. BAL samples were obtained from 10 NSCLC patients and 23 non-cancer control subjects. Following removal of macrophages by adherence purification, cells were stained with a panel of mAbs (Table 4.3.5) and analysed by flow cytometry. **A**) Flow cytometric dot plot showing FSC and SSC properties of BAL cells showing gated lymphocytes. **B)** and **C)** Flow cytometric dot plots showing 6B11 and CD3 expression by gated BAL lymphocytes from a patient with NSCLC (B) and a non-cancer control patient (C). Left panels shows unstained cells and right panels show anti-6B11 and anti-CD3 mAb-stained cells. **D)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and control subjects that expressed 6B11 and CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction. p value compares the mean iNKT cell frequencies in NSCLC patients and non-cancer controls.
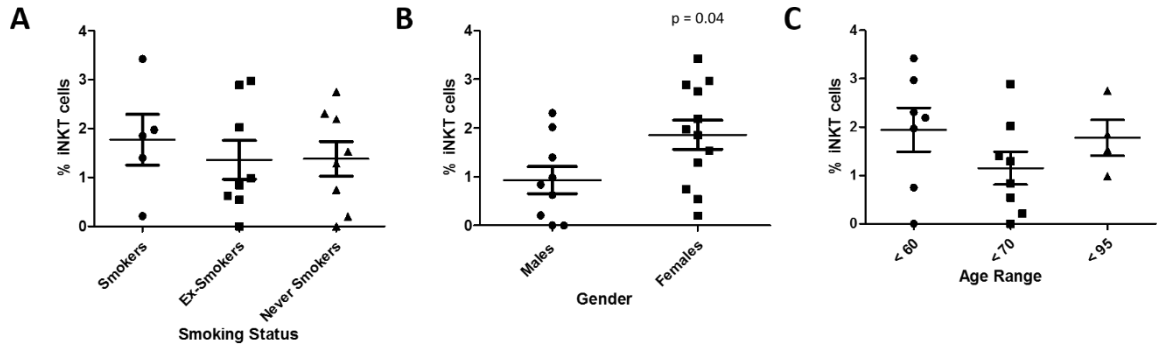
Figure 4.9: Analysis of non-cancer controls for variations in iNKT cell frequencies due to smoking status, gender and age. BAL samples from non-cancer controls were plated overnight to remove macrophages. Following staining with mAbs (Table 4.3.5), cells were analysed by flow cytometry. **A)** iNKT cell frequencies (As % of T cells) in BAL samples sorted according to smoking status. Smokers were defined as current smokers or ex-smokers of less than one year (n = 5), Ex-smokers were defined as previous smokers with more than one year off cigarettes (n = 9). Never smokers were defined as those who have smoked less than 100 cigarettes in their lifetime (n = 9). Statistical analysis was performed using a one-way ANOVA analysis with post hoc Bonferrnoi's multiple comparison test. **B)** iNKT cell frequencies in BAL samples from men (n = 9) and women (n = 13), compared by a t test with Welch's correction. **C)** iNKT cell frequencies in BAL samples from non-cancer patients aged < 60 (n = 7), 60 – 70 (n = 8) and 70 – 95 (n = 4). Statistical analysis was performed using a one-way ANOVA analysis with post hoc Bonferroni's multiple comparison test. p value compares the mean iNKT cell frequencies in men and women.

Non-cancer controls were also sorted by gender. BAL samples from women were found to have increased numbers of iNKT cells compared with men (p = 0.04) (Figure 4.9B). Non-cancer controls were split into three different age ranges, to see whether iNKT cell frequencies decrease with increasing age. Statistical analysis was performed using a one-way ANOVA with Bonferroni's Multiple Comparison test which found that iNKT cells did not decrease with increasing age (Figure 4.9C).

IV.IV.X. Circulating T cell frequencies are decreased in lung cancer patients

To ascertain if the phenotypic changes to lymphocyte populations that occurred in the lungs of patients with NSCLC are reflected in blood, PMBCs from patients with NSCLC and healthy donors were phenotyped. Cells were stained with anti-CD3 mAb and analysed by flow cytometry (Figure 4.10). After gating on lymphocytes, dead cells and doublets were excluded. Cells were examined for their expression of CD3 (Figure 4.10A and B). Absolute numbers were calculated as described in Section III.II.I.ii. Figure 4.10C shows that the frequencies of circulating T cells were significantly increased in NSCLC patients compared to controls (p = 0.03). Figure 4.10D shows the absolute numbers of circulating T cells in blood samples from NSCLC patients and healthy controls. While there was no difference in the absolute numbers between the two cohorts, the range of frequencies of T cells in NSCLC patients was significantly different to the range found in healthy controls (p = 0.0269).
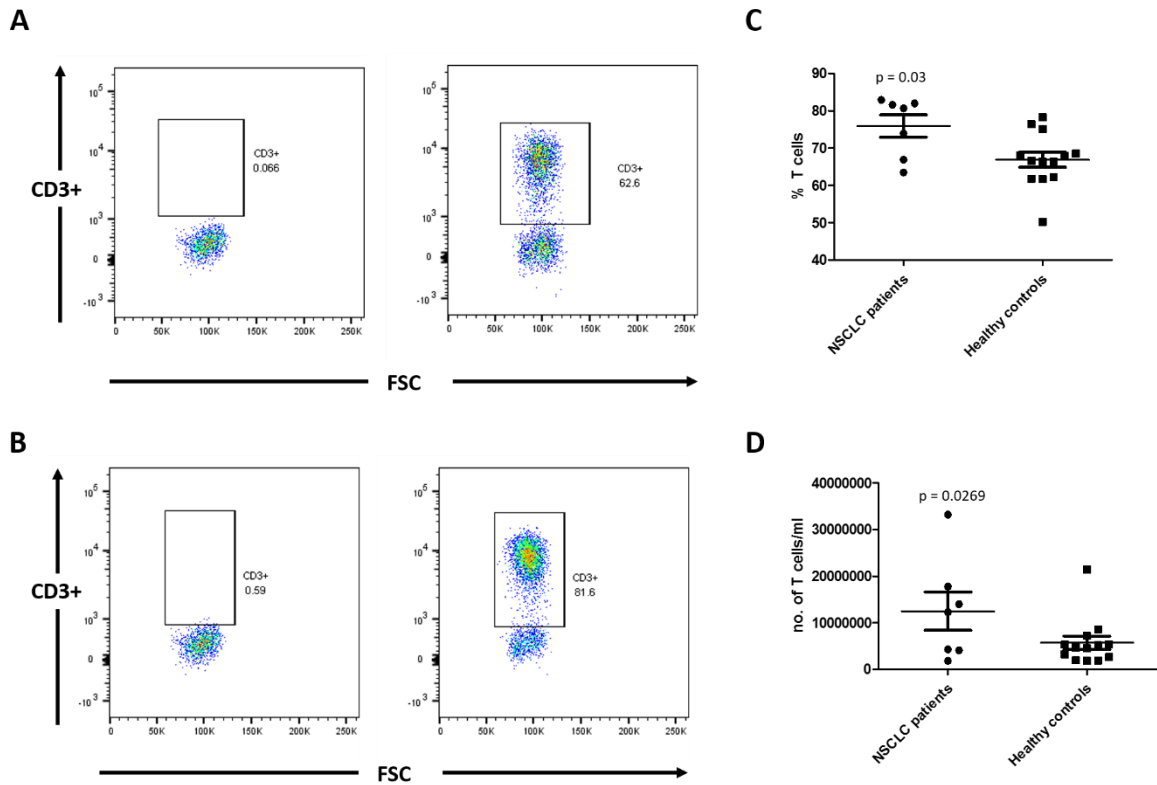
Figure 4.10: Circulating T cell frequencies are higher in patients with NSCLC compared to healthy control subjects. Blood samples were obtained from 7 NSCLC patients and 13 healthy controls. Following PBMC preparation, cells were stained with a panel of mAbs (Table 4.3.5) and analysed using flow cytometry. **A and B)** Flow cytometric dot plots showing CD3 expression by gated blood lymphocytes from a patient with NSCLC **(A)** and a healthy control subject **(B)**. Left panels shows unstained cells and right panels show anti-CD3 mAb-stained cells. **C)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and healthy control subjects that expressed CD3. **D)** Mean (± SEM) absolute numbers of lymphocytes from NSCLC patients and healthy control subjects that expressed CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction. p = 0.03 is comparing mean T cell frequencies in NSCLC patients and healthy controls. p value compares the variance of absolute T cell numbers in NSCLC patients and healthy controls.

IV.IV.XI. CD4$^+$, CD8$^+$ and CD4$^-$CD8$^-$ circulating T cell frequencies are unaltered in lung cancer patients

T cells from blood samples were further analysed to assess potential variations in CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ T cells between lung cancer patients and healthy controls (Figure 4.11). T cells were stained with anti-CD4 and anti-CD8 mAbs and analysed by flow cytometry. Cells are represented as percentages of T cells that expressed CD4, CD8 or neither and as absolute numbers of CD4$^+$, CD8$^+$ and double negative T cells per ml of blood. Statistical analysis was performed using a t test with Welch's correction, and no statistically significant variation was found (Figure 4.11B).

VI.IV.XII. Circulating B cell frequencies are similar in lung cancer patients and healthy controls

PMBCs from patients with NSCLC and healthy donors were phenotyped. Blood samples were layered over lymphoprep and the buffy coats were extracted by centrifugation. Cells were stained with anti-CD19 mAb and analysed by flow cytometry (Figure 4.12). After gating on lymphocytes, dead cells and doublets were excluded. Cells were examined for their expression of CD19 (Figure 4.12A and B). Figure 4.12C shows that the frequencies of circulating B cells were similar in NSCLC patients compared to healthy controls. Figure 4.12D shows the absolute numbers of circulating B cells in blood samples are similar in NSCLC patients and healthy controls. The range of frequencies of B cells in NSCLC patients was significantly different to the range found in healthy controls (p = 0.0026).
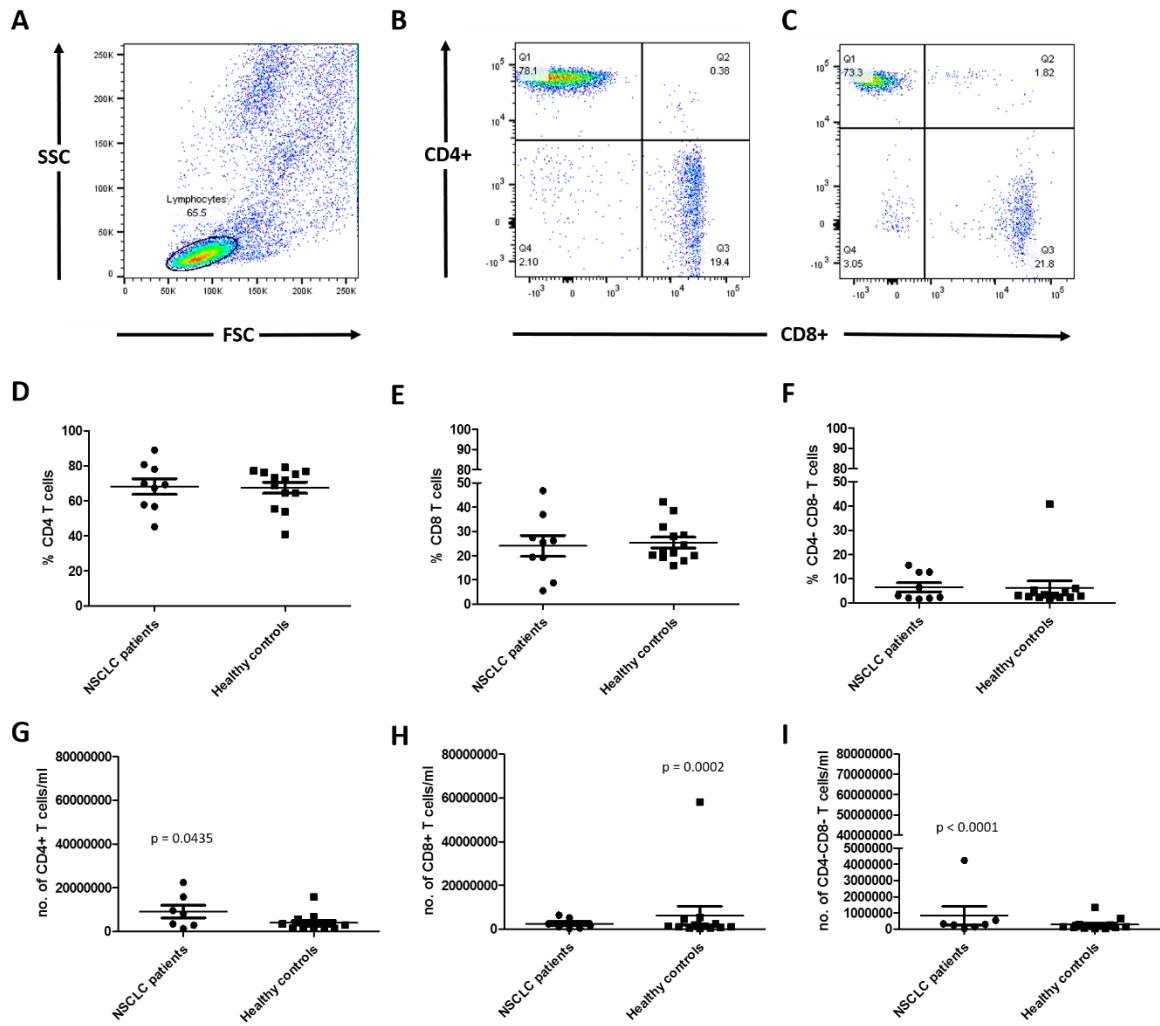
Figure 4.11: Flow cytometric analysis of CD4⁺ and CD8⁺ expression by PMBCs from patients with NSCLC and healthy control subjects. Blood samples were obtained from 9 NSCLC patients and 13 healthy controls. Following PBMC preparation, cells were stained with a panel of mAbs (Table 4.3.5.) and analysed using flow cytometry. **A)** Flow cytometric dot plot showing FSC and SSC properties of PMBCs showing gated lymphocytes. **B)** and **C)** Flow cytometry dot plots showing CD4 and CD8 expression by T cells in a BAL sample from a NSCLC patient **(B)** and a non-cancer control subject **(C)**. **D) – F)** Mean (± SEM) percentages of gated CD3⁺ cells in blood samples from NSCLC patients and non-cancer control subjects that expressed CD4 **(D)**, CD8 **(E)** and neither **(F)**. **G) – I)** Mean (± SEM) total cell counts of gated CD3⁺ cells in blood samples from NSCLC patients and non-cancer control subjects that expressed CD4 **(G)**, CD8 **(H)**, and neither **(I)**. Statistical analysis was performed using a two-tailed t test with Welch's correction. p values compare the variance of numbers of CD4⁺, CD8⁺ and double negative T cell numbers in NSCLC patients and healthy controls.
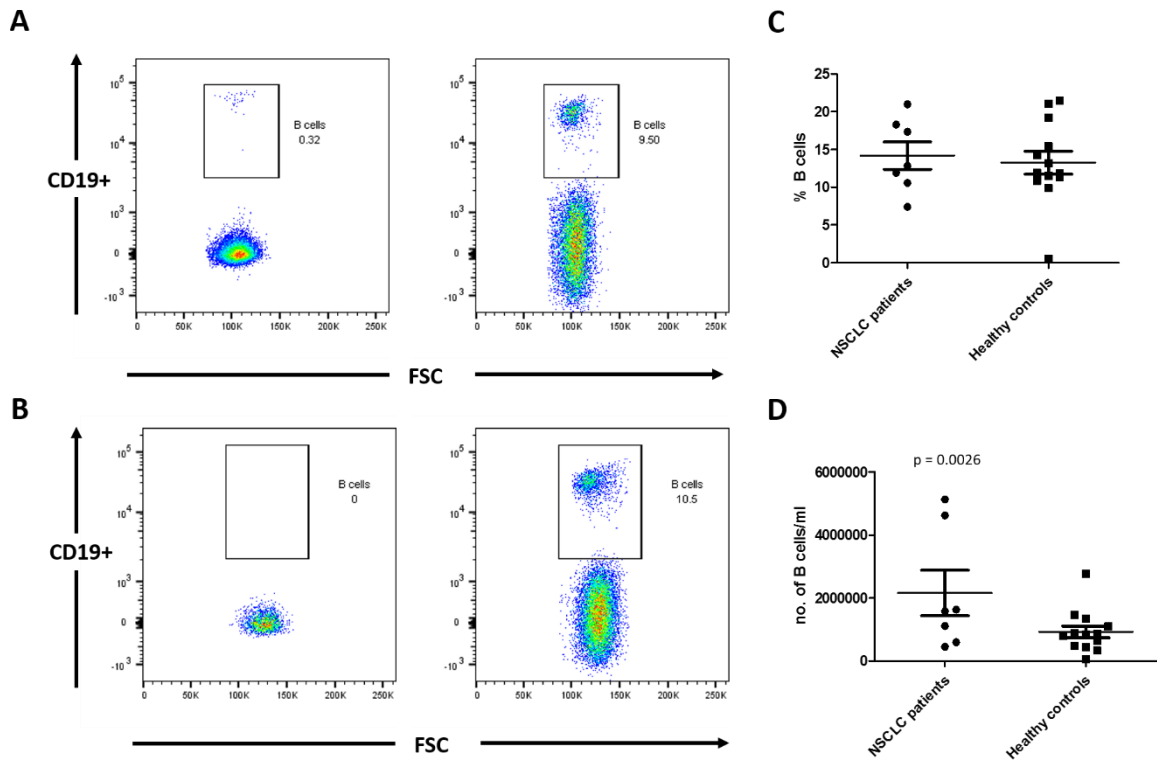
Figure 4.12: Analysis of blood samples from lung cancer patients and healthy controls to assess variations in B cell frequencies. Blood samples were obtained from 7 NSCLC patients and 13 healthy controls. Following PBMC preparation, cells were stained with a panel of mAbs (Table 4.3.5) and analysed using flow cytometry. **A** and **B)** Flow cytometric dot plots showing CD19 expression by gated blood lymphocytes from a patient with NSCLC **(A)** and a healthy control subject **(B)**. Left panels shows unstained cells and right panels show anti-CD19 mAb-stained cells. **C)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and healthy control subjects that expressed CD19. **D)** Mean (± SEM) absolute numbers of lymphocytes from NSCLC patients and healthy control subjects that expressed CD19. Statistical analysis was performed using a two-tailed t test with Welch's correction. p value compares absolute B cell numbers in NSCLC patients and healthy controls.

IV.IV.XIII. Circulating NK cell frequencies are significantly decreased in lung cancer patients

PMBCs from patients with NSCLC and healthy donors were phenotyped to assess any variations in circulating NK cells in NSCLC patients compared to healthy controls. Cells were stained with anti-CD56 and anti-CD3 mAbs and analysed by flow cytometry (Figure 4.13). After gating on lymphocytes, dead cells and doublets were excluded. Cells were examined for their expression of CD56 in the absence of CD3 (Figure 4.13A and B). Figure 4.13C shows that the frequencies of circulating NK cells were significantly decreased in NSCLC patients compared to healthy controls (p = 0.008). Figure 4.12D shows that the absolute numbers of circulating NK were similar in NSCLC patients and healthy controls.

IV.IV.XIV. Vδ1 T cells are depleted in NSCLC patients

PMBCs from patients with NSCLC and healthy donors were phenotyped to assess any variations in circulating Vδ1 T cells in NSCLC patients compared to healthy controls. Cells were stained with anti- Vδ1 and anti-CD3 mAbs and analysed by flow cytometry (Figure 4.14). After gating on lymphocytes, dead cells and doublets were excluded. Cells were examined for their expression of Vδ1 in the presence of CD3 (Figure 4.14A and B). Figure 4.14C shows that the frequencies of circulating Vδ1 T cells were significantly decreased in NSCLC patients compared to healthy controls (p = 0.03). Figure 4.14D shows that the absolute numbers of circulating Vδ1 T cells were similar in NSCLC patients and healthy controls.
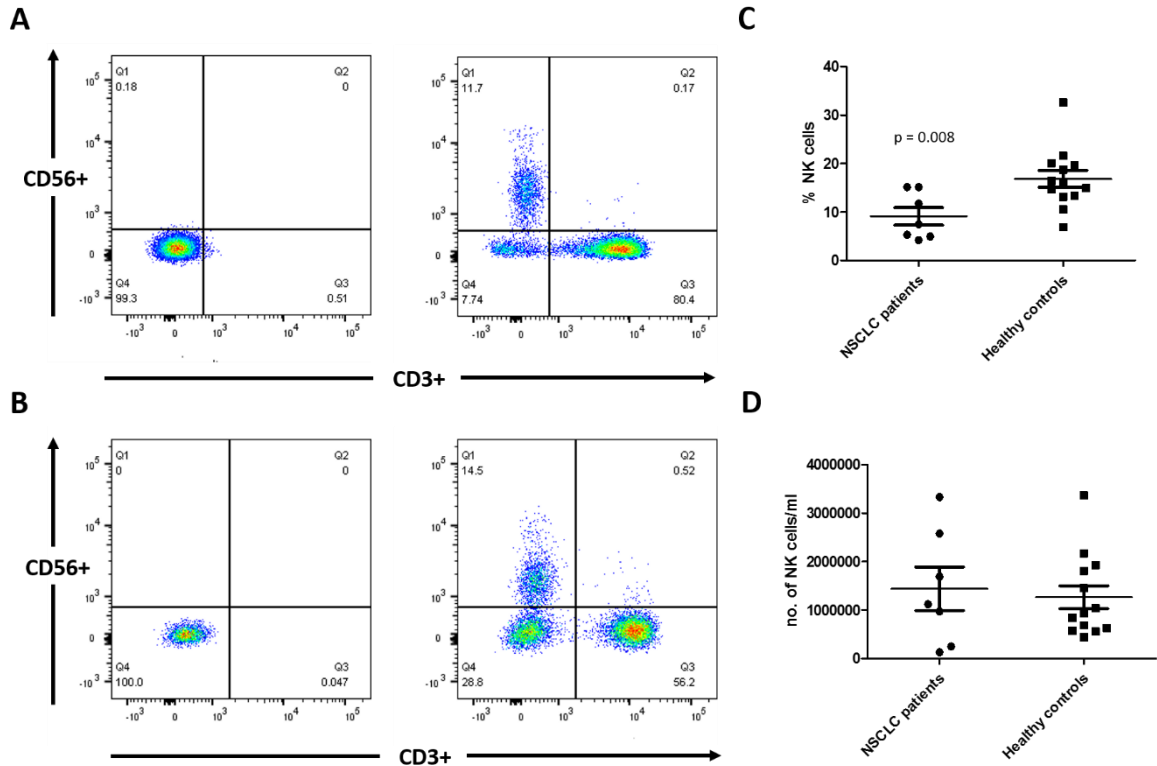
Figure 4.13: Analysis of blood samples from lung cancer patients and healthy controls to assess variations in NK cell frequencies. Blood samples were obtained from 7 NSCLC patients and 13 healthy controls. Following PBMC preparation, cells were stained with a panel of mAbs (Table 4.3.5) and analysed using flow cytometry. **A** and **B)** Flow cytometric dot plots showing CD56 expression by gated blood lymphocytes from a patient with NSCLC **(A)** and a healthy control subject **(B)**. Left panels shows unstained cells and right panels show anti-CD56 mAb-stained cells. **C)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and healthy control subjects that expressed CD56. **D)** Mean (± SEM) absolute numbers of lymphocytes from NSCLC patients and healthy control subjects that expressed CD56. Statistical analysis was performed using a two-tailed t test with Welch's correction. p value compares mean NK cell frequencies in NSCLC patients and healthy controls.
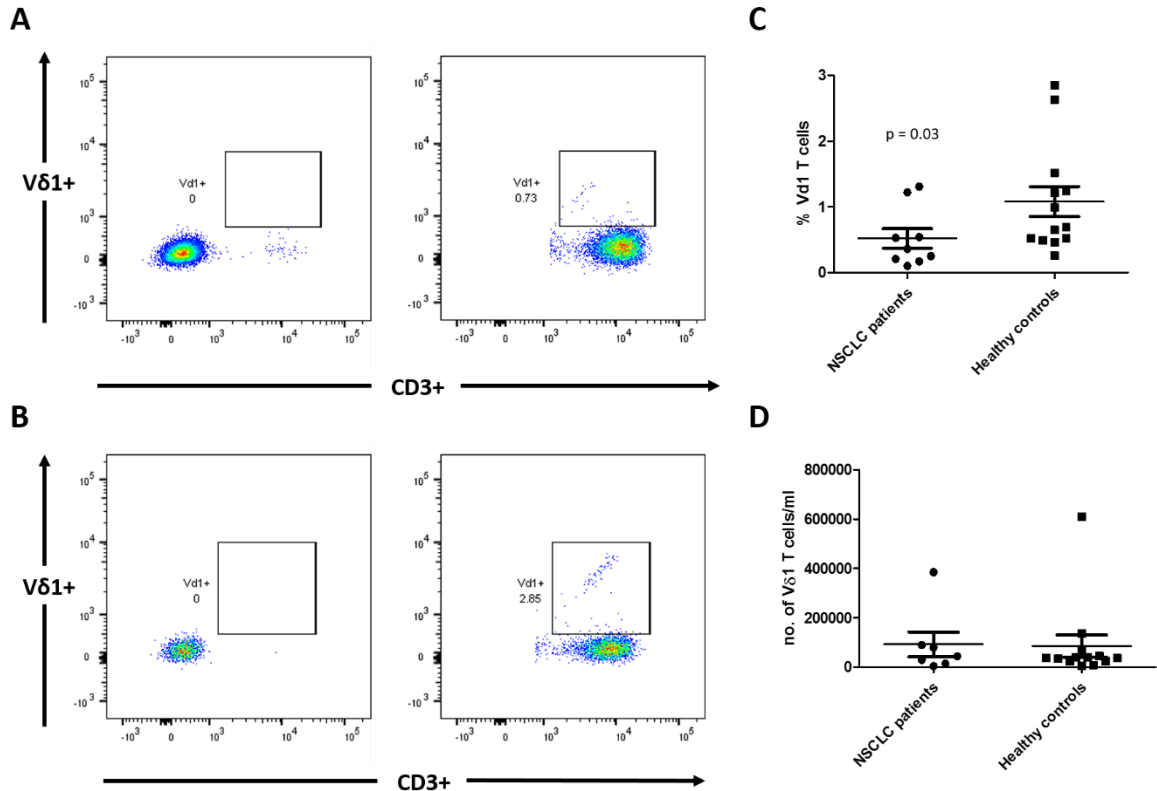
Figure 4.14: Analysis of blood samples from lung cancer patients and healthy controls to assess variations in Vδ1 T cell frequencies. Blood samples were obtained from 9 NSCLC patients and 13 healthy controls. Following PBMC preparation, cells were stained with a panel of mAbs (Table 4.3.5) and analysed using flow cytometry. **A** and **B)** Flow cytometric dot plots showing Vδ1 expression by gated blood lymphocytes from a patient with NSCLC **(A)** and a healthy control subject **(B)**. Left panels shows unstained cells and right panels show anti-Vδ1 and anti-CD3 mAb-stained cells. **C)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and healthy control subjects that expressed Vδ1 and CD3. **D)** Mean (± SEM) absolute numbers of lymphocytes from NSCLC patients and healthy control subjects that expressed Vδ1 and CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction. p value compare mean Vδ1 T cell frequencies in NSCLC patients and healthy controls.

IV.IV.XV.   Circulating Vδ2 T cell frequencies are similar in lung cancer patients and healthy controls

PMBCs from patients with NSCLC and healthy donors were phenotyped to assess any variations in circulating Vδ2 T cells in NSCLC patients compared to healthy controls. Cells were stained with anti- Vδ2 and anti-CD3 mAbs and analysed by flow cytometry (Figure 4.15). After gating on lymphocytes, dead cells and doublets were excluded. Cells were examined for their expression of Vδ2 in the presence of CD3 (Figure 4.15A and B). Figure 4.15C and D shows that the frequencies and absolute numbers of circulating Vδ2 T cells were similar in NSCLC patients and healthy controls. However, the range of frequencies of Vδ2 T cells in NSCLC patients was significantly different to the range found in healthy controls ($p < 0.0001$).

IV.IV.XVI.   Circulating Vδ3 T cell frequencies are similar in lung cancer patients and healthy controls

PMBCs from patients with NSCLC and healthy donors were phenotyped to assess any variations in circulating Vδ3 T cells in NSCLC patients compared to healthy controls. PBMCs were stained with anti- Vδ3 and anti-CD3 mAbs and analysed by flow cytometry (Figure 4.16). After gating on lymphocytes, dead cells and doublets were excluded. Cells were examined for their expression of Vδ3 in the presence of CD3 (Figure 4.16A and B). Figure 4.16C and D shows that the frequencies and absolute numbers of circulating Vδ3 T cells were similar in NSCLC patients and healthy controls. The range of frequencies of Vδ3 T cells in NSCLC patients was significantly different to the range found in healthy controls ($p < 0.0001$).
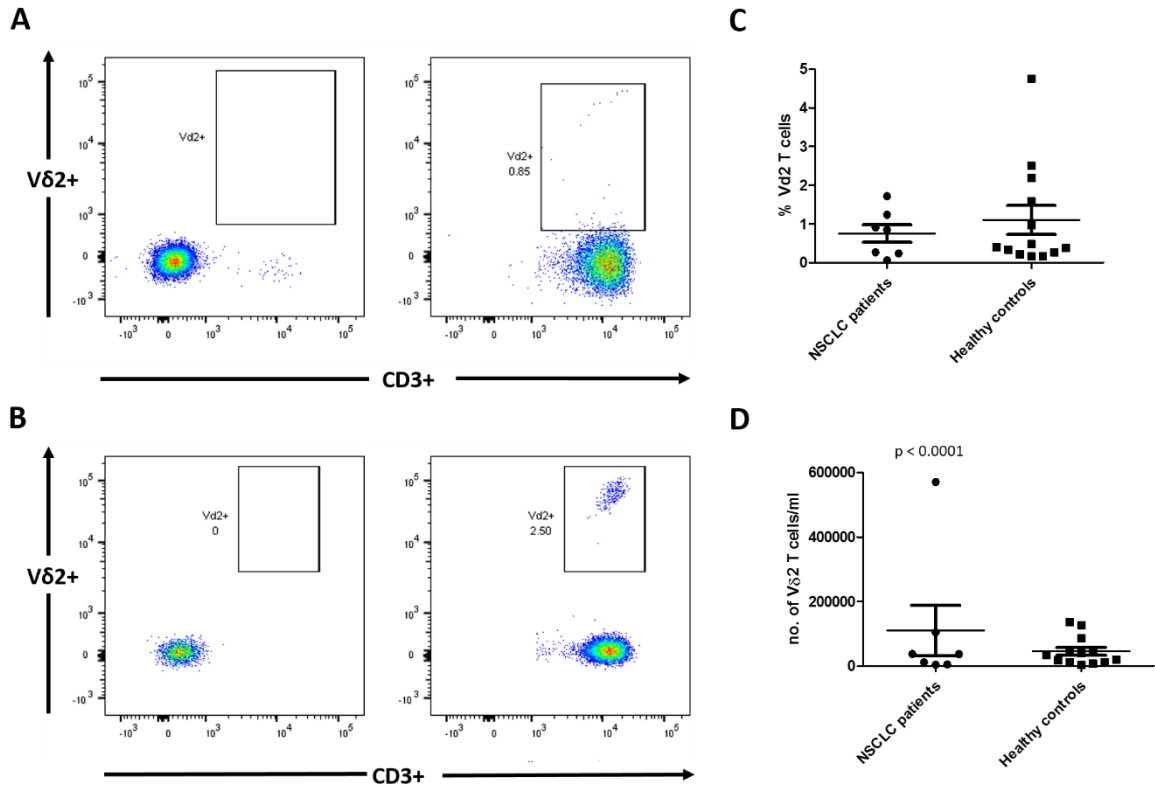
Figure 4.15: Analysis of blood samples from lung cancer patients and healthy controls to assess variations in Vδ2 T cell frequencies. Blood samples were obtained from 7 NSCLC patients and 13 healthy controls. Following PBMC preparation, cells were stained with a panel of mAbs (Table 4.3.5) and analysed using flow cytometry. **A** and **B)** Flow cytometric dot plots showing Vδ2 expression by gated blood lymphocytes from a patient with NSCLC **(A)** and a healthy control subject **(B)**. Left panels shows unstained cells and right panels show anti-Vδ2 and anti-CD3 mAb-stained cells. **C)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and healthy control subjects that expressed Vδ2 and CD3. **D)** Mean (± SEM) absolute numbers of lymphocytes from NSCLC patients and healthy control subjects that expressed Vδ2 and CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction. p value compares the variance in absolute Vδ2 T cell numbers in NSCLC patients compared with healthy controls.
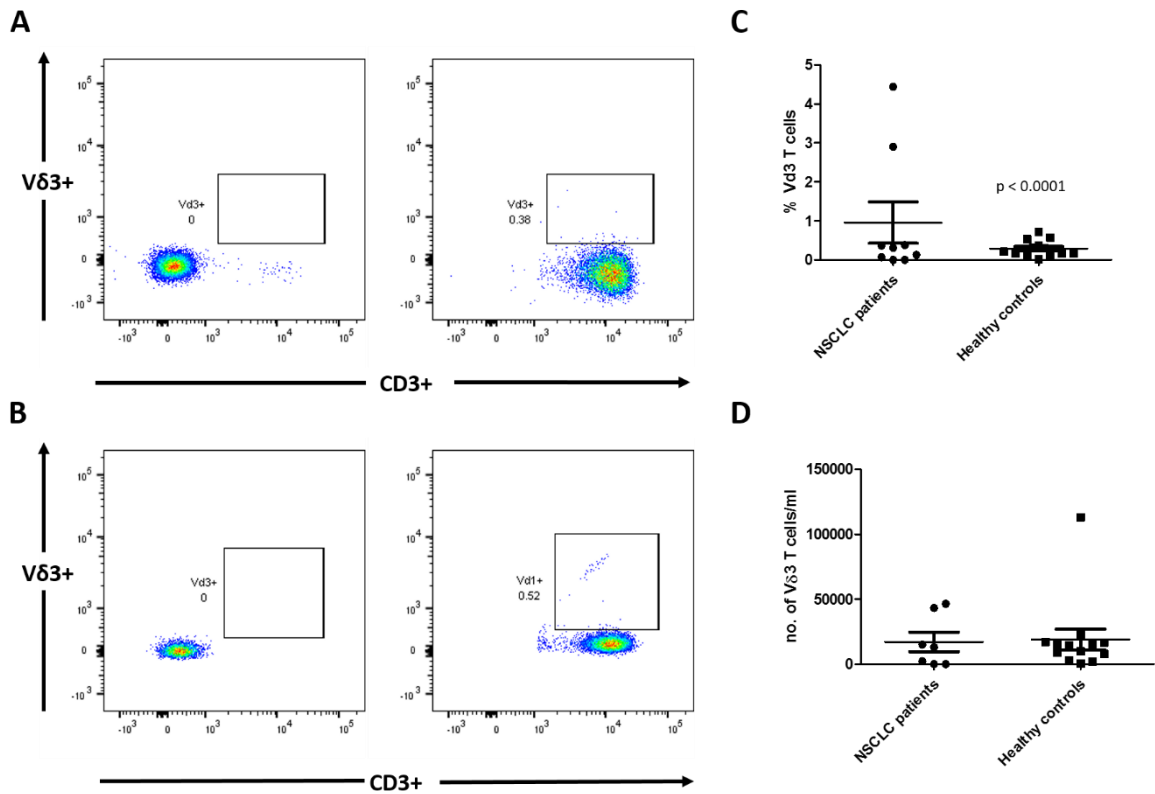
Figure 4.16: Analysis of blood samples from lung cancer patients and healthy controls to assess variations in Vδ3 T cell frequencies. Blood samples were obtained from 9 NSCLC patients and 13 healthy controls. Following PBMC preparation, cells were stained with a panel of mAbs (Table 4.3.5) and analysed using flow cytometry. **A** and **B)** Flow cytometric dot plots showing Vδ3 expression by gated blood lymphocytes from a patient with NSCLC **(A)** and a healthy control subject **(B)**. Left panels shows unstained cells and right panels show anti-Vδ3 and anti-CD3 mAb-stained cells. **C)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and healthy control subjects that expressed Vδ3 and CD3. **D)** Mean (± SEM) absolute numbers of lymphocytes from NSCLC patients and healthy control subjects that expressed Vδ3 and CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction. p value compares the variance of Vδ3 T cells in NSCLC patients and healthy controls.

IV.IV.XVII. Circulating iNKT cell frequencies are decreased in lung cancer patients

To ascertain if the phenotypic changes to iNKT cell frequencies that occurred in the lungs of patients with NSCLC are reflected in blood, PMBCs from patients with NSCLC and healthy donors were phenotyped. Cells were stained with anti-6B11 and anti-CD3 mAb and analysed by flow cytometry (Figure 4.17). After gating on lymphocytes, dead cells and doublets were excluded. Cells were examined for their expression of 6B11 and CD3 (Figure 4.17A and B). Figure 4.17C shows that the frequencies of circulating iNKT cells were significantly decreased in NSCLC patients compared to controls (p = 0.007). Figure 4.17D shows that the absolute numbers of circulating iNKT cells in blood samples from NSCLC patients and healthy controls were similar.

IV.IV.XVIII. Circulating iNKT cells are decreased in female lung cancer patients compared with their healthy counterparts

To investigate whether gender or age could affect iNKT cell frequencies, blood samples were collected from cancer patients and from healthy controls were, analysed by flow cytometry, and sorted accorded to gender and age range (Figure 4.18). Samples were sorted by gender and analysed using a t test with Welch's correction. There were no gender variations between iNKT cell frequencies in cancer patients (Figure 4.18A) and healthy controls (Figure 4.18B). Healthy women were found to have increased numbers of iNKT cells compared with female lung cancer patients (p < 0.05) (Figure 4.18D). Samples were split into two different age ranges, to see whether iNKT cell frequencies decrease with increasing age. Statistical analysis was performed using a t test with Welch's correction which found that iNKT cell frequencies do not decrease with increasing age in cancer patients (Figure 4.18E) or healthy controls (Figure 4.18F).
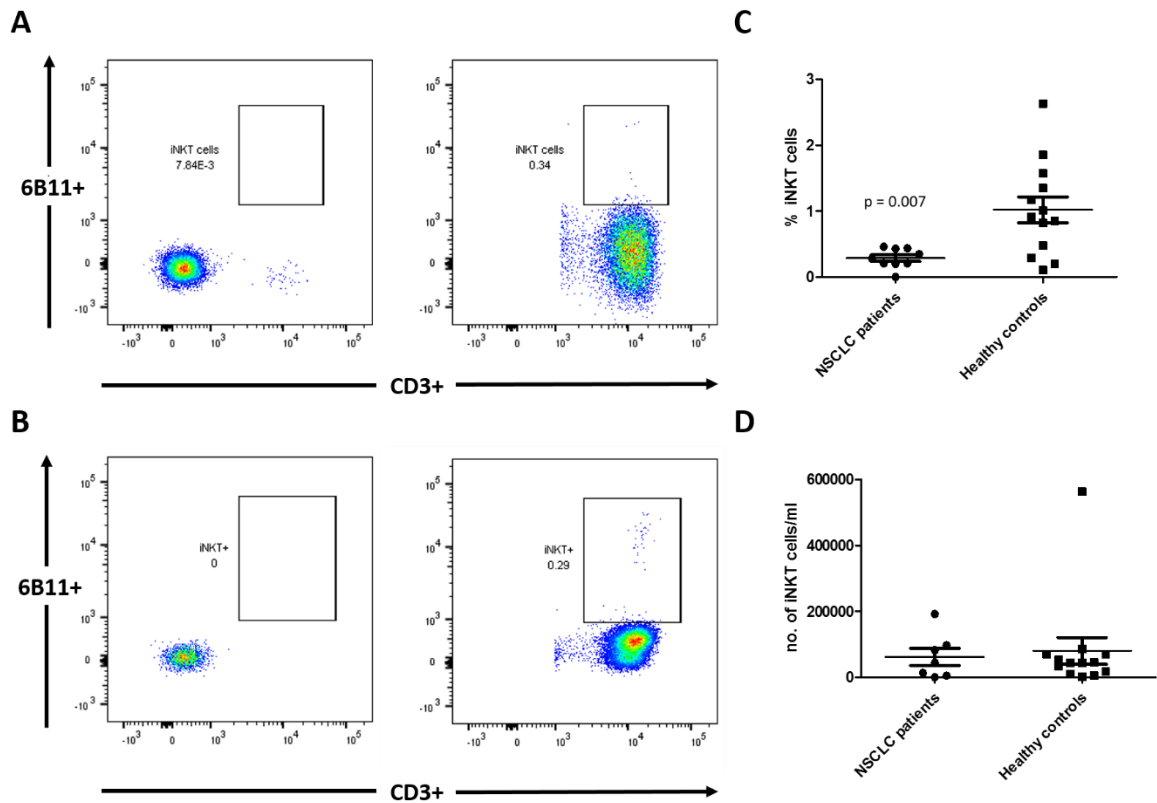
Figure 4.17: Analysis of blood samples from lung cancer patients and healthy controls to assess variations in iNKT cell frequencies. Blood samples were obtained from 9 NSCLC patients and 13 healthy controls. Following PBMC preparation, cells were stained with a panel of mAbs (Table 4.3.5) and analysed using flow cytometry. **A** and **B)** Flow cytometric dot plots showing 6B11 expression by gated blood lymphocytes from a patient with NSCLC **(A)** and a healthy control subject **(B)**. Left panels shows unstained cells and right panels show anti-6B11 and anti-CD3 mAb-stained cells. **C)** Mean (± SEM) percentages of lymphocytes from NSCLC patients and healthy control subjects that expressed 6B11 and CD3. **D)** Mean (± SEM) absolute numbers of lymphocytes from NSCLC patients and healthy control subjects that expressed 6B11 and CD3. Statistical analysis was performed using a two-tailed t test with Welch's correction. p value compares mean iNKT cell frequencies in NSCLC patients and healthy controls.
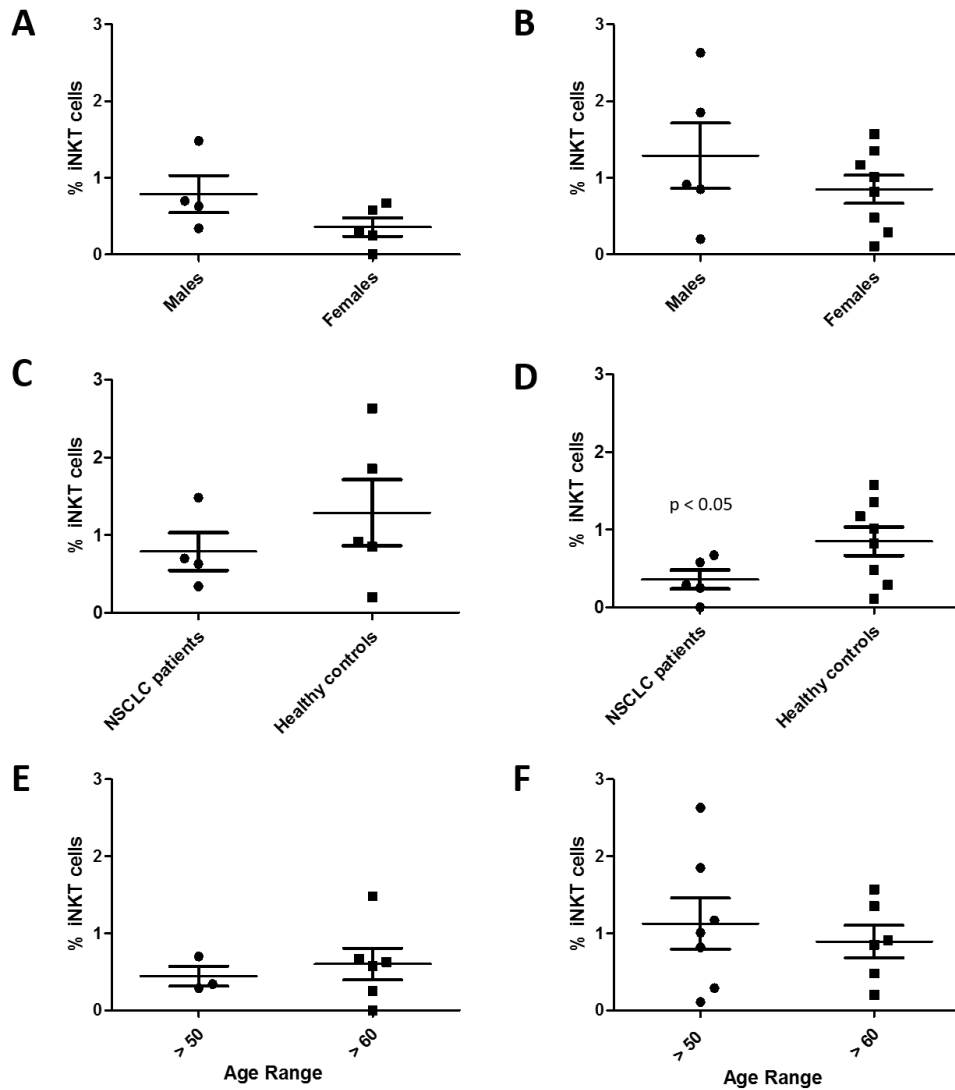
Figure 4.18: Analysis of NSCLC patients and healthy controls for variations in iNKT cell frequencies due to gender, disease status and age. Blood samples were obtained from 9 NSCLC patients and 13 healthy controls. Following PBMC preparation, cells were stained with a panel of mAbs (Table 4.3.5.) and analysed using flow cytometry. **A)** iNKT cell frequencies (As % of T cells) in blood samples from NSCLC patients sorted into males (n = 4) and females (n = 5). **B)** iNKT cell frequencies in blood samples from healthy controls sorted according into males (n = 5) and females (n = 8). **C)** iNKT cell frequencies in blood samples from males sorted into NSCLC patients (n = 4) and healthy controls (n = 5) **D)** iNKT cell frequencies in blood samples from females sorted into NSCLC patients (n = 5) and healthy controls (n = 8). **E)** iNKT cell frequencies in blood samples from NSCLC patients aged < 60 (n = 3) and > 60 (n = 6). **F)** iNKT cell frequencies in blood samples from healthy controls aged < 60 (n = 7) and > 60 (n = 6). Statistical analysis was performed using a two-tailed t test with Welch's correction. p value compares mean iNKT cell frequencies in female NSCLC patients and female healthy controls.

IV.IV.XIX. Frequencies of iNKT cells in lung cancer are comparable using BAL or blood samples

To determine if the depletion of iNKT cells found in BAL samples from NSCLC patients is similar to that found in blood samples from NSCLC patients, iNKT cell frequencies were analysed from both blood and BAL samples from different patients. iNKT frequencies in BAL samples from lung cancer patients were compared with those in blood samples from lung cancer patients (Figure 4.19A). Statistical analysis was performed using a t test with Welch's correction which found that there was no significant variation between the two means. To assess whether similar results could be seen in controls, BAL and blood samples were again compared against one another (Figure 4.19B). No statistically significant difference was observed between the two populations. For both analyses the range of frequencies were significantly different (p = 0.02 for NSCLC patients and p < 0.0001 for healthy and non-cancer controls). These results suggest that blood samples could be used as prognostic biomarkers for changes in iNKT cell frequencies due to lung cancer.
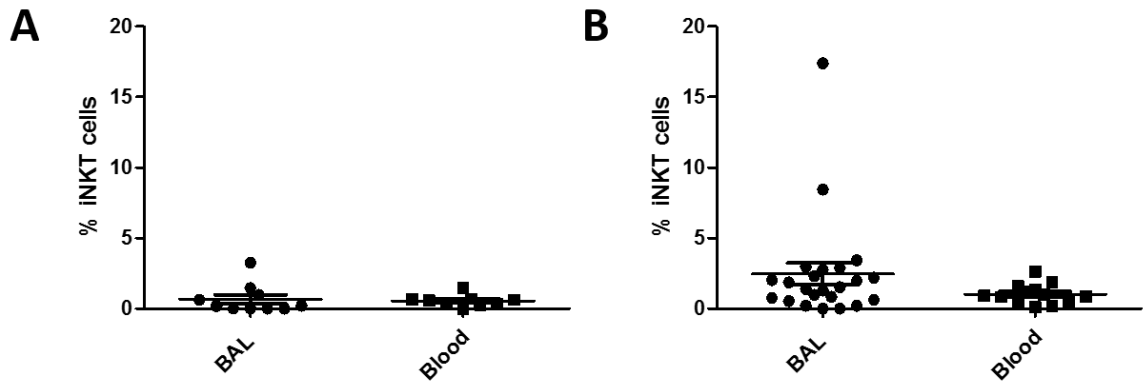
Figure 4.19: Comparison of iNKT cell frequencies from blood and BAL samples. To determine whether these different analyses could give comparable results, iNKT cell frequencies in cancer patients and non-cancer/healthy controls from blood and BAL samples were compared. **A)** Mean (+/-) SEM percentages of iNKT cells in cancer patients. n = 10 for BAL samples and n = 9 for blood samples. **B)** Mean (+/-) SEM percentages of iNKT cells in non-cancer and healthy controls. n = 23 for BAL samples and 13 for blood samples. Statistical analysis was performed using a t test with Welch's correction. iNKT cells are expressed as percentage of T lymphocytes.

IV.V.   Discussion

TILs are known to play critical roles in tumorigenesis and anti-tumour immunity. Lymphocytes can secrete proangiogenic factors, growth factors and survival factors that facilitate tumour growth, angiogenesis and metastasis (Colotta *et al*., 2009, DeNardo *et al*., 2010, Grivennikov *et al*., 2010, Qian and Pollard, 2010). Deficits in particular T cells and NK cells have also been shown to aid tumour progression (Kim *et* al., 2007, Teng *et al*., 2008). By identifying the ratios of immune cell populations within cancer patients, targeted therapies to impede tumorigenesis may be designed by adoptive transfer with anti-tumour cells which could overcome low infiltration of tumours by CTLs which correlates with poor prognosis in many cancers.

The first aim of this study was to obtain lymphocytes from BAL samples from NSCLC patients and non-cancer controls in order to assess whether phenotypic variations existed between the two populations. BAL samples were taken from the non-malignant lung of patients who were coming in for clinically indicated BALs to confirm their cancer status. BAL samples from non-cancer patients were taken from patients who were free from a known (or ensuing) malignancy, sarcoidosis, HIV or Hepatitis C. T cells were examined on the basis of expression of CD3, and their frequencies were unchanged in NSCLC patients and non-cancer controls.

We also obtained blood samples from chemotherapy naïve NSCLC patients and healthy donors. Blood samples from NSCLC patients did not come from the same patients from whom the BAL samples were obtained. Flow cytometric analysis of PBMCs from these cohorts indicated that T cell frequencies were significantly higher in NSCLC patients than in healthy controls. However, analysis of absolute numbers of T cells between these cohorts found that there was no difference in the means of these cohorts but that the spread of values from NSCLC patients were significantly different to those from healthy controls.

These results suggest that T cell frequencies are similar in the lungs of NSCLC patients and non-cancer controls. However, the non-cancer controls were undergoing the BAL collection for a

clinical indication and it is possible that an underlying condition could be affecting their T cell frequencies also. In addition, it is possible that T cell frequencies in the non-malignant lung do not represent those in the malignant lung. Unfortunately BAL samples could not be taken from the malignant lung as these patients had undergone a biopsy immediately prior so that any BAL sample from the malignant lung would have been contaminated by blood. Blood sample analysis from different NSCLC patient and healthy control cohorts indicate that circulating T cells are higher in NSCLC patients than healthy controls. This finding contradicts a study by Wang and colleagues that found that circulating T cells were significantly decreased in NSCLC patients compared with healthy controls (Wang *et al.*, 2013).

The role of $CD4^+$ T cells in tumorigenesis is not fully understood. Retrospective studies on lung carcinomas indicate that high levels of tumour-infiltrating $CD4^+$ T cells are associated with improved clinical outcome(Wakabayashi *et al.*, 2003), whereas similar infiltrations in breast and renal cancers are associated with decreased survival (DeNardo *et al.*, 2008 and Siddiqui *et al.*, 2007). Another study demonstrated that high levels of stromal $CD4^+$ and $CD8^+$ T cells were correlated with improved survival in NSCLC, even in the absence of other clinicopathological prognostic factors such as histology, tumour status, and staging (Al-Shibli *et al.*, 2008). A study by Ruffini and colleagues showed that the presence of $CD8^+$ T cells in surgical specimens from NSCLC patients were associated with improved survival in lung squamous cell carcinoma (Ruffini *et al.*, 2009). We investigated if there was an association between $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ T cell frequencies and NSCLC. The proportions of BAL-derived T cells expressing $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ T cells were unchanged in NSCLC patients compared with non-cancer controls. Similar frequencies of these T cell sub-populations were found in blood samples from lung cancer patients and healthy controls (Figure 4.11). Absolute numbers of these T cell subpopulations were also similar in NSCLC patients and healthy controls, although a slight increase in CD4 T cell numbers was observed in NSCLC patients compared with healthy controls. When the CD4 data was analysed using an unpaired t test, it was found that $CD4^+$ T cells were significantly increased

in blood samples from NSCLC patients compared with healthy controls. Wei and colleagues demonstrated that absolute numbers of circulating $CD4^+$ T_{REG} cells are increased in NSCLC patients compared with healthy controls (Wei et al., 2015). These cells could be contributing to the increase in absolute T cell numbers in the peripheral blood of NSCLC patients that were examined in the present study, T_{REG} cells were not enumerated.

B cells are thought to be a critical constituent of tumour immunity (Tan and Coussens, 2007). Murine studies have shown that mice with B cell deficits are resistant to several types of cancer, suggesting that B cells contribute to cancer progression (Inoue et al., 2006, De Visser et al., 2005 and Shah et al., 2005). Zapata and colleagues demonstrated how mice expressing TNF-receptor associated factor 3 (TRAF3), a recently identified tumour suppressor, in lymphocytes had significantly increased frequencies of squamous cell carcinomas as a result of chronic inflammation (Zapata et al., 2009). This study showed that TRAF3 caused B cells to become hyper-reactive to antigens and TLR agonists, promoting auto-immunity, inflammation and cancer (Zapata et al., 2009). Although murine studies have described a pro-tumoural role to B cells, various studies in humans have shown a positive correlation between B cell numbers and survival (Germain et al., 2014, Al-Shibli et al., 2008 and Pelletier et al., 2001). We investigated whether B cell frequencies and numbers in BAL and blood samples from NSCLC patients and controls. B cell frequencies were not altered in BAL or blood samples from NSCLC patients, although a slight increase in B cell numbers was observed in NSCLC patients compared with healthy controls. If an unpaired t test was used to analyse the data, a significant increase in B cell frequencies in blood samples from NSCLC patients was observed compared with heathy controls. Welch's correction is used in this study because of the difference in sample size and because it cannot be assumed that the variance is equal between the two populations. Therefore the significance from using the unpaired t test could be because of the different sample sizes between the two populations. These results do not support a role for B cells in lung tumorigenesis.

NK cells are capable of recognising and killing cancer cells *in vivo*, and studies have shown that mice with depleted NK cells are more susceptible to tumorigenesis (Kim *et* al., 2007, Teng *et al*., 2008). A prospective cohort study found that people with low NK cell activity had an increased risk of cancer (Imai *et al*., 2000). In addition, NSCLC infiltration by NK cells has been shown to be a positive factor of prognosis (Platonova *et al*., 2011 and Carrega *et al*., 2008). To investigate whether NK cells are altered in our NSCLC patients, BAL samples from lung cancer patients and non-cancer controls were analysed by multi-colour flow cytometry (Figure 4.4). This analysis found that NK cell frequencies in the lung are similar in NSCLC patients and non-cancer controls. In comparison, the frequencies of circulating NK cells were significantly lower in NSCLC patients compared to healthy controls, although absolute numbers of NK cells were similar between the two populations. NK cells in blood may be trafficking and this decrease in NK cell frequencies could be because they have migrated to the lung.

γδT cells are thought to play important roles in anti-tumour immunity. Vδ1 T cells recognise MHC class I-related molecules MICA, MICB and ULPBs that are expressed on many epithelial and haematopoietic tumour cells (Poggi *et al*., 2004, Groh *et al*., 1999 and Groh *et al*., 1998). Many studies have demonstrated the cytotoxic capabilities of *ex-vivo* expanded γδT cell lines and clones from lung, breast, ovary, renal and pancreatic cancers against tumour cells lines and freshly isolated tumour cells (Groh *et al*., 1999, Maeurer *et al*., 1996, Ferrarini *et al*., 1996, and Choudhary *et al*., 1995). We found no difference in Vδ1 T cell frequencies in BAL samples from NSCLC patients and non-cancer controls were observed. However, comparison of blood samples from NSCLC patients and healthy controls showed that Vδ1 T cell frequencies were lower in NSCLC patients, although absolute numbers were similar. It was observed that there was a possible outlier in the NSCLC BAL samples. Excluding this value from the analysis leads to a statistically significant decrease in frequency of Vδ1 T cells in lung cancer patients compared with non-cancer controls.

Vδ2 T cells are thought to directly mediate anti-tumour immunity. A meta-analysis looking at 13 clinical trials found that Vδ2 T cell-based immunotherapy improved overall survival (Buccheri *et al.*, 2014), this in addition to its low toxicity grade make Vδ2 T cells an attractive prospect for novel immunotherapies. BAL samples from NSCLC patients and non-cancer controls were assessed for variations in Vδ2 T cell frequencies. We did not find any significant variation between the two populations. A similar result was found using blood samples from NSCLC patients and healthy controls. These results suggest that Vδ2 T cells are not involved in NSCLC tumorigenicity.

Vδ3 T cells make up the majority of non-Vδ1 and non-Vδ2 T cells in humans. While there is currently no known ligand specificities for these cells, Vδ3 T cells have been shown to be expanded in B cell chronic lymphocytic leukaemia (Bartkowiak *et al.*, 2002) and HIV infection (Kabelitz *et al.*, 1997). A study by Mangan and colleagues demonstrated how expanded Vδ3 T cells can recognise and kill CD1d$^+$ cells *in vitro* (Mangan *et al.*, 2013), making them a candidate for cancer immunotherapy. Analysis of the frequencies of Vδ3 T cells in BAL samples did not discern any variations between NSCLC patients and non-cancer controls. In addition, blood sample analysis did not demonstrate any depletion of Vδ3 T cells in lung cancer patients compared with healthy controls. Interestingly frequencies of Vδ3 T cells in BAL samples were as much as 10 times higher than those found in the peripheral blood. These results suggest that Vδ3 T cells are not involved in lung tumorigenesis.

A clear role for iNKT cells I anti-tumour immunity bas been documented. iNKT cells are among the first responders in many types of neoplastic and infectious malignancies. Many studies have documented their role in immune surveillance in early stage tumours and chemically induced cancers (Taniguchi *et al.*, 2010, Berzofskey *et al.*, 2009, Dhodapkar *et al.*, 2009, Swann *et al.*, 2007, Godfrey and Kronenberg, 2004, and van der Vliet *et al.*, 2004). iNKT immunity against many tumour models is demonstrated following activation by αGalCer presented by a CD1d$^+$

APC (Berzofskey *et al*., 2009, Dhodapkar *et al*., 2009 and Swann *et al*., 2007). Defects in iNKT frequencies have been documented in many cancer types including lung, colon, breast, prostate, head and neck squamous cell carcinoma and melanoma among others (Dhodapkar *et al*., 2009, Molling *et al*., 2007, Crough *et al*., 2004 and Tahir *et al*., 2001). To assess whether iNKT cells are depleted in NSCLC, BAL samples were analysed for variations between NSCLC patients and non-cancer controls (Figure 4.8). A significant reduction in iNKT cell frequency in lung cancer patients was observed; this observation was also seen in the analysis of blood samples from NSCLC patients and healthy controls (Figure 4.17). These results indicate that iNKT cells are significantly depleted in NSCLC. These results suggest that iNKT cells are critical for anti-tumour immunity in NSCLC, and that immunotherapies to target NSCLC will need to target this depletion for successful treatment.

Smoking continues to be a high risk factor for developing lung cancer, with the causal relationship well-established at a $10 - 20$ fold increased risk (Brownson *et al.,* 1998). Hogan and colleagues found that peripheral blood iNKT cells are significantly decreased in smokers compared with controls, and that iNKTs exposed to cigarette smoke showed defects in cytokine production and cytotoxicity (Hogan *et al*., 2011a). We analysed iNKT frequencies in BAL samples from non-cancer controls and sorted the samples according to smoking status. This analysis did not show any variations in iNKT frequencies between smokers, ex-smokers and never smokers. Analysis of BAL samples from the same source have shown that cigarette smoking affects alveolar macrophage function against *Mycobacterium tuberculosis* (O''Leary *et al*., 2014).

Studies have shown that women tend to have higher levels of iNKTs than men (Kee *et al*., 2012 and Exley *et al*., 2011). To assess if gender variations in iNKT cell frequencies exist, BAL and blood samples from control subjects were analysed. BAL samples from non-cancer controls did show a significantly higher level of iNKTs in men than women, however this was not observed in blood samples.

Peralbo and colleagues demonstrated that iNKT cell frequencies are decreased in healthy elderly people (Peralbo *et al.,* 2006). Neither blood nor BAL sample analysis showed a decrease in iNKT cell frequencies with increasing age. A likely explanation is that the age brackets used in this study were too close, whereas the study by Peralbo *et al.* compared the iNKT cell frequencies in 25 – 35 year olds with those in 77 – 88 year olds.

Finally in order to determine whether blood iNKT cell frequencies can be used as a biomarker of BAL iNKT cell frequencies, cancer BAL samples were compared against cancer blood samples. Non-cancer BALs were also compared with blood samples from healthy controls. Statistical analysis did not show any difference between the comparison groups indicating that iNKT cell frequencies in blood mirror iNKT cell frequencies in the lung.

In conclusion, the only phenotypic difference that was observed in both blood and BAL samples was in iNKT cell frequency. iNKT cell frequencies were found to be decreased in the lungs and peripheral blood of NSCLC patients compared with control subjects. These results suggest a role for iNKT cells in anti-NSCLC immunity, and that their depletion aids lung tumorigenesis. These results also suggest a potential for adoptive iNKT cell-based immunotherapy in treating NSCLC. iNKT cells are CD1d-restricted cells so it must first be determined whether NSCLC cells are CD1d-positive, if future iNKT cell-based immunotherapy is to be effective.

V. Induction of CD1d in NSCLC Cell Lines

V.I.   Introduction

NSCLC does not appear to provoke an immune response (Dasanu *et al.*, 2012), however Tsuji and colleagues suggest that the presence of tumour-specific T lymphocytes in some cases of the disease would indicate that it is subject to immune surveillance (Tsuji *et al.*, 2009). There is also some evidence to suggest that the tumour microenvironment develops immunosuppressive mechanisms to aid in the evasion of immunosurveillance (Woo *et al.*, 2002). Immunosuppressive T regulatory cells and cytokines have been described in patients with NSCLC that could have the ability to suppress an efficient host immune response (Meloni *et al.*, 2006).

iNKT cells are critical for an effective anti-tumour response in mice and humans (Metelitsa *et al.*, 2003, Crowe *et al.*, 2002, Cui *et al.*, 1997 and Kawano *et al.*, 1997). iNKT cells recognise CD1d-bearing molecules directly; CD1d molecules present glycolipids to iNKT cells, thereby contributing to the protective nature of these T cells (Vincent *et al,*. 2003). CD1d is expressed in certain tumour types such as prostate cancers and some neurologic tumours, and as such these cells can be targeted for NKT-mediated cell killing. However, most solid tumours in human and mice are CD1d negative. Patients with solid tumours are also found to have lower numbers of circulating iNKT cells. In addition, we have found that iNKT cell frequencies are lower in the blood and BAL of NSCLC patients. Studies by Algarra and Jäger have shown that tumours can develop the ability to downregulate MHC class I molecules (Algarra *et al.*, 2004 and Jäger *et al.*, 2001), thereby evading killing by CTLs.

A study of murine breast cancer cell lines found that highly metastatic cells had a 4.72-fold downregulation of CD1d expression compared with cells that were less prone to metastasis (Hix *et al.*, 2011). Downregulation of CD1d expression is associated with decreased iNKT anti-tumour

immunity in both murine and human cancers (Haraguchi *et* al., 2006, Metelitsa *et* al., 2003 and Kawano *et al.*, 1998). Downregulation of *CD1d* is also associated with increasing malignancy in breast cancer and HPV-transformed cervical carcinoma (Hix *et al.*, 2011 and Miura *et al.*, 2010). CD1d expression was also shown to be decreased in the adipose tissue of patients with colorectal cancer (Lynch *et al.*, 2009). These studies suggest that the downregulation of CD1d is an important mechanism in aiding immune evasion and metastasis.

Aberrant epigenetic marks are associated with tumorigenesis, and are widespread, along with genetic mutations. The potential reversibility of these marks makes them an attractive candidate for novel therapies. Reversal of DNA hypermethylation or histone deacetylation and associated gene silencing has potential to prevent immune evasion and tumour metastatic progression.

DNMT inhibitors work by inhibiting the action of DNMTs and thereby reversing DNA methylation. 5-Aza-2'-Aeoxycytidine (DAC) is a FDA-approved DNMT inhibitor that causes DNA hypomethylation and gene reactivation through chromatin remodelling. It has been shown to be a powerful therapeutic for myelodysplastic syndrome as well as established leukaemias (Cashen *et al.*, 2010, Blum *et al.*, 2007 and Issa *et al.*, 2004). The study by Issa *et al*. demonstrated that DAC could safely be used in the treatment of hematopoietic malignancies with a significantly reduced drug dose (Issa *et al.*, 2004).

HDACs silence gene expression through the removal of acetylation marks. Following the discovery that Trichostatin (TSA) can prevent this action, and in doing so allow the reactivation of the silenced gene through chromatin remodelling, as well as causing cell cycle arrest, HDAC inhibitors have been developed and studied as anti-cancer therapies (Vendetti and Rudin, 2013). Suberanilohydroxamic acid (SAHA) is one of two FDA-approved HDAC inhibitors that was first approved for the treatment of cutaneous T cell lymphoma. Preclinical studies of NSCLC suggest

that HDAC inhibitors can induce apoptosis, and a phase II trial of SAHA in relapsed patients with NSCLC achieved stable disease in 57% of patients (Traynor *et al.*, 2009).

We hypothesize that CD1d expression by NSCLC cells is decreased and may be restored by treatment with epigenetic targeting therapies, and that this could make NSCLC cells susceptible to killing by iNKT cells-mediated cytolytic degranulation.

V.II.   Objectives

The main aims of this chapter are as follows:

1) To assess CD1d expression in NSCLC cell lines

2) To establish NSCLC cell lines that over-express CD1d

3) To generate iNKT cell lines for use in functional and translational studies

4) To determine whether epigenetic targeting therapies can induce CD1d expression in NSCLC

5) To determine whether induction of CD1d expression is due to direct changes at the CD1d promoter region

V.III. Materials and Methods

V.III.I. CD107a degranulation assay

Cytolytic degranulation is a cellular process where apoptosis-inducing molecules are released from secretory vesicles within the cells. Lysosomal-associated membrane protein -1 (LAMP-1 or CD107a) is a marker of degranulation following T or NK cell stimulation (Betts *et al.*, 2003). Under normal conditions CD107a resides in vesicles within cells. When these cells come into contact with target cells CD107a is up-regulated on the cell surface. This up-regulation correlates with both cytokine secretion, and NKT-mediated lysis of target cells (Alter *et al.*, 2004 and Hogan *et al.*; 2011).

NSCLC lines were pulsed with either αGalCer or 7Dw8.5 overnight. iNKT cells were added the following day. Cells were stained with a mAB specific for CD107a (Table 5.3.1) and incubated for 1 hour at 37°C. Monensin was added to the each well and the plate was incubated for 3 hours at 37°C. After a total of 4 hours stimulation time, cells were washed in PBS and stained with a panel of mAbs (Table 5.3.1). Cells were analysed immediately using a flow cytometer.

Table 5.3.1 Panel of antibodies used in CD107a degranulation assay

mAb	CD3	6B11	CD107a	eFluor 506
Flurochrome	Pacific Blue	PE	FITC	Dead cell stain

V.III.IV. Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays are immunoprecipitation techniques that investigate the interaction between protein and DNA. They are used to determine whether specific proteins are associated with specific genomic regions. They are also used to determine the precise region in the genome that various histone modifications are associated with, thereby indicating the target of the histone modifiers. A549 cells were treated with either DMSO, or 250 ng/ml TSA for 24 hours.

Formaldehyde was added to allow the DNA and associated proteins to crosslink. Samples were sonicated thrice to shear the DNA into fragments. Cross-linked DNA fragments associated with the proteins of interest (Table 5.3.2) were selectively immunoprecipitated using protein-specific antibodies (Table 3.23). Primers were specifically designed to study the *CD1d* promoter by searching for the reference sequence of the *CD1d* gene using the Pubmed gene database. Primer sequences were designed to amplify a specific region within the CD1d promoter region. Primer information is listed in Table 5.3.3. PCR was performed on the samples as described in Section III.II.III.iv., and the samples were run out on an agarose gel.

Table 5.3.2: Histone post-translational modifications of interest for ChIP assay

Histone Marks	Abbreviation
Pan-acetylated histone H3	α-H3Ac (pan)
Pan-acetylated histone H4	α-H4Ac (pan)
Histone H3 acetylated at lysine 9	α-H3K9Ac
Histone H3 acetylated at lysine 9 and 14	α-H3K9/K14Ac
Histone H3 monomethylation at lysine 4	α-H3K4 me
Histone H3 dimethylation at lysine 4	α-H3K4 me2
Histone H3 dimethylation at lysine 9	α-H3K9 me2
Histone H3 acetylated at lysine 9 and phosphorylated at serine 10	α-H3K9Ac/pS10
Histone H4 trimethylation at lysine 20	α-H4K20 me3
Histone H3 trimethylation at lysine 27	α-H3K27 me3

Table 5.3.2: Primer information for ChIP sequencing

Primer name	Sequence	Annealing Temp	No. of cycles
CD1d ChIP (bp)	Forward 5'-CCACCTAGAGACATGTACTGC-3' Reverse 5'-CGCTCACTTCAGTAGGTTTC-3'	56°C	40

V.IV.   Results

V.IV.I.   Analysis of NSCLC cell lines for the expression of CD1d

Immunoediting is the process whereby tumour cells alter the expression of MHC class I molecules and their ability to present tumour antigens in an attempt to evade immunosurveillance (Algarra *et al.*, 2004 and Jäger *et al.*, 2001). CD1d is an MHC class I-like molecule that contributes to the anti-tumour activities of iNKT cells through presentation of glycolipid antigens. For iNKT cell-based immunotherapies to be successful in treating NSCLC, it is important to determine whether these cells express CD1d.

A549 and SK-MES-1 cell lines were assessed by flow cytometry for the expression of cell-surface CD1d (Figure 5.1). CD1d expression is expressed on the bi-exponential scale. CD1d expression was undetectable in both cell lines. Hela mock and Hela CD1d cell lines were assessed as negative and positive controls respectively. Lack of CD1d expression in both A549 and SK-MES-1 cell lines could be one cause of the lack of immunogenicity that is ascribed to NSCLC (Thomas and Hassan, 2012).

V.IV.II.   Generation of NSCLC cell lines over-expressing CD1d

To test whether upregulation of CD1d expression in NSCLC can lead to increased cytotoxicity by iNKT cells, transfected NSCLC lines were established. A549 and SK-MES-1 cells were transfected with CD1d using the FUGENE protocol according to the manufacturer's instructions. Transient transfection was verified using RT-PCR (Figure 5.6A and 5.6C). Once it was verified that it was possible to transfect A549 and SK-MES-1 cells with CD1d, a stable or long-term transfection was established in a SK-MES-1 cell line, as verified by flow cytometry (Figure 5.2D). Despite multiple
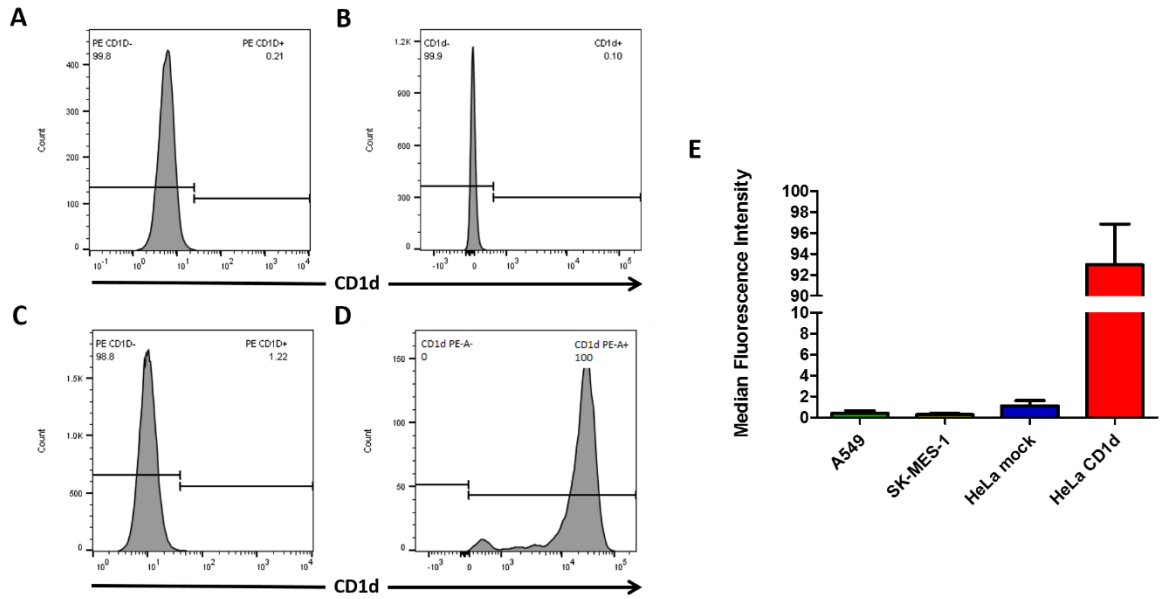
Figure 5.1: Analysis of cancer cell lines for cell-surface expression of CD1d. Representative flow cytometric histograms showing expression by A549 **(A)**, SK-MES-1 **(B)**, mock-transfected HeLa **(C)** and CD1d-transfected HeLa cells **(D)**. **E)** Bar chart showing median fluorescence intensities of CD1d staining by each cell type. Results are means of three different experiments.
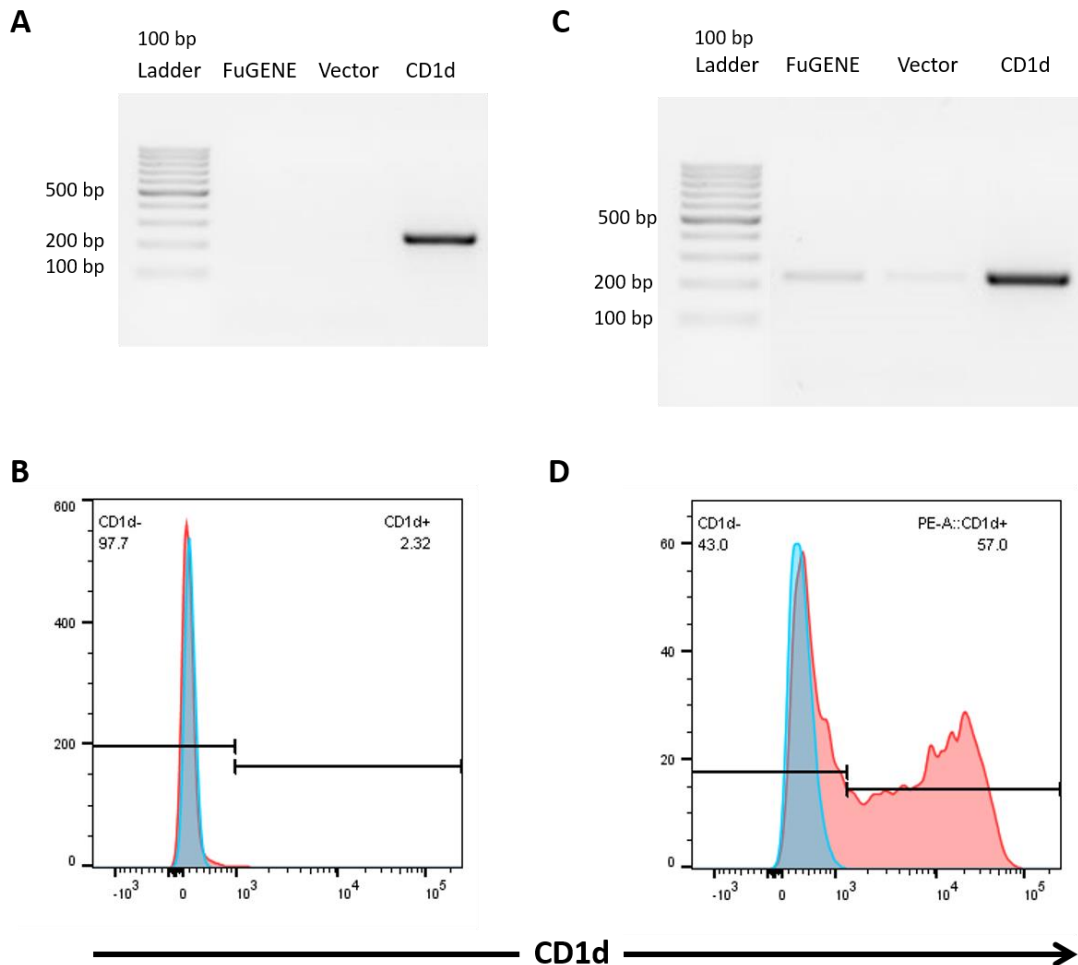
Figure 5.2: NSCLC cell lines were transfected with CD1d using the FuGENE protocol. A549 **(A and B)** and SK-MES-1 **(C and D)** cells were transfected with an expression vector containing either the *CD1d* gene or empty vector using the FuGENE protocol. Cells were solubilised for analysis of CD1d mRNA by RT-PCR **(A and C)**. Cells were labelled with a mAb specific for CD1d (red) or were left unstained as controls (blue), and cell surface CD1d protein expression was examined by flow cytometry **(B and D)**. A549-transfection experiments were repeated 3 times, SK-MES-1-transfection experiments were performed once.

attempts, a stably transfected A549 cell line was not established (Figure 5.2B). Following stable transfection of SK-MES-1, the cells were sorted to ensure a homogeneous population (> 99% purity) (Figure 5.3).

V.IV.III.   Generation of iNKT cell lines

iNKT cell lines were generated for use in co-culture experiments with NSCLC lines. PBMCs were isolated from buffy packs obtained from the IBTS at St. James's Hospital in Dublin. iNKT cells were separated from PBMCs using positive selection with anti-iNKT microbeads as described in section III.II.VI. The unlabelled cells were discarded and the iNKT^{+} cells were collected and sorted using a MoFlo[TM] cell sorter. Sorted cells were cultured in cRPMI media with IL-2 in the presence of irradiated PMBCs. αGalCer was added to stimulate iNKT cell expansion. Following a period of four weeks iNKT cells were stained with mAbs specific for CD3, CD4, CD8 and 6B11 and assessed by flow cytometry to determine purities (Figure 5.4). iNKT cell lines were generated with purities of >99% (Figure 5.4A). Most iNKT cell lines were heterogeneous for CD4 and CD8, however one cell line was mostly CD4^{+} with few CD8^{+} cells (Figure 5.4B).

V.IV.IV.   Up-regulation of CD1d by tumour cells increases their susceptibility to degranulation by iNKT cells

iNKT cells were co-cultured with a CD1d-transfected SK-MES-1 cell line and its parent SK-MES-1 cell line (Figure 5.5). Hela mock and Hela CD1d cell lines were used as negative and positive controls respectively. Cells were pulsed for 24 hours with either αGalCer or 7Dw-8.5, two glycolipids shown to stimulate iNKT cells when bound to CD1d. PMA and ionomycin were used as a positive control of degranulation. After four hours, cells were stained with a panel of mAbs (Table 5.3.1) and were examined by flow cytometry to assess the proportion of iNKT cells expressing CD107a. iNKT cells that were co-cultured with CD1d-positive SK-MES-1 cells that were pulsed with either αGalCer ($p < 0.001$) or 7Dw-8.5 ($p < 0.001$) showed a statistically significant induction of CD107a. In contrast iNKT cells cultured with untransfected SK-MES-1
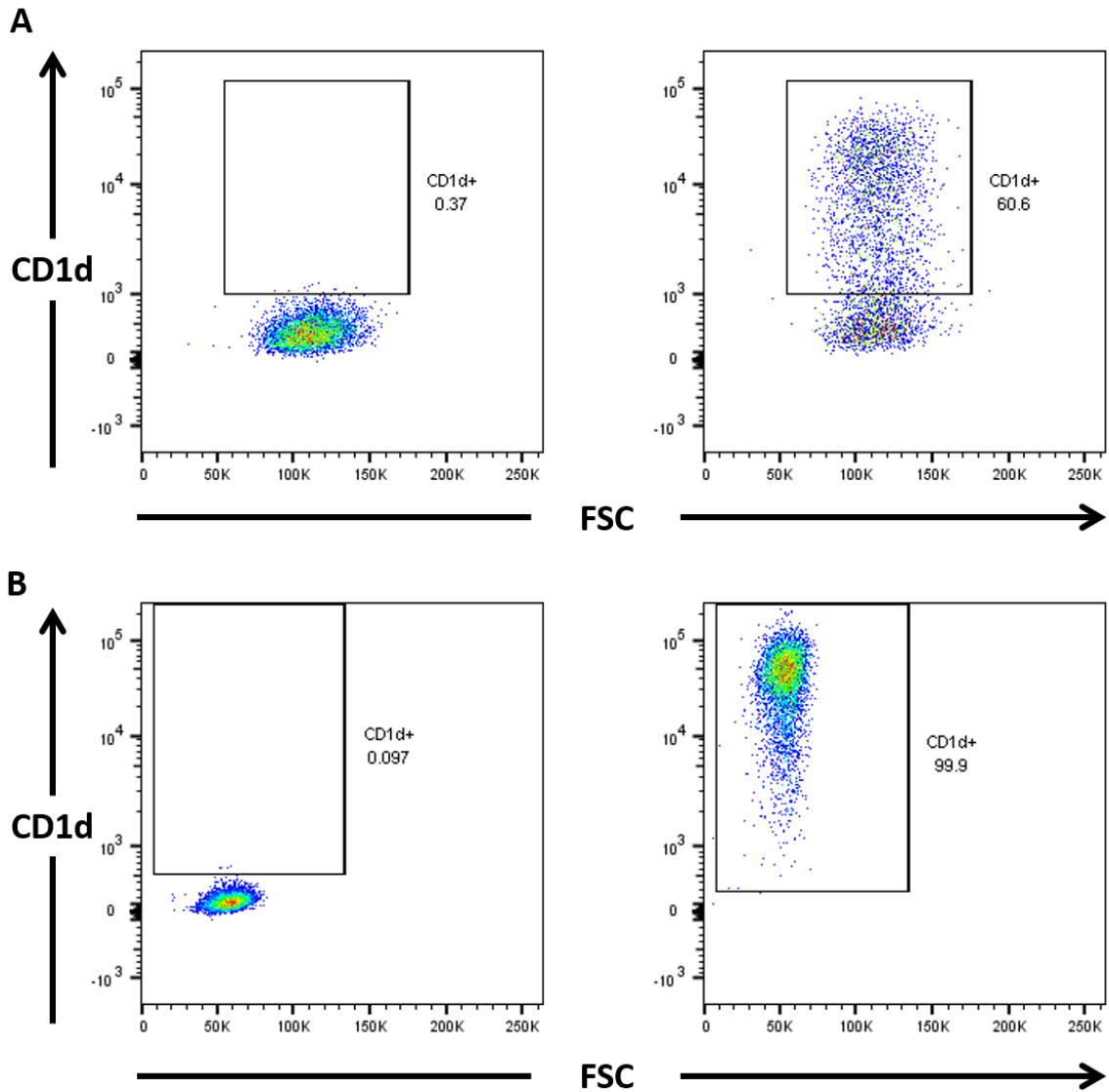
Figure 5.3: Purification of stably transfected SK-MES-1 cells over-expressing CD1d. SK-MES-1 cells were stably transfected with CD1d and subsequently analysed for CD1d expression. **A)** Flow cytometry dot plots showing CD1d expression by transfected SK-MES-1 cells (right) and FMO control (left). Transfected cells were then sorted into a homogenous CD1d-expressing population. To verify that the SK-MES-1 cells transfected with CD1d were homogeneous, cells were stained with a mAb specific for CD1d and analysed by flow cytometry. **B)** Flow cytometric dot plots showing CD1d expression by sorted cells (right) and FMO control (left).
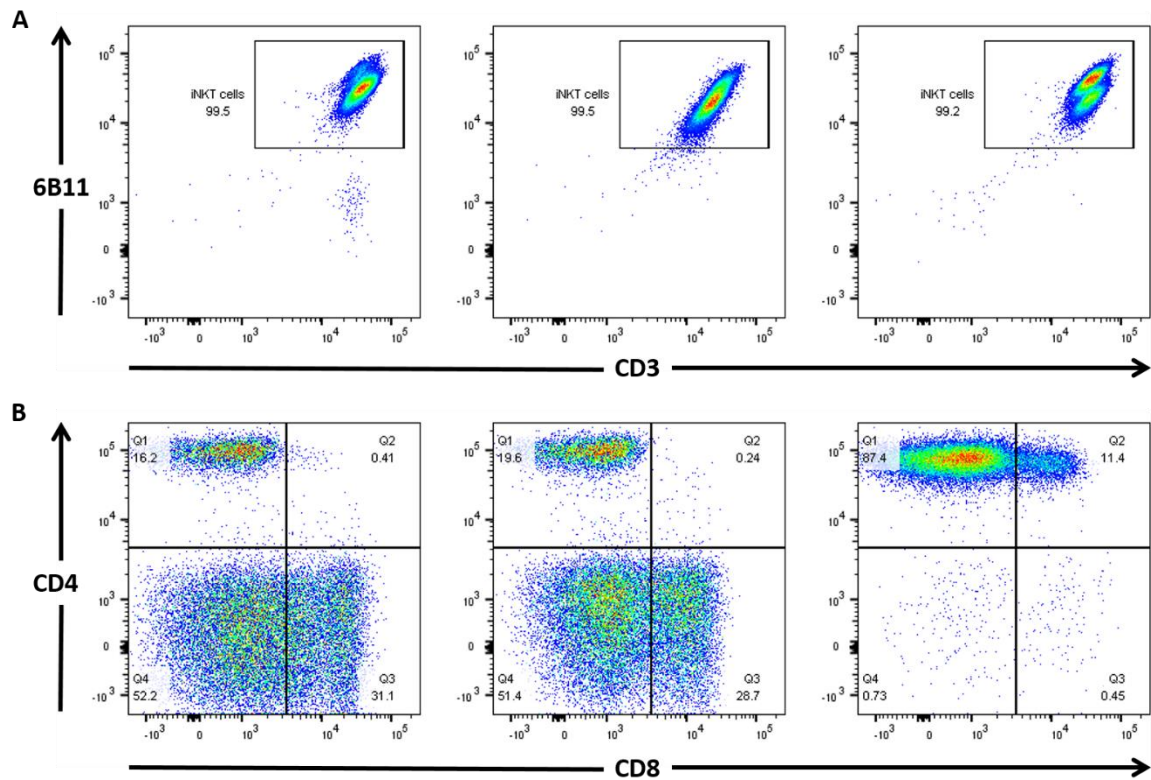
Figure 5.4: Flow cytometric analysis of iNKT cell lines generat from PBMCs. iNKT cells were sorted from PBMCs and stimulated with αGalCer. After four weeks in culture, cells were stained with mAbs specific for CD3, CD4, CD8 and 6B11 and analysed by flow cytometry. **A)** Flow cytometric dot plots showing 6B11^{+}CD3^{+} iNKT cells from three different donors. **B)** Flow cytometric dot plots showing CD4^{+} and CD8^{+} subpopulations from the same three donors.

cells pulsed with either glycolipid did not result in a significant induction of CD107a. iNKT cells that were cultured with CD1d-transfected HeLa cells pulsed with both glycolipids also showed a statistically significant induction of CD107a (p < 0.001). In contrast iNKT cells cultured with mock-transfected HeLa cells pulsed with either glycolipid did not result in a significant induction of CD107a. These results are consistent with the hypothesis that increased expression of CD1d by tumour cells can lead to cytolytic degranulation by iNKT cells.

V.IV.V. Histone acetylation is involved in the regulation of CD1d Expression in NSCLC cell lines

CD1d expression is often downregulated in tumour cells as a method of immune evasion (Hix *et al.*, 2012, Algarra *et al.*, 2004 and Jäger *et al.*, 2001). In order to ascertain whether aberrant epigenetic marks are involved in the downregulation of CD1d expression NSCLC, cell lines were treated with a panel of epigenetic targeted therapies.

To assess whether histone acetylation is involved in the regulation of CD1d expression, A549 and SK-MES-1 cell lines were treated with TSA, a HDAC inhibitor, at 250ng/ml for 24 hours. RNA was extracted and quantified following treatment. RT-PCR was performed in order to ascertain whether transcription of CD1d had occurred (Figure 5.6). β-actin was used as a loading control since it is both commonly and widely expressed at high levels in all cells. Changes in CD1d expression were expressed as ratios of CD1d mRNA against β-actin and plotted as mean ± the standard error of the mean (SEM). In both cell lines a statistically significant upregulation of CD1d was observed following treatment with TSA (Figure 5.6).

A549 and SK-MES-1 cell lines were also treated with SAHA, another HDAC inhibitor, at 5 µM for 24 hours. RNA was extracted and quantified following treatment. RT-PCR was performed in order to ascertain whether transcription of CD1d had occurred (Figure 5.7). Changes in CD1d expression were expressed as ratios of CD1d mRNA against β-actin and plotted as mean ± the
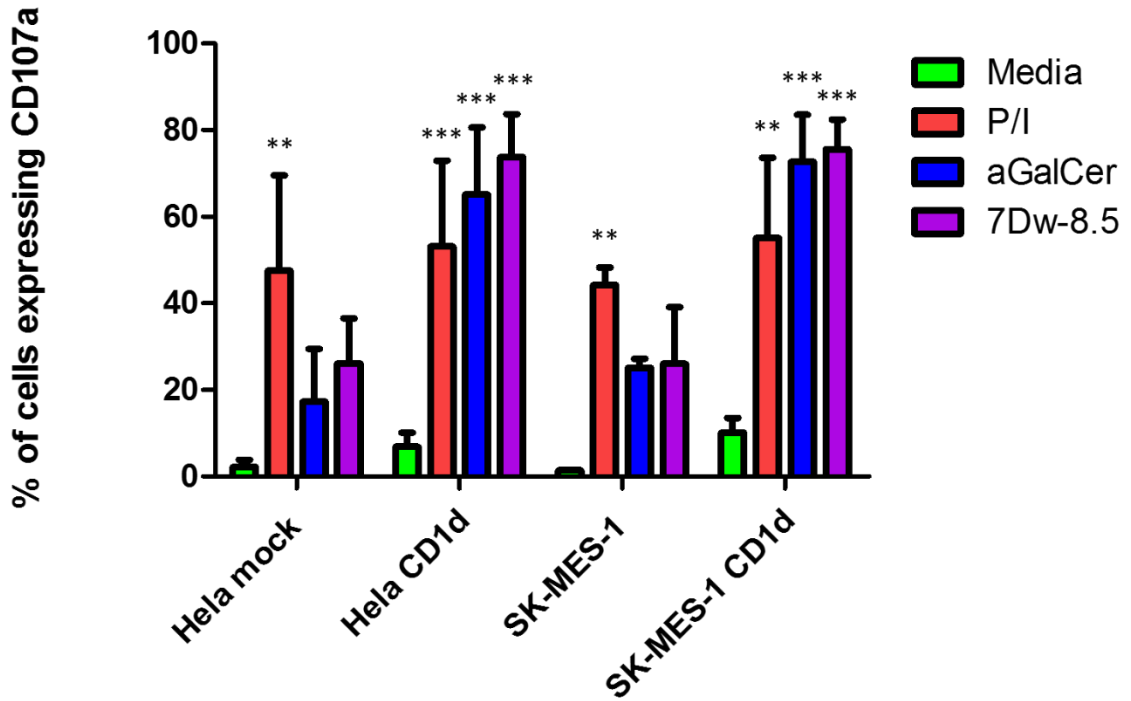
Figure 5.5: Cancer cells expressing transfected CD1d induce cytolytic degranulation by iNKT cells. Mock-transfected Hela cells, CD1d-transfected Hela cells and SK-MES-1 cells, and untransfected SK-MES-1 cells were pulsed for 24 hours with medium alone, glycolipids αGalCer or 7Dw-8.5, and then co-cultured iNKT cells. Cells were stained with a panel of mAbs (Table 5.3.1). Cytolytic degranulation by the iNKT cells was examined by measuring the cell surface mobilisation of CD107a. iNKT stimulation with PMA and ionomycin was used as a positive control. Statistical analysis was performed using a two- way ANOVA analysis with Bonferroni's Post Test. Each experiment was done in triplicate.

Figure 5.6: TSA induces the up-regulation of CD1d expression in NSCLC cell lines at the mRNA level. A) RT-PCR analysis of CD1d expression by untreated (UT) A549 cells and A549 cells treated for 24 hours with 250 ng/ml TSA. **B)** Mean (± SEM) ratios of expression of CD1d and β-actin by untreated and TSA-treated A549 cells from 3 experiments. **C)** Quantitative RT-PCR analysis of CD1d expression by untreated (UT) SK-MES-1 cells and SK-MES-1 cells treated for 24 hours with 250 ng/ml TSA. **B)** Mean (± SEM) ratios of expression of CD1d and β-actin by untreated and TSA-treated SK-MES-1 cells from 3 experiments.

standard error of the mean (SEM). Again, both cell lines showed a statistically significant upregulation of CD1d following treatment with SAHA.In order to determine whether this upregulation could be maintained over time, A549 and SK-MES-1 cell lines were treated with SAHA as before and then washed to remove SAHA and left for a period of 24 hours. CD1d mRNA was quantified by RT-PCR following this period of rest (Figure 5.8). Upregulation of CD1d in response to SAHA treatment was observed in both cell lines. In A549 cells, induction of CD1d expression was still statistically significant after 24 hours of culture in the absence of SAHA. In SK-MES-1 cells, the upregulation of CD1d expression was not maintained after removal of SAHA. These results indicate that it may be possible to circumvent the downregulation of *CD1d* in lung cancer cells.

V.IV.VI. Methylation is involved in regulation of CD1d expression in NSCLC cell lines

To assess whether DNA methylation is involved in the regulation of CD1d expression, A549 cells were treated with a DNMT inhibitor, DAC. Cells were treated with two concentrations of DAC at 200 nM and 1 μM for a period of 48 hours. Both the media and the drugs were replaced every 24 hours. After 48 hours CD1d mRNA was examined by RT-PCR (Figure 5.9). An induction of CD1d expression was observed at both concentrations, although this increase in expression was only significant at the higher concentration (p = 0.037).

Gemcitabine hydrocholoride (GEM), a chemotherapeutic agent, has been shown to function as a DNMT inhibitor with equivalent activity to DAC (Gray *et al.*, 2012). A549 cells were treated with GEM at 200 nM and 1 μM for a period of 48 hours (Figure 5.9). The media and drugs were replaced every 24 hours. CD1d was significantly induced at both 200 nM (p = 0.0019) and 1 μM (p < 0.0001). The reactivation of expression of CD1d following treatment with DAC and GEM would suggest that this is epigenetically regulated at the methylation level. Although GEM
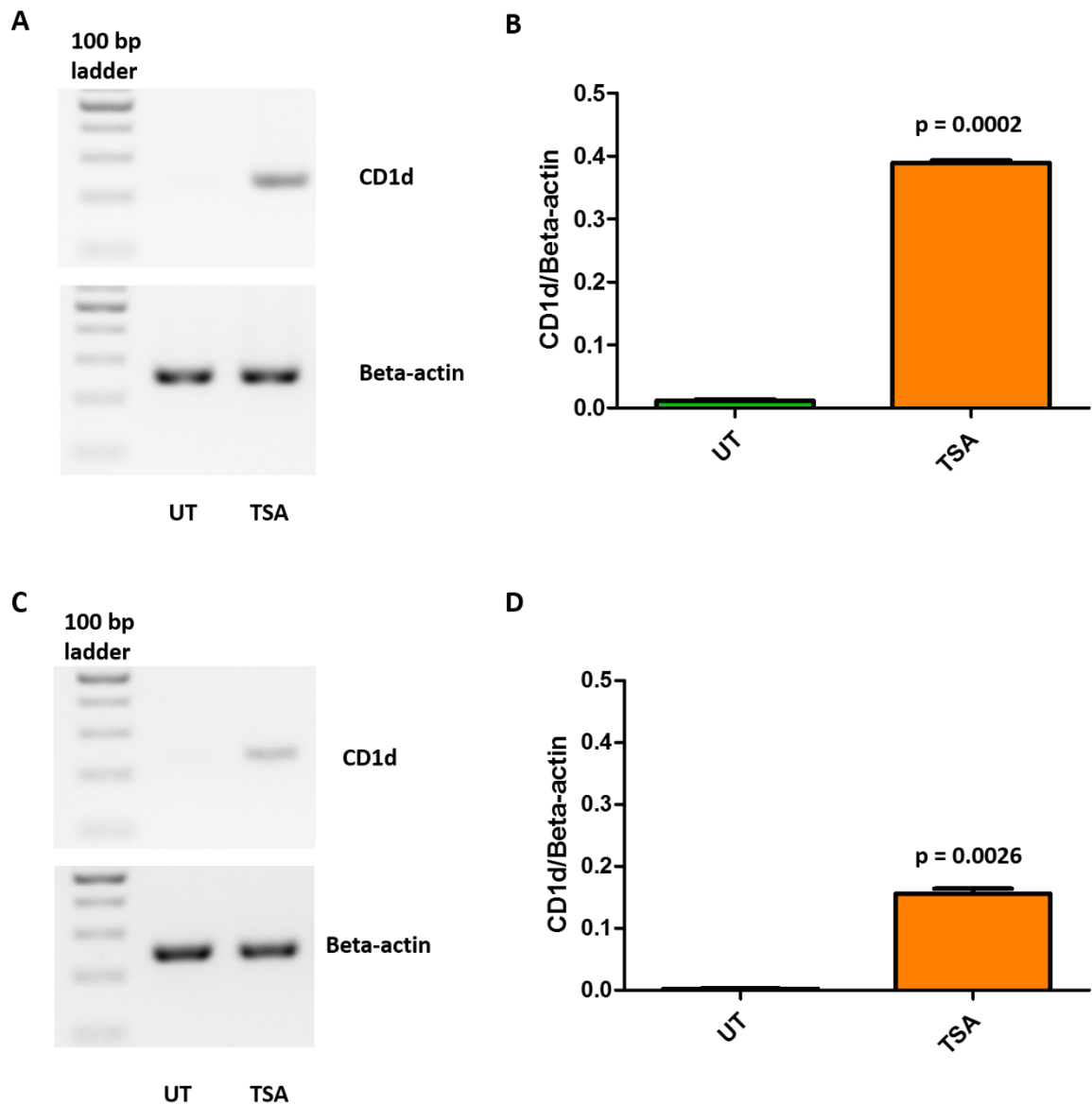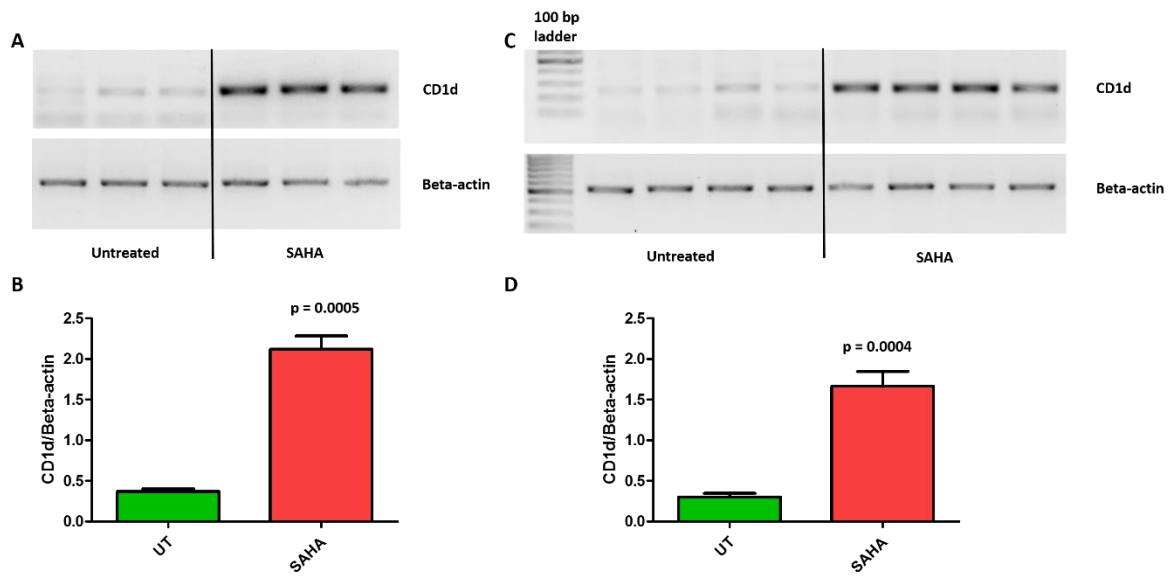
Figure 5.7: SAHA induces the up-regulation of CD1d expression in NSCLC cell lines at the mRNA level.

A) RT-PCR analysis of CD1d expression by untreated (UT) A549 cells and A549 cells treated for 24 hours with 5 µM SAHA. **B)** Mean (± SEM) ratios of expression of CD1d and β-actin by untreated and SAHA-treated A549 cells from 3 experiments. **C)** Quantitative RT-PCR analysis of CD1d expression by untreated (UT) SK-MES-1 cells and SK-MES-1 cells treated for 24 hours with 5 µM SAHA. **B)** Mean (± SEM) ratios of expression of CD1d and β-actin by untreated and SAHA-treated SK-MES-1 cells from 4 experiments.

Figure 5.8: SAHA-treated A549 but not SK-MES-1 cell lines maintain increased levels of CD1d mRNA following a 24 hour recovery period. NSCLC cell lines were cultured in medium alone (UT) or with 5 µM SAHA for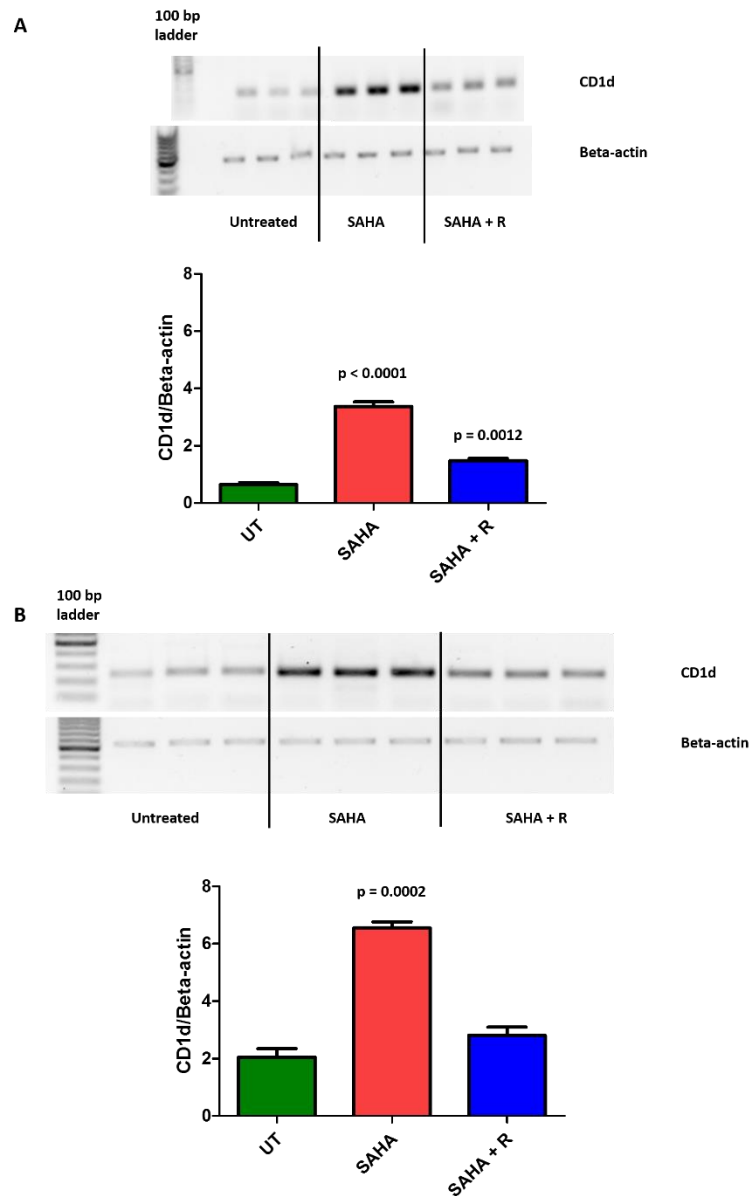 a period of 24 hours. Cells were washed to remove SAHA and re-cultured in medium alone for a further 24 hours. Gene expression was measured by quantitative RT-PCR following treatment, and again after the 24 hour recovery period. CD1d was expressed as the ratios of CD1d to β-actin. **A)** Agarose gel electrophoresis of CD1d and β-actin PCR products from amplifications of untreated, SAHA-treated and SAHA treated and rested A549 cells (top), and histogram showing mean (± SEM) ratios of expression of CD1d and β actin for 3 experiments. **B)** Agarose gel electrophoresis of CD1d and β-actin PCR products from amplifications of untreated, SAHA-treated and SATA treated and rested SK-MES-1 cells (top), and histogram showing mean (± SEM) ratios of expression of CD1d and β actin for 3 experiments.
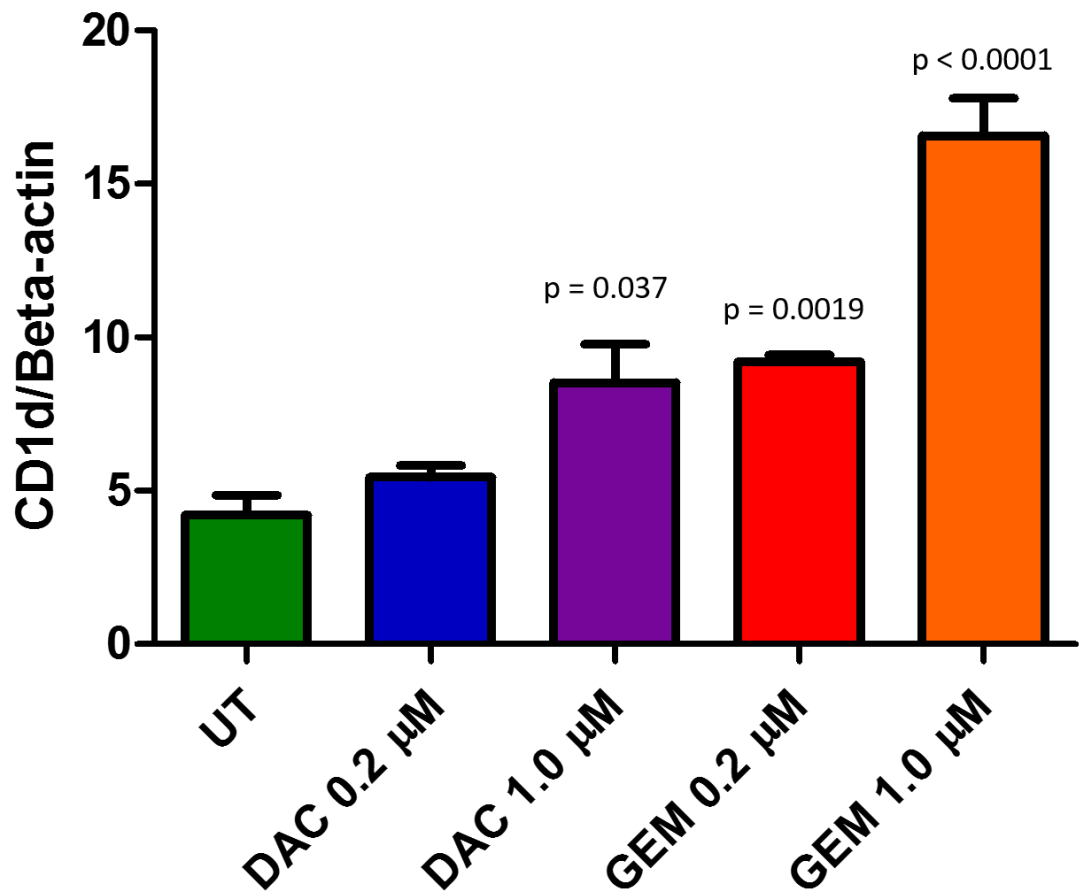
Figure 5.9: DNMT inhibitors induce an upregulation of CD1d mRNA in A549 cells. A549 cells were treated for 48 hours with 5-aza-2'-deoxycytidine (DAC) or Gemcitabine (GEM) at 200 nM and 1 µM. Quantitative RT-PCR analysis of CD1d expression by untreated (UT) A549 cells and A549 cells treated for 48 hours with 0.2 µM DAC, 1 µM DAC, 0.2 µM GEM and 1 µM GEM. Expression of CD1d against β-actin is plotted as mean (± SEM) for 3 experiments.

shows a stronger induction of CD1d expression, DAC has been approved by the FDA and as a result all remaining experiments were conducted using DAC.

V.IV.VII.   Regulation of CD1d expression by A549 cells is due to direct chromatin remodelling

The increase in CD1d expression following treatment with epigenetic targeted therapies could be a consequence of chromatin remodelling at the *CD1d* promoter. To confirm that the observed effects for induction of CD1d expression following treatment with a HDAC inhibitor was due to increased hyperacetylation at the *CD1d* promoter, ChIP analysis was carried out in A549 cells treated with TSA (Figure 5.10). A549 cells were treated with 250 ng/ml TSA for a period of 24 hours. The DNA was fragmented and immunoprecipitated with the antibodies specific for the histone marks listed in Table 5.3.2. A no antibody control was used as a negative control. Primers were specifically designed to study the *CD1d* promoter, as described in section V.III.IV., and are listed, along with the optimal annealing temperature in Table 5.3.3.

Treatment with TSA resulted in an increase of PCR product, indicating enhanced histone hyperacetylation at the *CD1d* promoter. Histone acetylation is associated with gene expression, indicating that the *CD1d* promoter region was silenced in A549 cells due to aberrant histone deacetylation. Treatment with TSA prevented gene silencing by HDACs allowing for the gene to be expressed. Both lysine 9 and 14 were hyperacetylated following treatment. These are known activating marks of expression. In addition, a decrease in expression was observed at the histone H3 lysine 4 monomethylation repressive mark with a simultaneous increase in levels of an activating mark, H3K4me2. Methylation marks are also associated with gene silencing; a decrease in the expression of a known repressive methylation mark, H3K9me2, further indicates that gene expression has been induced. These modifications demonstrate that changes in CD1d expression occurs as a result of direct changes to its promoter region.

The experiments in this chapter were performed as a proof of principle. The results indicate that CD1d expression is epigenetically regulated as a result of chromatin modifications. In NSCLC, aberrant histone acetylation patterns are involved in the downregulation of CD1d expression. By treating NSCLC cells with HDAC inhibitors, CD1d expression can restored. These data suggest that epigenetic targeting therapy may be used to sensitise NSCLC cells to iNKT cell-mediated cytolytic degranulation by inducing CD1d expression.
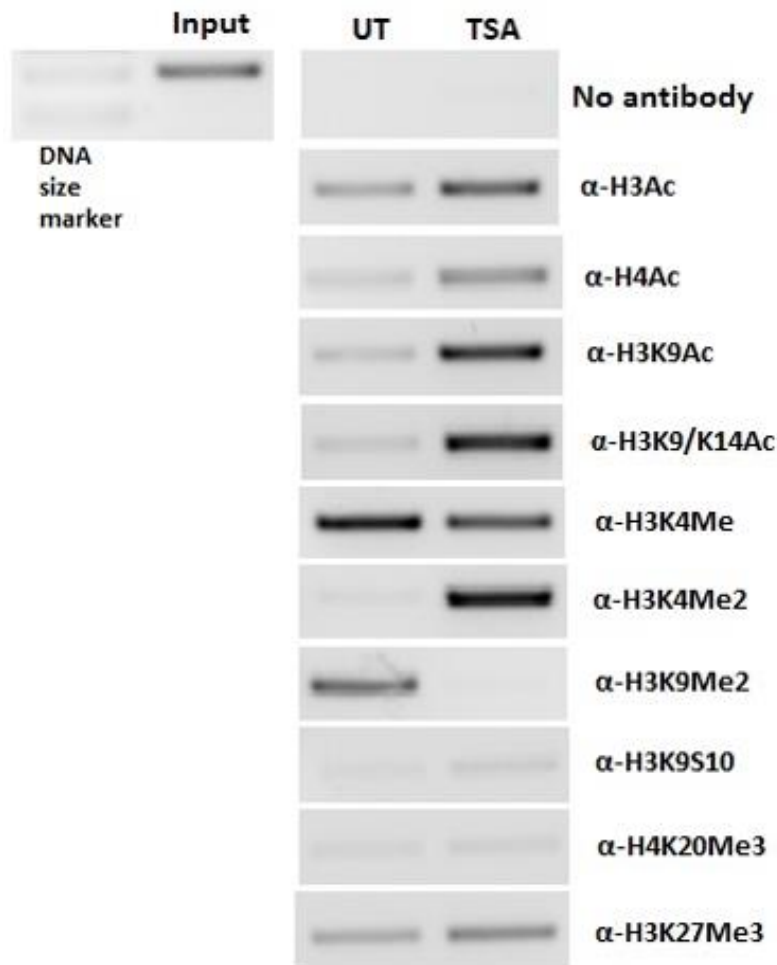
Figure 5.10: Chromatin remodelling is directly involved at the promoter level in CD1d following treatment with HDAC inhibitor TSA. Analysis of the CD1d promoter was performed using a ChIP assay. A549 cells were treated with DMSO as an untreated (UT) control or with 250 ng/ml TSA for 24 hours. 1% Formaldehyde was added to allow for crosslinking. Samples were sonicated thrice to shear the DNA into fragments. Cross-linked DNA fragments associated with the proteins of interest (Table 5.3.2) were selectively immunoprecipitated using protein-specific antibodies (Table 3.23). UT samples were compared against TSA-treated samples to determine any changes to the histone marks.

V.V.   Discussion

To determine whether NSCLC cells can be targeted for direct iNKT cell-based cytotoxicity, it must be determined if these cells express CD1d. CD1d is a MHC class I-like molecule that presents glycolipids to iNKT cells, as well as some Vδ1 and Vδ3 T cells (Bai *et al*., 2012 and Mangan *et al*., 2013). iNKT cells can directly recognise and kill CD1d-bearing tumour cells. Some tumour types such as prostate cancer, myelomonocytic leukaemia cells and some neurologic tumours express CD1d and as such can be targeted for NKT-mediated cell killing (Nowak *et* al, 2010, Dhodapkar *et al*., 2004 and Metelitsa *et al*., 2003). However, CD1d is downregulated in many tumour types (Hix *et al*., 2012, Spanoudakis *et* al., 2009, Song *et* al., 2008 and Chang, *et al*., 2006).

iNKT cells were first shown to be necessary for the elimination of tumours in a mouse murine model (Cui *et al*., 1997). Mice with iNKT cell deficiencies were unable to reject tumours in an IL-12-mediated fashion. While it was previously believed that the anti-tumour effect of IL-12 was mediated through NK cells and T cells, this study found that iNKT cells were an essential target of IL-12. αGalCer is a marine sponge-derived glycolipid that was identified as a ligand for iNKT cells (Kawano *et al*., 1997). iNKT cells activated by presentation of αGalCer by dendritic cells (Kawano *et al*., 1998) can kill tumour cells in mice. Cytotoxic activity against B cell melanoma was observed when mice were injected with αGalCer but not with the vehicle control. This study showed that αGalCer-activated iNKT cells can kill tumour cells. Adoptive transfer of αGalCer-pulsed dendritic cells in a murine model showed that targeted delivery of αGalCer triggered optimal stimulation of iNKT cells, which was maintained after the challenge had dissipated (Macho-Fernandez *et al*., 2014).

The first phase I study assessed the IV administration of αGalCer in patients with refractory solid tumours. While the study found only minimal toxicity in patients, no anti-tumour immunity was observed. Disease stabilisation was detected in 7 out of a total of 24 patients (Giaccone *et al*., 2002). IV-injected monocyte-derived dendritic cells pulsed with αGalCer showed sustained

expansions of iNKT cells in 5 patients with advanced cancer up to six months after inoculation (Chang *et al.*, 2005). Another study evaluating the administration of iNKT cells with αGalCer-pulsed APCs demonstrated that of the eight patients with head and neck squamous carcinoma tested, three had partial but significant responses, four had stable disease, and one had progressive disease. These studies indicate the potential of iNKT immunotherapy for cancer.

As mentioned previously, iNKT cells directly recognise and lyse tumour cells in a CD1d-dependent manner. However, most human and mouse solid tumours are CD1d negative. Increasing evidence has suggested that cancer cells are capable of downregulating MHC class I molecules, including CD1d, in an attempt to evade immunosurveillance (Algarra *et al.*, 2005 and Jäger *et al.*, 2001). A study of breast cancer cells found that highly metastatic cells had decreased expression of CD1d compared with breast cancer cells less prone to metastasis (Hix *et al.*, 2011). Other studies have demonstrated that demonstrated that advanced cancer cells lose CD1d expression. Both early and intermediate stage myeloma cells express CD1d and can be targeted for iNKT-mediated cytotoxicity, but this expression is lost on advanced myeloma cells (Spanoudakis *et al.*, 2009). These studies suggest that loss of CD1d expression on cancer cells is a mechanism by which tumours can evade immunosurveillance.

The aims of this chapter were to assess whether NSCLC cell lines express CD1d, and so identify whether they are a possible target for iNKT cell-mediated killing; to establish iNKT cell lines for functional and translational studies; to establish NSCLC cell lines that over-express CD1d; to determine whether CD1d expression can be induced in NSCLC cell lines.

Adenocarcinoma (A549) and squamous carcinoma (SK-MES-1) cell lines were examined for the presence of CD1d. Flow cytometry analysis showed that both cells lines had undetectable levels of CD1d expression (Figure 5.1), re-iterating previous studies that suggest that CD1d expression is downregulated in cancer as a method of immune evasion (Algarra *et al.*, 2004 and Jäger *et al.*, 2001). Furthermore some tumours are heterogenous for CD1d expression (Metelitsa *et al.*,

2011); this study suggests that only a minority of patients have CD1d-positive tumours. Therefore, screening NSCLC cell lines for the presence of cell-surface CD1d should allow for determination of their CD1d status, and whether these cells are homogeneous or heterogeneous for the expression of CD1d. If CD1d expression can be induced in NSCLC cell lines, these tumours could be targets for iNKT immunotherapy. We decided to generate an NSCLC cell line over-expressing CD1d for use in future iNKT co-culture experiments.

Activated iNKT cells have been shown to be directly cytotoxic against CD1d-bearing tumour cells *in vitro*, and previous studies have shown a positive correlation between tumour expression of CD1d and sensitivity to iNKT-mediated anti-tumour immunity (Haraguchi *et al.*, 2006 and Metelitsa, 2003). In order to examine whether induced *CD1d* expression by NSCLC cells can be targeted for CD1d iNKT cell-mediated cytotoxicity, NSCLC cell lines over-expressing CD1d were needed as a positive control. A549 and SK-MES-1 cell lines were transiently transfected with CD1d using the FuGENE protocol to assess whether such a transfection was feasible. Transient transfection of CD1d was established in both NSCLC cell lines as verified by RT-PCR. A stable transfection of A549 was not successful despite multiple attempts, however a stable of SK-MES-1 transfectant cell line over-expressing CD1d protein was successfully established.

The stably transfected SK-MES-1 cell line, referred to as SK-MES-1 CD1d was heterogeneous for CD1d expression. In order for it to be used as a positive control in iNKT co-culture experiments, cells were sorted using MoFlo™ cell sorter. Following the sort, the SK-MES-1 CD1d cell line had a purity of >99%; this CD1d expression remained stable throughout the course of this study. As a negative control, the parent SK-MES-1 cell line would be used for comparative analysis.

In order to test our hypothesis that cancer cell lines over-expressing CD1d would be more susceptible to iNKT cell-mediated cytotoxicity, highly pure iNKT cell lines were needed. iNKT cells were enriched from PBMCs of healthy donors using anti-iNKT microbeads. Labelled cells were sorted and stimulated with αGalCer in the presence of irradiated PBMCs and IL-2. After

four weeks of culture, cells were stained with mAbs specific for CD3, CD4, CD8 and 6B11, and assessed by flow cytometry (Figure 5.4). iNKT cell populations were homogeneous, with purities of >99%. Each population was also analysed for CD4$^+$ and CD8$^+$ subpopulations. The majority of iNKT cell lines were heterogeneous for CD4 and CD8, but one cell line was mainly composed of CD4$^+$ iNKT cells with few CD8$^+$ cells. These phenotypes were retained for up to 12 months.

To determine whether the SK-MES-1 cell line transfected with CD1d was more susceptible to iNKT cell-mediated killing than its parent SK-MES-1 cell line, iNKT cells were co-cultured with SK-MES-1 cells, mock-transfected HeLa cells and CD1d-transfected HeLa and SK-MES-1 cells. Cancer cell lines were pulsed with αGalCer or 7Dw-8.5 for 24 hours before co-culturing them with iNKT cells. Following a four hour co-culture, cells were stained with a panel of mAbs (Table 5.3.1) and analysed by flow cytometry. When iNKT cells were co-cultured with mock-transfected HeLa cells or SK-MES-1 cells in the absence or presence of glycolipids, no up-regulation of CD107a was observed. In comparison, iNKT cells co-cultured with either CD1d-transfected HeLa or SK-MES-1 cells showed a significant induction of CD107a. These results corroborate accepted evidence that iNKT cells kill in a CD1d-restricted manner. These experiments suggest that CD1d induction in NSCLC cells makes them susceptible to iNKT cell killing *in vivo*.

Epigenetics is defined as the sum of all stable and inheritable changes to gene expression that do not occur as a result of changes to the underlying DNA sequence (Probst *et al*., 2009). Chromatin structure defines the condition in which DNA is organised and allows for transcriptional repression or activation of genes (Sharma *et al.*, 2010). Epigenetic mechanisms such as DNA CpG methylation and histone post-translational modifications (HPTMs) affect chromatin structure and gene expression by changing the accessibility of transcription factors to the promoter regions of genes. Aberrant acetylation patterns also contribute to cancer proliferation in various cancers, including gastric and lung (Cortez and Jones, 2008), and over-expression of HDACs have been observed in lung cancer (Bartling *et al*., 2005). If CD1d

expression can be induced by epigenetic modification of NSCLC cell lines, these tumours could be targets for iNKT cell immunotherapy.

To assess whether CD1d could be epigenetically targeted, A549 and SK-MES-1 cell lines were treated with a panel of HDAC and DNMT inhibitors, and CD1d expression was examined at the mRNA level by RT-PCR. Treatment with HDAC inhibitor TSA showed a statistically significant increase in CD1d expression for both cell lines. SAHA is another HDAC inhibitor that affects the same histones as TSA. Treatment with SAHA resulted in a significant increase in CD1d expression for both cell lines. While this result is promising, for such a treatment to have clinical relevance in NSCLC patients, induction of CD1d needs to be maintained after the cessation of treatment. To evaluate whether such induction was transient or could be maintained over a period of time, cell lines were treated with SAHA, which was then removed and cells were cultured in media alone. Analysis by RT-PCR showed that the induction of CD1d expression was only maintained in A549 cells. HDAC inhibitors prevent the action of HDACs which silence genes by removing acetyl groups from histones. These results indicate corroborate evidence that HDACs are over-expressed in lung cancer.

A549 cells were examined by RT-PCR to investigate for the effects of DAC and GEM on the expression of CD1d at the mRNA level (Figure 5.9). DAC is a DNMT inhibitor that has been shown to affect DNA methylation patterns in cancer (Nguyen *et al*., 2002). A previous study by our group has shown that GEM bears a strong resemblance to DAC structurally. This study subsequently showed that GEM also appears to act as a DNA methyltransferase inhibitor (Gray *et al*., 2012). CD1d expression was upregulated at 1 µM DAC, and at 200 nM and 1 µM GEM, indicating that aberrant DNA methylation patterns affect the expression of *CD1d* in NSCLC. It would appear that treatment with DNMT inhibitors remove some of the hypermethylation of the promoter resulting in an upregulation of *CD1d* expression.

The results from the ChIP assay indicate that HDAC inhibitor TSA directly remodels the promoter region of *CD1d*. As mentioned previously, HDACs silence genes by removing acetyl groups from histones. HDAC inhibitors prevent this action allowing for re-acetylation of histones. This modifies chromatin structure allowing for transcription factors to access the promoter regions of genes. The assay shows hyperacetylation at histones H3 and H4, as well as at histone H3 lysines 9 and 14. Histone methylation can be associated with either transcriptional activation or repression. Methylation of histone H3 lysine 4 (HeK4Me) indicates transcriptional activation. Hypermethylation is seen at this mark following treatment with TSA. In addition, there is an increase of the histone H3 lysine 4 dimethylation marker, and a decrease at histone H3 monomethylation at the lysine 4 marker. Conversely, dimethylation of histone H3 lysine 9 (H3K9Me2) indicates transcriptional repression. In this experiment the levels of H3K9Me2 decrease after treatment with TSA. These results show that HDAC inhibition causes a conformational change in the chromatin landscape, allowing histone post-translational modifications to occur at the promoter region of CD1d. These results indicate that HDACs play an important role in the regulation of the *CD1d* gene.

In conclusion, NSCLC cells have undetectable amounts of CD1d, which could be a mechanism whereby these tumours can evade the immune system. The stably transfected SK-MES-1 cell line generated using the FuGENE protocol has been used to study the anti-tumour effects of iNKT cells against NSCLC, and showed that increased CD1d expression correlates with increased iNKT cell mediated killing *in vitro*. Downregulation of CD1d expression In NSCLC cell lines is the result of aberrant HDAC and DNMT activity. Treatment with either HDAC or DNMT inhibitors promotes the upregulation of CD1d expression in these cells lines. The induction of CD1d expression was still evident following a recovery period where cells were not exposed to HDAC inhibitor SAHA, showing that the increase in CD1d expression can be sustained over time. Finally it was demonstrated that changes in CD1d expression in NSCLC occur as the result of direct conformational changes to the promoter region of *CD1d*. The next chapter will look to further

these results by treating NSCLC cell lines with DNMT and HDAC inhibitors to see whether CD1d can be increased at the protein level, and whether such an increase leads to increased susceptibility to iNKT cell-mediated killing.

VI. Epigenetic induction of CD1d in NSCLC cell lines induces cytolytic degranulation by iNKT cells

VI.I. Introduction

Lung cancer is the leading cause of cancer deaths worldwide, accounting for approximately 8.2 million deaths in 2012 alone (Torre *et al.*, 2015). It is the most commonly diagnosed cancer, with a five year net survival of 15.7% for newly diagnosed patients. In advanced cases this drops to 3.5%, and median survival for advanced lung cancer patients is one year. Even if radical surgery is a possibility, more than 40% of patients will develop recurrences and ultimately succumb to the disease (Jemal *et al.*, 2011). Platinum-based therapy is the current gold standard of care (Pujol *et al.*, 2006), however, most patients do not benefit from it, and many who do eventually develop resistance.

Immunotherapy has become increasingly popular as a treatment for NSCLC. Immunotherapy is a targeted treatment with a long-lasting response that has few toxic side effects and has recently become the fourth most common modality for treating malignant tumours after surgery, radiotherapy and chemotherapy (Dougan and Dranoff, 2009). There are three types of immunotherapy that are currently applied for the treatment of NSCLC: supportive immunotherapy, active immunotherapy, and passive immunotherapy. Supportive immunotherapy involves the use of mAbs such as anti-VEGF, which has been shown to improve survival in patients with non-surgical NSCLC (Yang *et al.*, 2010). Two mAbs, cetuximab and bevacizumab, which target EGRF and VEGF respectively, have been included as first line therapies for NSCLC. Active immunotherapies include vaccinations such as Belagenpumatucel-L (Lucanix), an allogeneic tumour cell vaccine that inhibits the inhibition of T cells, B cells and dendritic cells by TGF-β, and Tecemotide (Liposomal BLP25), a peptide vaccine that targets the

membrane-associated glycoprotein, MUC-1, rely on specific activation of the host immune system. Clinical trials of vaccinations within the last year have not shown significant clinical outcomes despite their ability to prime and expand tumour antigen-specific T cells (Thomas and Giaccone, 2015). Finally, adoptive immunotherapy involving T cells and dendritic cells belongs to passive immunotherapy. First used for relapses after allogeneic bone marrow transplantation in leukaemia patients, it has been shown to achieve therapeutic benefit in solid cancers (Zheng *et al*., 2013).

Adoptive immunotherapy involves the removal of blood cells from a patient, treatment of specific blood cells *ex vivo* followed by transfer back to the patient. Current adoptive immunotherapies encompass many different cell types such as TILs, γδT cells, LAK cells, CIK cells. TILs have high affinity TCRs against tumour antigens and recognise tumour antigens in the context of MHC molecules (Wu *et al*., 2012). These cells can be expanded efficiently *ex vivo* and their tumour reactivity can be tested *in vitro* (Nguyen *et al*., 2010), however, this type of adoptive therapy is limited as TILs have to be obtained through surgery or biopsy. γδT cells can recognise tumours in an MHC-unrestricted manner, and these cells can also directly influence adaptive immunity by acting as APCs (Yoshida *et al*., 2011 and Hayday, 2009). γδT cells have cytotoxic activity against a variety of cancers including renal tumours, head and neck squamous carcinoma and colon cancer (Alexander *et al*., 2008, Viey *et al*., 2008, Corvaisier *et al*., 2008 and Viey *et al*., 2005). They also rapidly release IFN-γ and TNF-α. LAK cells are generated from T cells exposed to IL-2 and can lyse NK-resistant tumour cells (Sinkovics and Horvath, 2005). CIK cells are heterogeneous lymphocytes that have possess MHC-unrestricted anti-tumour immunity (Mesiano *et al*., 2012 and Lin and Hui, 2010). These cells are generated *in vitro* by incubating PBMCs with anti-CD3 mAb, IL-2, IL-1α, and IFN-γ (Hontscha *et al*., 2011). Clinical trials have shown that CIK cells can prevent recurrence and to improve quality of life and progression free survival in several solid tumours (Hui, 2012, Ma *et al*., 2012, Zhang *et al*., 2012 and Hontscha *et al*., 2011).

Dendritic cells are a heterogeneous group of cells that link the innate and adaptive immune systems. Mature dendritic cells enhance anti-tumour immunity in lung cancer by the stimulation of CD8^{+} T cells via IL-12 secretion but studies have demonstrated that the maturation of dendritic cells in NSCLC is suppressed by IL-10, VEGF and TGF-β (Shen *et al.*, 2010 and Bergeron *et al.*, 2006). It has been suggested that immature dendritic cells play a role in tumour progression through facilitating tumour migration, invasion and epithelial-to-mesenchymal transition (Schneider *et al.*, 2011). Dendritic cells are currently being investigated as *in vitro* vehicles to develop vaccines against lung cancer. Studies have demonstrated that tumour antigen-pulsed dendritic cells can increase the numbers of CD4^{+} T cells, CD8^{+} T cells and NK cells (Kato *et al.*, 2011, Zhong *et al.*, 2011, Bergeron *et al.*, 2006 and Chen *et al.*, 2001). In addition, αGalCer-pulsed dendritic cells has been demonstrated to result in sustained activation of iNKT cells *in vivo* (Macho-Fernandez *et al.*, 2014).

Adoptive immunotherapy has shown great promise in the treatment of cancer but many factors can influence its efficiency. Cytokines play critical roles in host anti-tumour immunity, but different adoptive immunotherapies have shown diverse cytokine profiles. In a phase I study of adoptive immunotherapy against advanced NSCLC using zoledronate-expanded γδ T cells, IFN-γ was not found to be an indicator of improvement (Sakamoto *et al.*, 2011). Another phase I clinical trial of γδ T cells and NSCLC showed that patients with elevated serum IFN-γ levels had stable disease while those with normal IFN-γ levels developed progressive disease (Nakajima *et al.*, 2010). It has also been reported that low-dose TNF-α enhances T-cell infiltration and overall survival in solid tumours, as well as substantially improving adoptive T cell therapy for anti-tumour vaccination (Johansson *et al.*, 2012).

Immunosuppressive cells in the tumour microenvironment such as MDSCs (Lu *et al.*, 2011) and CD4^{+} T$_{REG}$ cells (Yao *et al.*, 2012) have been shown to inhibit the anti-tumour activity of activated immune cells. A study of adoptive NK cell transfer found that non-responders had higher

frequencies of peripheral CD4$^+$FoxP3$^+$ T$_{REG}$ cells than responders (Salagianni *et al*., 2011). In a murine model of melanoma Kodumudi *et al*. found that administration of myeloablative chemotherapy or total body irradiation prior to adoptive transfer of T cells could reduce or eliminate immunosuppressive cell populations (Kodumudi *et al*., 2012). Lymphopenia induced by total body irradiation was rapidly followed by reconstitution of both T$_{REG}$ and MDSC populations, with enhanced ability to inhibit CD8$^+$ T cells. Total body irradiation with docetaxol improved the efficacy of adoptive immunotherapy and induced a significant reduction in tumour size as well as enhanced survival. Tumour regression correlated with the persistence of adoptively transferred T cells as well as CTL activity (Kodumudi *et al*., 2012).

The previous chapter found that NSCLC cell lines had undetectable amounts of CD1d, a MHC class I-like molecule that is required for iNKT cell-mediated cytotoxicity. We showed that transfection of a squamous cells cell line with CD1d resulted in their increased susceptibility to iNKT cell-mediated cytolytic degranulation. Treatment with DNMTi and HDACi were able to increase CD1d expression in adenocarcinoma and squamous cell carcinoma cell lines, which we demonstrated was the result of direct changes to the CD1d promoter. Those results demonstrated that it is possible to induce CD1d expression in NSCLC cell lines using epigenetic targeted therapies, and that increased CD1d expression can make NSCLC cells susceptible to cytotoxicity by iNKT cells. However, the first results chapter showed that iNKT cells are decreased in BAL and blood from NSCLC patients therefore induction of CD1d would need to be done in combination with adoptive transfer of iNKT cells, possibly with αGalCer-pulsed dendritic cells as in other iNKT cell-based immunotherapies (Macho-Fernandez *et al*., 2014, Chang *et al*., 2005, Ishikawa *et al*., 2005 and Dhodapkar *et al*., 2004).

DAC is a DNMTi that is used for the treatment of myelodysplastic syndromes and acute myeloid leukaemia. The previous chapter showed that treatment with DAC can induce CD1d at the mRNA level in both A549 and SK-MES-1 cell lines, so we wanted to investigate whether high

dose DAC could increase CD1d expression in NSCLC cell lines at the protein level. Past trials with high doses of DAC have been plagued by extreme toxicities (Abele *et al*.; 1987), however, and transient low doses of DAC have been shown to sustain long-term anti-tumour effects and are at least temporarily associated with their ability to maintain their targeting of DNMTs and alterations of gene expression (Tsai *et al*., 2012), therefore we tested lower doses of DAC to see whether they could also induce CD1d expression. A study by Yang and colleagues demonstrated that HDACi SAHA and TSA can induce CD1d expression in lung adenocarcinoma cell lines (Yang *et al*., 2012). This study showed that A549 cells treated with either 1 µM TSA or 5 µM SAHA showed an increase of CD1d at the mRNA level. Cameron and colleagues showed that using DNMTi and HDACi in combination can reactivate several hypermethylated genes so we decided to investigate whether using DAC and SAHA in combination could increase CD1d expression in NSCLC cell lines. The overall objective of this chapter was to perform preclinical evaluations of DAC and SAHA treatments in NSCLC cell lines to see whether induction of CD1d expression is possible and whether such an increase can increase NSCLC susceptibility to iNKT cell-mediated cytolytic degranulation.

VI.II. Objectives

The previous chapter demonstrated the feasibility of inducing CD1d expression in NSCLC cell lines using epigenetic targeted therapy and showed as a proof of principle that NSCLC cell lines over-expressing CD1d were more susceptible to cytolytic degranulation by iNKT cells. This chapter aimed to compare the efficacies of various epigenetic treatments at inducing CD1d, and to test whether up-regulation of CD1d is accompanied by increased cytolytic degranulation by iNKT cells.

The main aims of this chapter are:

1) To test the efficacy of high dose DAC treatment at inducing CD1d expression in NSCLC cell lines

2) To test the efficacy of transient, low dose DAC treatment at inducing CD1d expression in NSCLC cell lines

3) To test the efficacy of DAC and SAHA combined treatment at inducing CD1d expression in NSCLC cell lines

4) To assess whether increased expression of CD1d induced by epigenetic targeted treatments of NSCLC cell lines increases iNKT cell cytolytic degranulation

VI.III. Materials and Methods

VI.III.I. High Dose DAC Treatment

A549 and SK-MES-1 cells were treated with DAC at 100 nM, 1 µM and 5 µM to assess whether CD1d expression could be up-regulated at the protein level. DAC was diluted in MeOH to either 100 nM, 1 µM or 5 µM. Cells were treated one of the three concentrations of DAC, or PBS. MeOH was used as a vehicle control. The media was replaced after 72 hours and cells were given a recovery period of either 2, 5 or 7 days (Figure 6.3.1). Following this recovery period cells were assessed for expression of CD1d by flow cytometry.
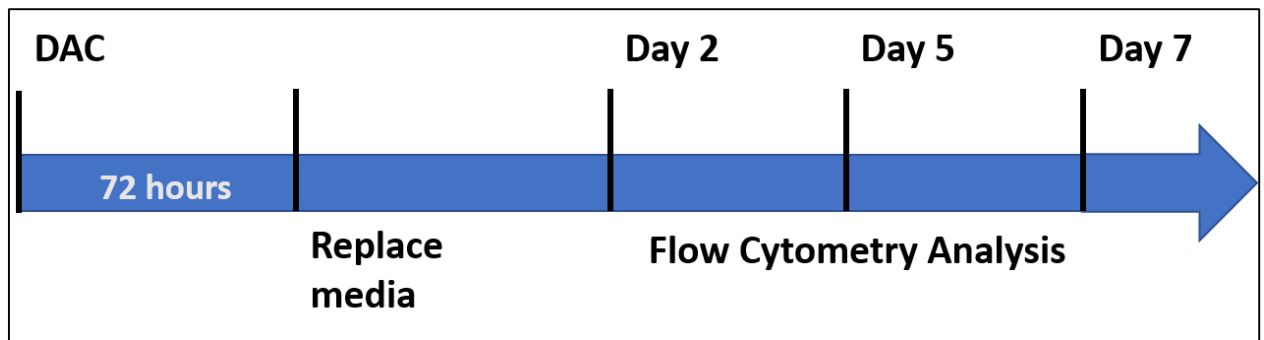
Figure 6.3.1 High Dose DAC Treatment to Induce CD1d Expression in NSCLC

VI.III.II. Transient DAC Treatment

A549 and SK-MES-1 cells were treated with transient low doses of DAC at 50 nM, 100 nM or 1 µM for 72 hours. DAC was diluted in MeOH as before. Cells were treated with one of the three concentrations of DAC, or PBS. MeOH was used as a vehicle control. Both the media and the treatments were replaced every 24 hours (Figure 6.3.2). Cells were assessed for CD1d expression by flow cytometry immediately after treatment and again following a recovery period of 4 days.
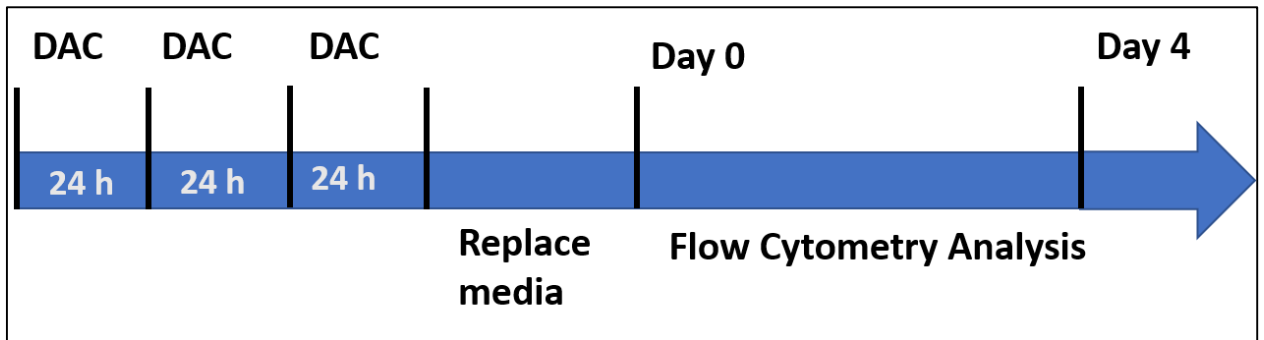
Figure 4.3: Transient Low Dose DAC Treatment to Induce CD1d Expression in NSCLC

VI.III.III.   Combination DAC + SAHA Treatment

A549 and SK-MES-1 cells were treated with both drugs for a period of 72 hours as depicted in Figure 6.3.3 below. DAC was diluted in MeOH to final concentrations of 100 nM, 500 nM and 1 µM. SAHA was diluted to a final concentration of 5 µM in DMSO. Cells were treated with one of the three concentrations of DAC with SAHA, or PBS. MeOH was used as a vehicle control. Both the drugs and media were replaced every 24 hours. Cells were assessed for presence of CD1d by flow cytometry directly following the treatment period, and again following a four day recovery period.
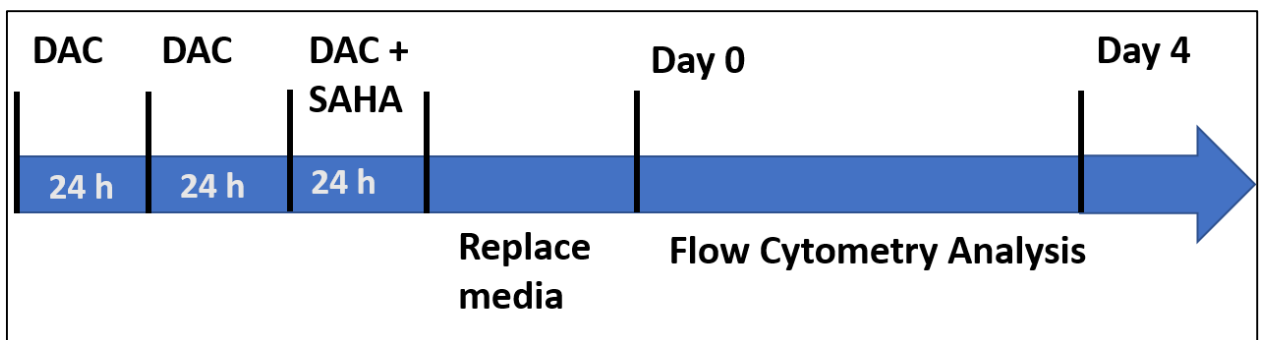

Figure 6.3.3: Combinatorial DAC + SAHA Treatment to Induce CD1d Expression in NSCLC

VI.IV.   Results

VI.IV.I.   High dose DAC treatment induces CD1d expression by SK-MES-1 cells

Experiments described previously demonstrated that treatment with DNMTi DAC led to an induction of CD1d expression at the mRNA level. In order to ascertain whether this induction could also occur at the protein level, SK-MES-1 cells were treated with PBS or DAC at 100 nM, 1 µM and 5 µM for 72 hours (Figure 6.1). Following this treatment, cells were given a rest period of either 2, 5 or 7 days to see whether an increase of CD1d was maintained over a period of time. MeOH vehicle was used as a negative control. At each time point cells were analysed by flow cytometry for the presence of CD1d. While there was an increase in CD1d expression on days 2 and 5, this was only significant at Day 5 for the higher concentrations of DAC ($p < 0.05$). These results show that treatment with DAC can induce CD1d expression in SK-MES-1 cells, and that this induction can be maintained for up to 5 days.

VI.IV.II.   Induction of CD1d by high dose DAC treatment in SK-MES-1 cells increases their susceptibility to iNKT cell degranulation

To assess whether the induction of CD1d by SK-MES-1 cells by DAC treatment can in turn induce cytotoxicity of iNKT cells, SK-MES-1 cells were treated with DAC at 100 nM, 1 µM and 5 µM as before. After 72 hours the cells were washed and cultured in medium for 4 days. The previous experiment found that a recovery period of 5 days showed optimal CD1d induction. Cells were then pulsed for 24 hours with αGalCer or 7Dw-8.5, and then co-cultured with iNKT cells with a mAb specific for CD107a. iNKT cells of greater than 99% purity were used for the co-culture (Figure 6.2A). Following
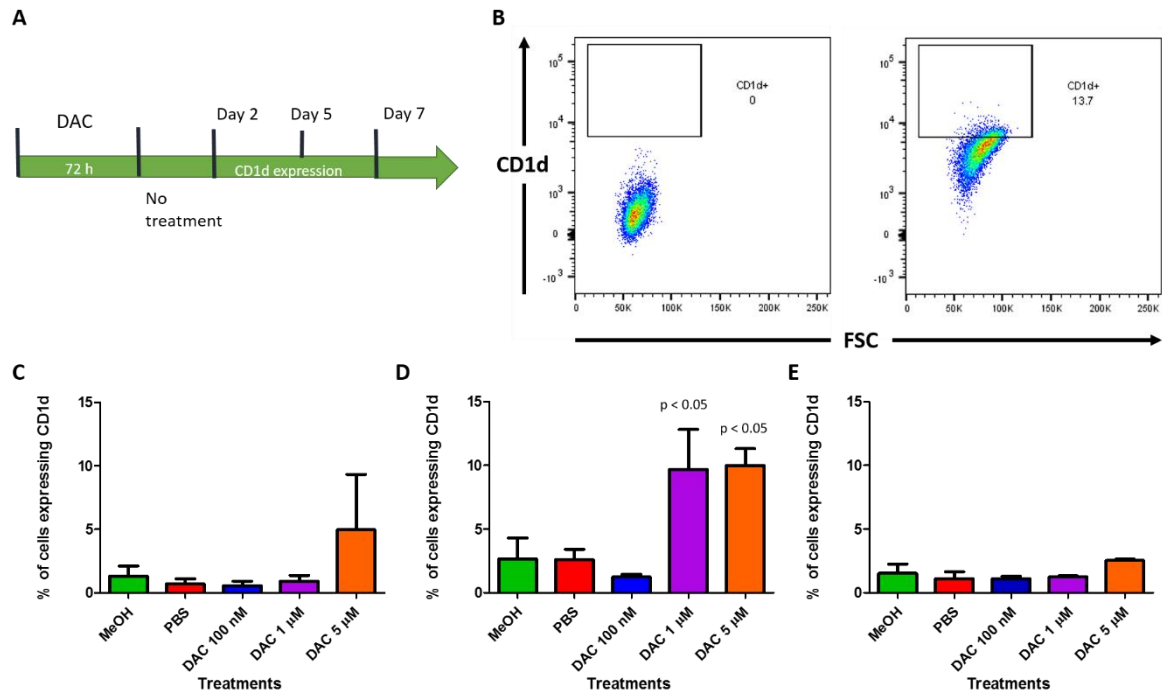
Figure 6.1: DAC induces up-regulation of CD1d in SK-MES-1 cell lines at the protein level. A) SK-MES-1 cells were given a once-off treatment of PBS or DAC at either 100 nM, 1 µM or 5 µM. MeOH was used as a vehicle control. After 72 hours the medium were replaced and cells were allowed a rest period of either 2 days, 5 days or 7 days, and then stained with mAb specific for CD1d and analysed using flow cytometry. **B)** Flow cytometry dot plots showing CD1d expression by an FMO control (left) and SK-MES-1 cells (right). **C- E)**. Mean (± SEM) percentages of SK-MES-1 cells expressing CD1d at days 2 **(C)**, 5 **(D)**, and 7 **(E)**. A one-way ANOVA with Dunnett's post hoc test was used to determine whether any significant increase in CD1d expression resulted following DAC treatment when compared to the vehicle control. Results are representative of three experiments.

Figure 6.2: iNKT cells degranulate in response to DAC-treated SK-MES-1 cell lines. SK-MES-1 cell lines were treated wit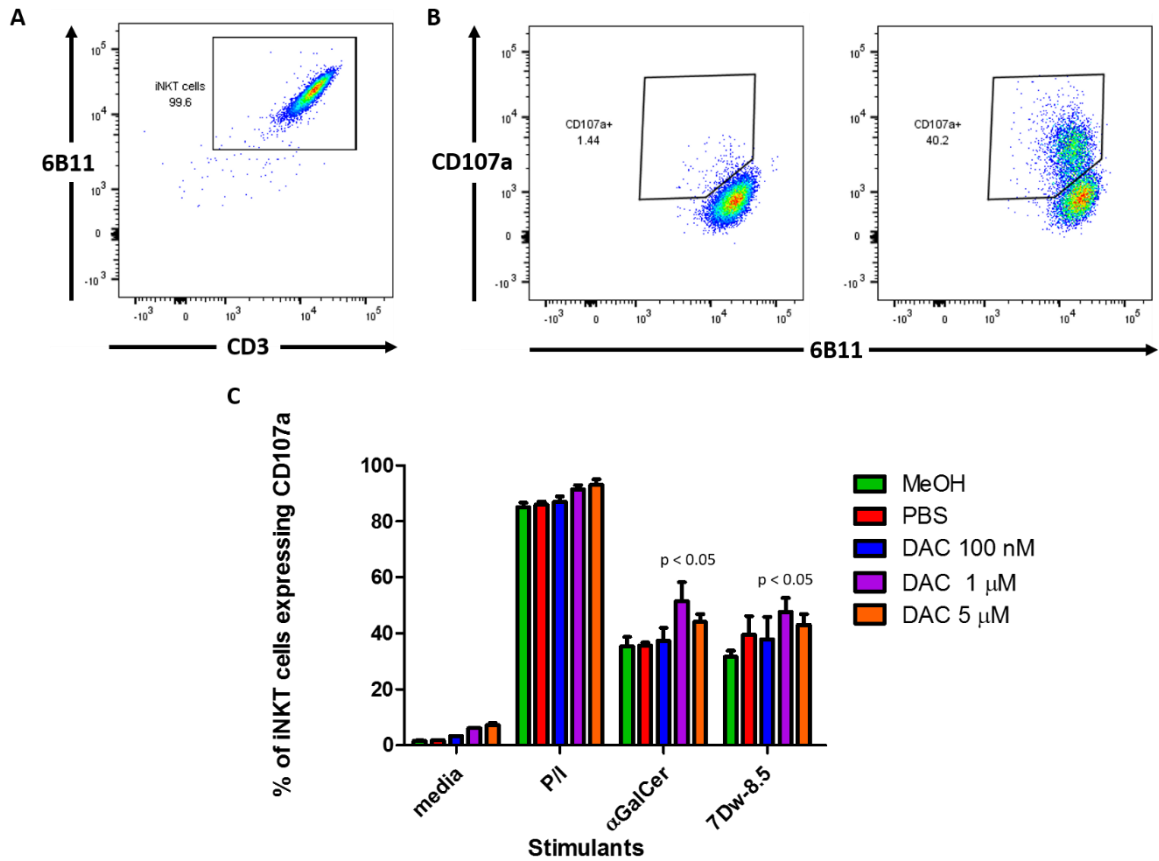h PBS or DAC (100 nM, 1 μM and 5 μM) for 72 h. MeOH was used as a negative control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing purity of iNKT cells as lymphocytes expressing CD3 and Vα24Jα18 T cell receptor, as detected by 6B11 antibody. **B)** Flow cytometry dot plots showing cell-surface CD107a expression by iNKT cells after exposure to vehicle (left) or DAC (right). **C)** Mean (± SEM) frequencies of iNKT cells that expressed CD107a after co-culture with DAC-treated SK-MES-1 cells pulsed with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated SK-MES-1 cells were compared to those exposed to SK-MES-1 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments.

a four hour co-culture, cells were stained with mAbs specific for CD3 and 6B11, and assessed by flow cytometry. iNKT cells co-cultured with SK-MES-1 cells treated with 1 µM DAC showed a statistically significant up-regulation of CD107a when stimulated with either αGalCer ($p < 0.05$) or 7Dw-8.5 ($p < 0.05$).

To determine whether particular iNKT sub-populations co-cultured with DAC-treated SK-MES-1 cells show an increase in degranulation, CD107a expression by CD4+ (Figure 6.3), CD8+ (Figure 6.4) and CD4-CD8- (Figure 6.5) cells was assessed by flow cytometry. SK-MES-1 cells were treated with DAC at 100 nM, 1 µM and 5 µM as before. After 72 hours the cells were washed and cultured in medium alone for four days. Cells were then pulsed for 24 hours with αGalCer or 7Dw8.5, and then co-cultured with iNKT cells stained with anti-CD107a mAb as before. Following a four hour co-culture, cells were stained with a panel of mAbs specific for CD4 and CD8, and assessed by flow cytometry. CD4+ cells co-cultured with SK-MES-1 cells treated with any concentration of DAC did not induce CD107a expression except when stimulated with PMA and ionomycin. (Figure 6.3C). CD8+ cells co-cultured with SK-MES-1 cells treated with 1 µM DAC and stimulated with either αGalCer ($p < 0.001$) or 7Dw-8.5 ($p < 0.001$) showed a statistically significant up-regulation of CD107a, as did CD8+ cells co-cultured with SK-MES-1 cells treated 5 µM DAC and stimulated with either αGalCer ($p < 0.05$) or 7Dw-8.5 ($p < 0.01$) (Figure 6.4B). CD4-CD8- cells co-cultured with SK-MES-1 cells treated with 1 µM DAC and pulsed with either αGalCer ($p < 0.01$) or 7Dw-8.5 ($p < 0.01$) showed a statistically significant up-regulation of CD107a (Figure 6.5B). Thus, epigenetic induction of CD1d by SK-MES-1 cells can target them for killing by CD8+ and CD4-CD8- iNKT cells, but not CD4+ iNKT cells *in vitro*.

Figure 6.3: CD4^{+} iNKT cells do not kill DAC-treated SK-MES-1 cell lines. SK-MES-1 cell lines were treated with PBS or DAC (100 nM, 1 μM and 5 μM) for 72 h. MeOH was used as a vehicle control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing frequencies of CD4^{+} and CD8^{+} within an iNKT cell line. **B)** Flow cytometry dot plot showing cell-surface CD107a expression by CD4^{+} iNKT cells after stimulation with medium (left) and P/I (right). **C)** Mean (± SEM) **f**requencies of CD4^{+} iNKT cells that expressed CD107a after co-culture with DAC-treated SK-MES-1 cells and stimulation with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated SK-MES-1 cells were compared to those exposed to SK-MES-1 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (***$p < 0.001$).
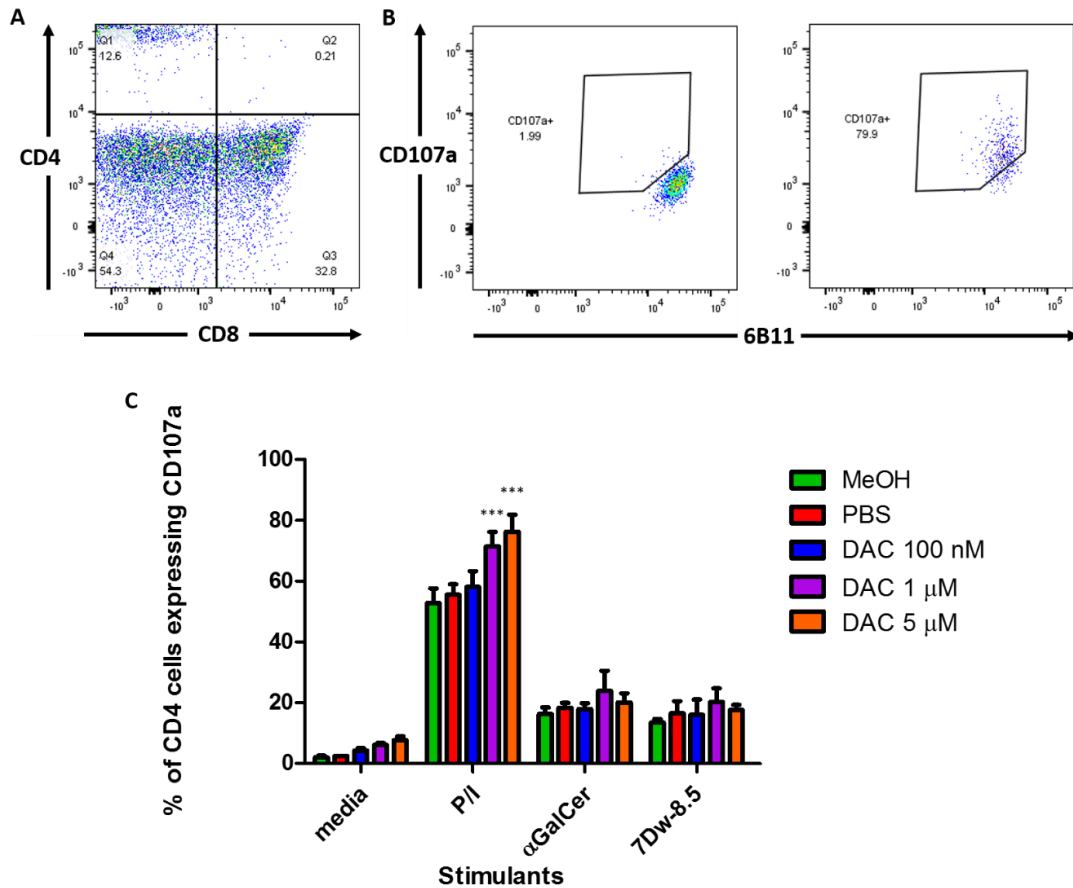
Figure 6.4: CD8⁺ iNKT cells kill DAC-treated SK-MES-1 cell lines. SK-MES-1 cell lines were treated with PBS or DAC (100 nM, 1 μM and 5 μM) for 72 h. MeOH was used as a vehicle control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing cell-surface CD107a expression by CD8⁺ iNKT cells after stimulation with medium (left) and P/I (right). **B)** Mean (± SEM) **f**requencies of CD8⁺ iNKT cells that expressed CD107a after co-culture with DAC-treated SK-MES-1 cells and stimulation with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated SK-MES-1 cells were compared to those exposed to SK-MES-1 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 6.5: CD4⁻CD8⁻ iNKT cells kill DAC-treated SK-MES-1 cell lines. SK-MES-1 cell lines were treated with PBS or DAC (100 nM, 1 μM and 5 μM) for 72 h. MeOH was used as a vehicle control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing cell-surface CD107a expression by CD4⁻CD8⁻ iNKT cells after stimulation with medium (left) and αGalCer (right). **C)** Mean (± SEM) frequencies of CD4⁻CD8⁻ iNKT cells that expressed CD107a after co-culture with DAC-treated SK-MES-1 cells and stimulation with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated SK-MES-1 cells were compared to those exposed to SK-MES-1 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (**$p < 0.01$).
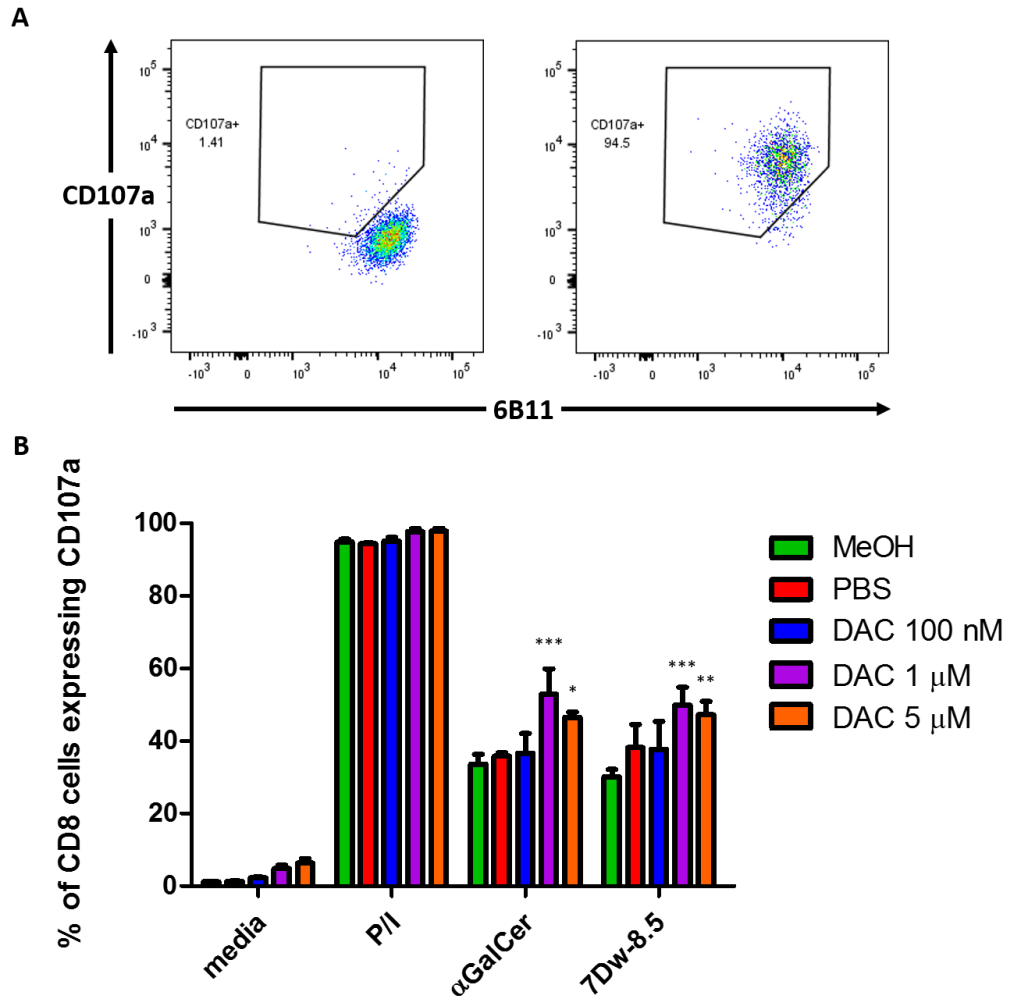
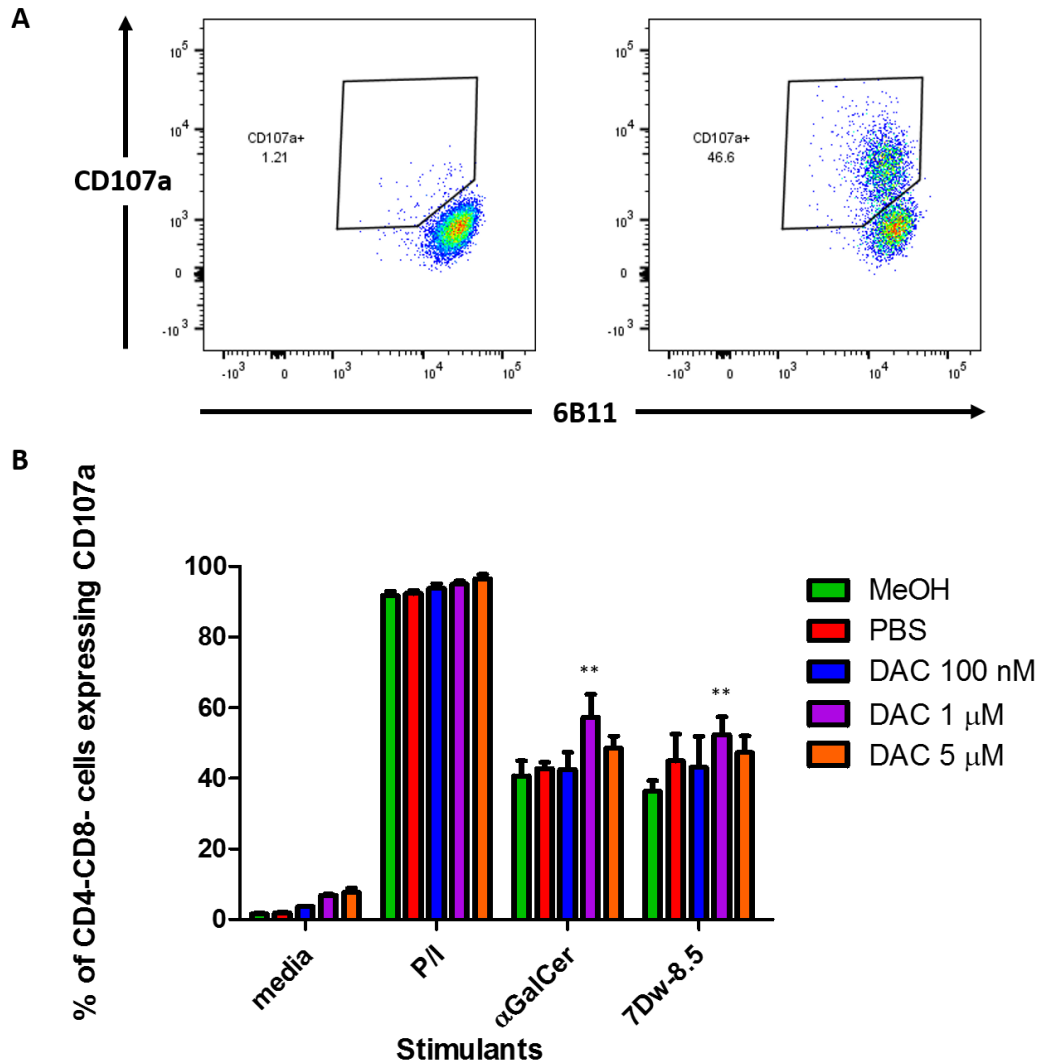### VI.IV.III. Transient low dose DAC treatment induces CD1d expression in SK-MES-1 cells

Past trials with high doses of DAC have been plagued by extreme toxicities (Abele *et al.*; 1987). Transient low doses of DAC have been shown to sustain long-term anti-tumour effects and are at least temporarily associated with their ability to maintain their targeting of DNMTs and alterations of gene expression (Tsai *et al.*, 2012). In order to test whether low doses of DAC could induce and maintain CD1d expression at the protein level, SK-MES-1 cells were treated with PBS or DAC at either 50 nM, 100 nM or 1 µM over 72 hours. Media and DAC were replaced every 24 hours. Cells were assessed for expression of CD1d by flow cytometry immediately following treatment and at four days post-treatment. An induction of CD1d was observed immediately following treatment (Figure 6.6C), but what is striking is the significant induction of CD1d at 1 µM DAC ($p = 0.0065$) at day 4 post-treatment (Figure 6.6D). In addition, the increased CD1d expression due to treatment with 1 µM DAC remained significant after 4 days of recovery ($p < 0.01$). These results show that transient low dose DAC treatment is accompanied by a prolonged induction of CD1d expression.

VI.IV.IV. Induction of CD1d by transient low dose DAC treatment in SK-MES-1 cells increases susceptibility to iNKT cell degranulation

To assess whether lower, less toxic concentrations of DAC can increase susceptibility of SK-MES-1 cells to cytoxicity by iNKT cells, SK-MES-1 cells were treated with PBS or DAC at 50 nM, 100 nM and 1 µM for 72 h. MeOH was used as a negative control. The media and treatments were replaced every 24 h. At three days post-treatment the cells were pulsed with glycolipids and then co-cultured with iNKT cells and a mAb specific for CD107a (Figure 6.7A). CD107a expression by iNKT cells were examined by flow cytometry (Figure 6.7B). iNKT cells co-cultured with SK-MES-1 cells treated with 50 nM DAC showed a statistically significant induction of CD107a when
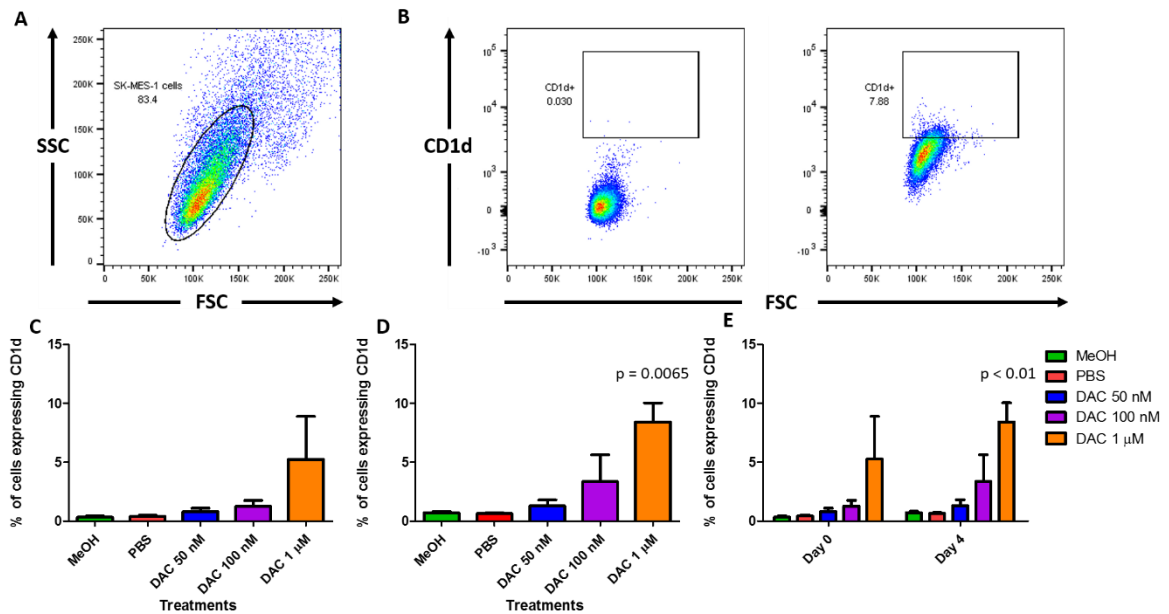
Figure 6.6: Transient low dose DAC treatment induces expression of CD1d in SK-MES-1 cell lines. SK-MES-1 cells were treated with PBS or DAC at either 50 nM, 100 nM or 1 µM for 72 hours. Both media and DAC were replaced every 24 hours. MeOH was used as a vehicle control. After 72 hours cells were stained with anti-CD1d mAb and analysed immediately by flow cytometry. Cells were also cultured in medium for four hours and then stained with a mAb specific for CD1d and analysed by flow cytometry. **A)** Flow cytometric dot plot showing FSC and SSC properties of gated SK-MES-1 cells. **B)** Flow cytometry dot plots showing CD1d expression in an FMO control (left) and SK-MES-1 cells (right). **C - D)** Mean (± SEM) frequencies of SK-MES-1 cells that expressed CD1d immediately following treatment **(C)** and at day 4 following treatment **(D)** with various concentrations of DAC. A one-way ANOVA with Dunnett's post hoc test was used to determine significance. **E)** Mean (± SEM) frequencies of SK-MES-1 cells that expressed CD1d immediately and at day 4 following treatment with various concentrations of DAC. A two-way ANOVA with Bonferroni's post hoc test was used to determine significance of treatments at day 4 compared to day 0. Results are representative of three experiments.
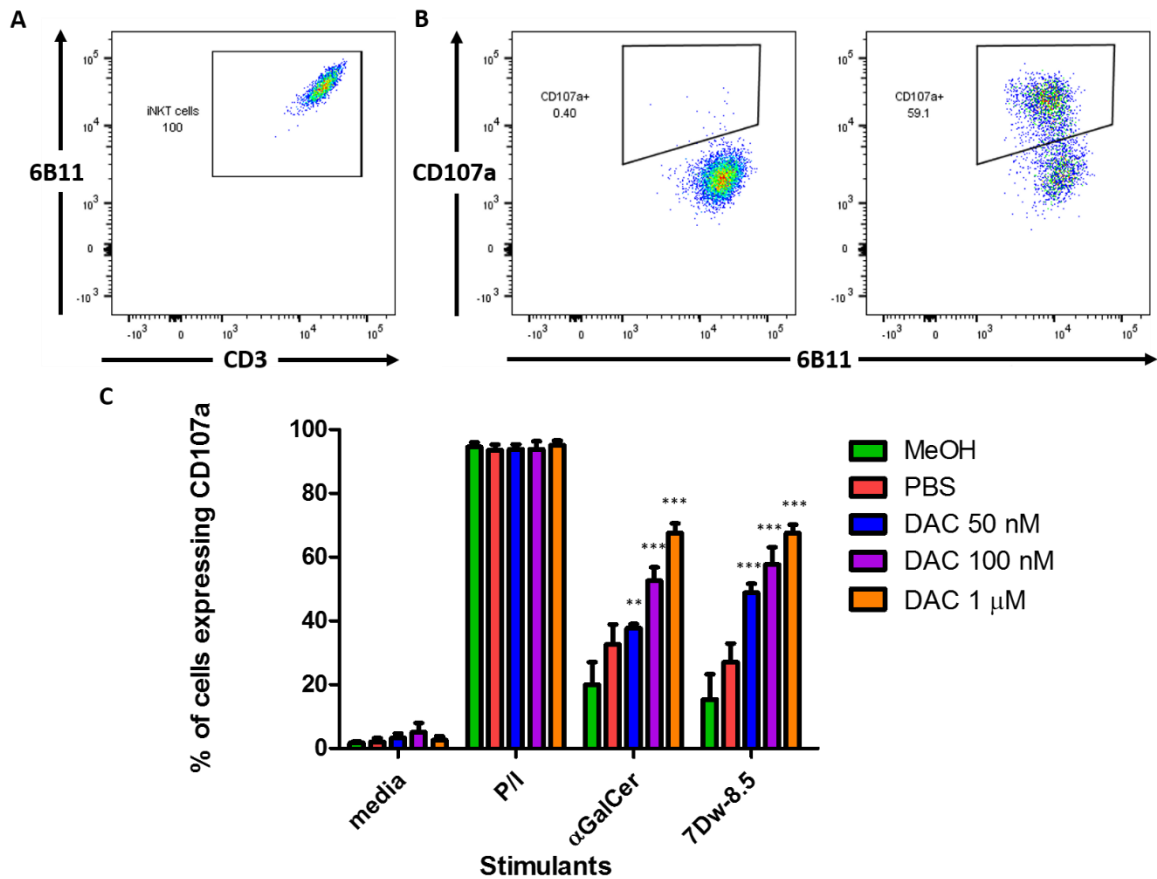
Figure 6.7: iNKT cells degranulate in response to low dose DAC-treated SK-MES-1 cells. SK-MES-1 cell lines were treated with PBS or DAC (50 nM, 100 nM and 1 µM) at 24, 48 and 72 hours. MeOH was used as a negative control. After a rest period of 3 days the cells were pulsed with either αGalCer or 7Dw8.5 for 24 hours, and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing purity of iNKT cells as lymphocytes expressing CD3 and Vα24Jα18 T cell receptor, as detected by 6B11 antibody. **B)** Flow cytometry dot plot showing cell-surface CD107a expression by iNKT cells after exposure to vehicle (left) or DAC (right). **C)** Mean (± SEM) frequencies of iNKT cells that expressed CD107a after co-culture with DAC-treated SK-MES-1 cells pulsed with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated SK-MES-1 cells were compared to those exposed to SK-MES-1 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments. (**p < 0.01, ***p < 0.001).

stimulated with αGalCer (p < 0.01) and 7Dw-8.5 (p < 0.001). SK-MES-1 cells treated with 100 nM or 1 µM DAC also showed increased susceptibility to iNKT cell degranulation when stimulated with αGalCer (p < 0.001) and 7Dw-8.5 (p < 0.001).

To determine whether iNKT sub-populations co-cultured with low dose DAC-treated SK-MES-1 cells show an increase in degranulation, CD107a expression by CD4$^+$ (Figure 6.8), CD8$^+$ (Figure 6.9) and CD4$^-$CD8$^-$ (Figure 6.10) cells was assessed by flow cytometry. SK-MES-1 cells were treated with DAC at 50 nM, 100 nM and 1 µM as before. After 72 hours the cells were washed and cultured in media alone for three days. Cells were pulsed for 24 hours with αGalCer and 7Dw8.5, and then co-cultured with iNKT cells. Following a four hour co-culture with a mAb specific for CD107a, cells were stained with mAbs specific for CD3, CD4, CD8 and Vα24Vβ11 and assessed by flow cytometry. CD4$^+$ iNKT cells co-cultured with SK-MES-1 cells treated with 50 nM, 100 nM or 1 µM DAC showed statistically significant up-regulation of CD107a compared to cells cultured with vehicle-treated SK-MES-1 cells (p < 0.05, p < 0.01 and p < 0.001 respectively) (Figure 6.8C). CD8$^+$ cells co-cultured with SK-MES-1 cells treated with 50 nM – 1 µM DAC and pulsed with either αGalCer or 7Dw-8.5 also showed a statistically significant up-regulation of CD107a (p < 0.05 and p < 0.001) (Figure 6.10B). Similarly CD4$^-$CD8$^-$ cells co-cultured with SK-MES-1 cells treated with 50 nM – 1 µM DAC and pulsed with either αGalCer or 7Dw-8.5 showed a statistically significant up-regulation of CD107a (p < 0.01 to p < 0.001) (Figure 6.10B). These results suggest that low dose DAC is superior to high dose DAC in its ability to make SK-MES-1 cells susceptible to iNKT cell cytolysis because, unlike high dose DAC, low dose DAC sensitises SK-MES-1 cells to CD4$^+$ iNKT cells.

Figure 6.8: CD4⁺ iNKT cells kill DAC-treated SK-MES-1 cell lines. SK-MES-1 cell lines were treated with PBS or DAC (50 nM, 100 nM and 1 μM) for 72 h. Media and treatments were replaced every 24 hours. MeOH was used as a vehicle control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing frequencies of CD4⁺ and CD8⁺ cells within an iNKT cell line. **B)** Flow cytometry dot plot showing cell-surface CD107a expression by CD4⁺ iNKT cells after stimulation with medium (left) and P/I (right). **C)** Mean (± SEM) **f**requencies of CD4⁺ iNKT cells that expressed CD107a after co-culture with DAC-treated SK-MES-1 cells and pulsed with glycolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated SK-MES-1 cells were compared to those exposed to SK-MES-1 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (*p < 0.05, **p < 0.01 and ***p < 0.001).
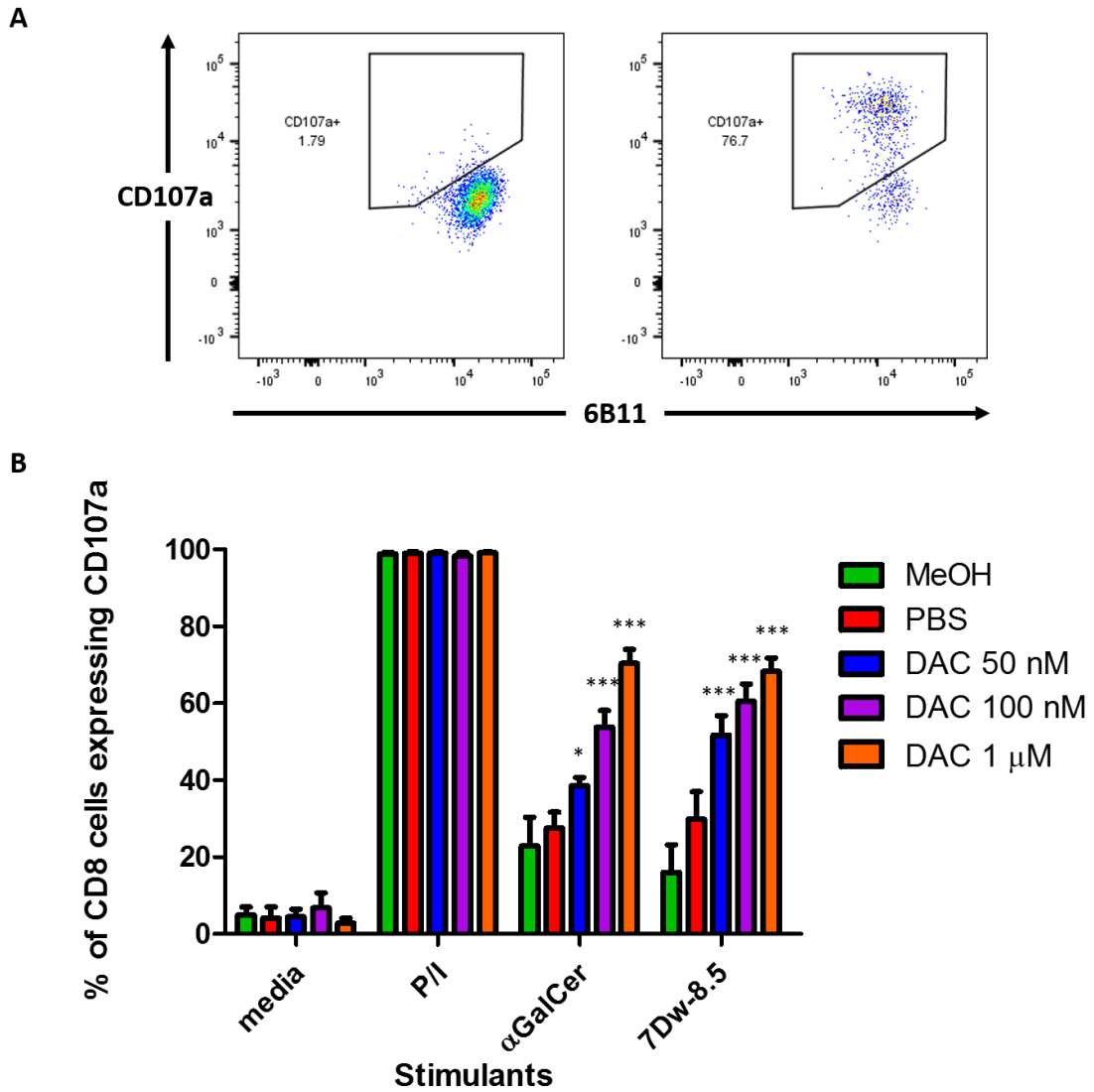
Figure 6.9: CD8⁺ iNKT cells kill DAC-treated SK-MES-1 cell lines. SK-MES-1 cell lines were treated with PBS or DAC (50 nM, 100 nM and 1 μM) for 72 h. Media and treatments were replaced every 24 hours. MeOH was used as a vehicle control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing cell-surface CD107a expression by CD8⁺ iNKT cells after stimulation with medium (left) and αGalCer (right). **B)** Mean (± SEM) **f**requencies of CD8⁺iNKT cells that expressed CD107a after co-culture with DAC-treated SK-MES-1 cells and stimulation with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated SK-MES-1 cells were compared to those exposed to SK-MES-1 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (*p < 0.05, and ***p < 0.001).
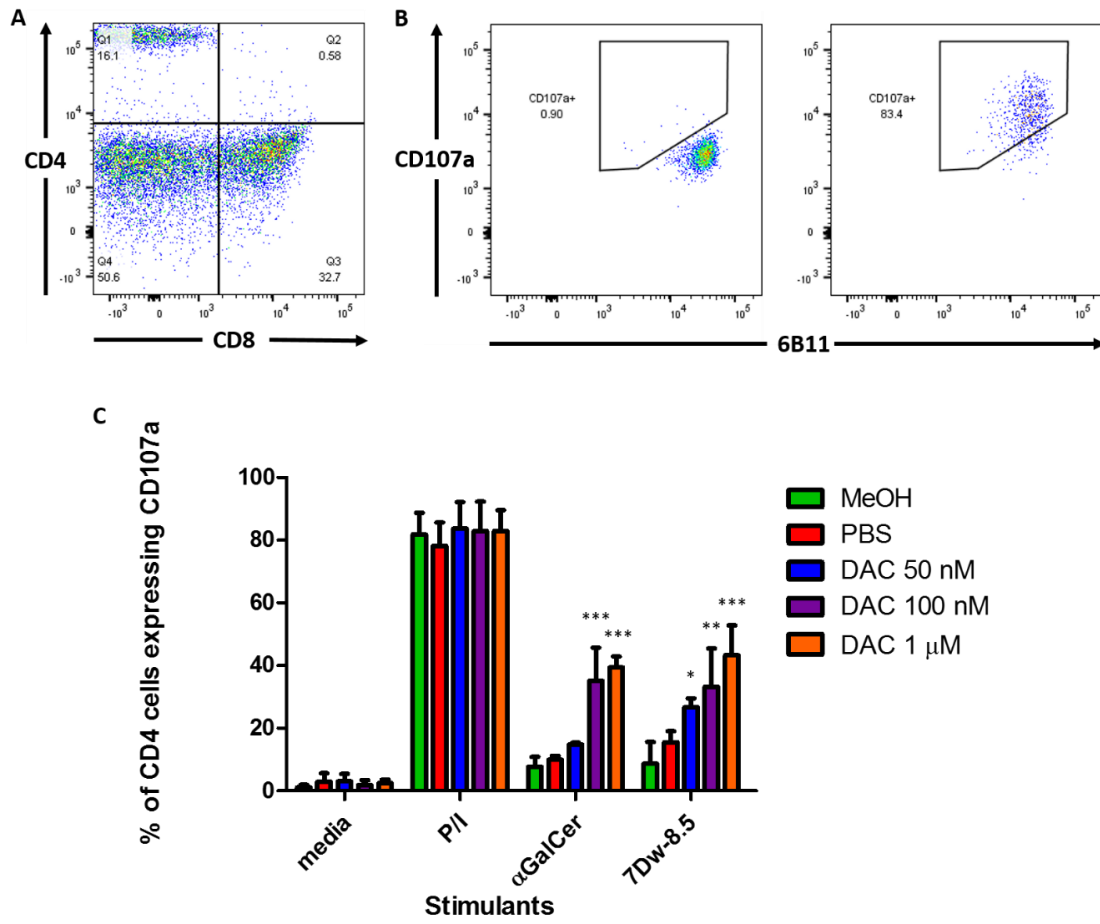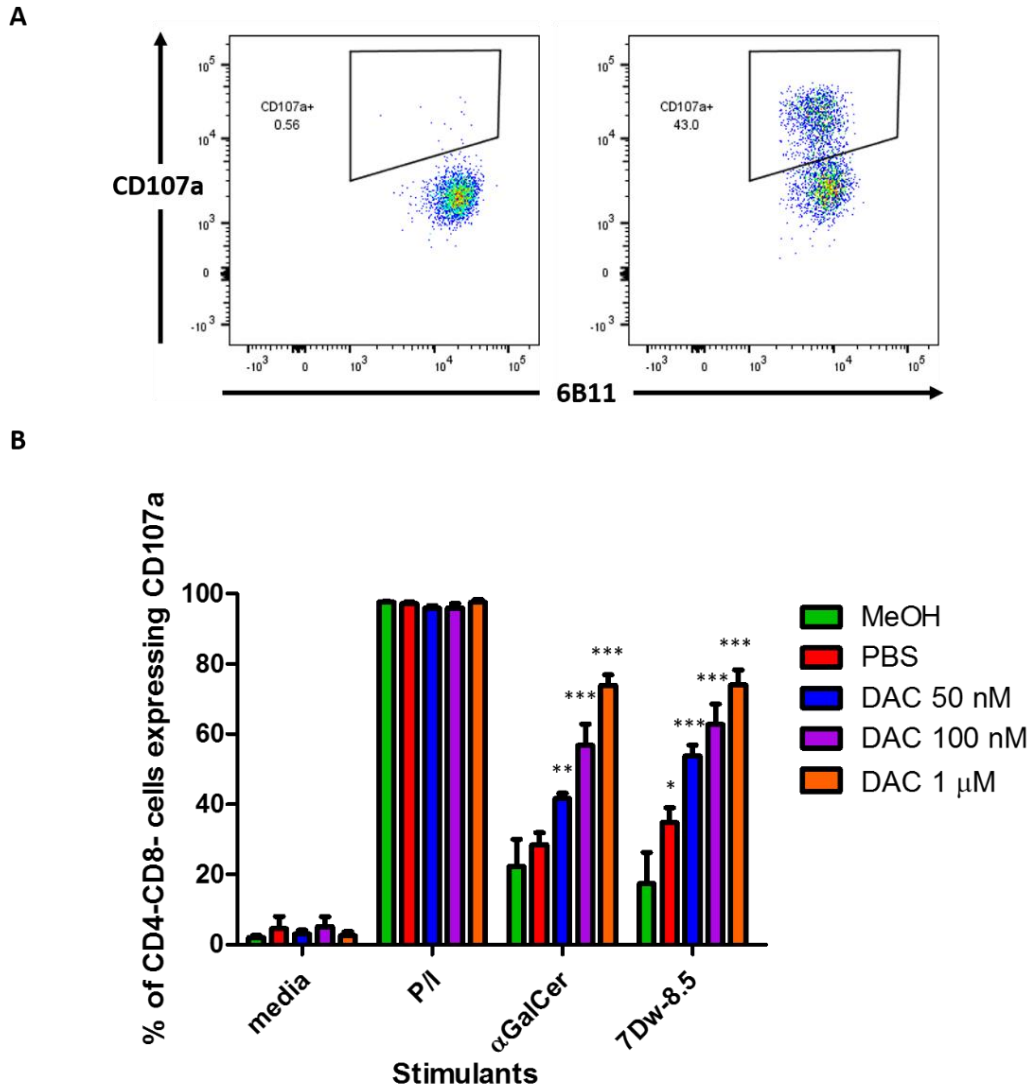
Figure 6.10: CD4⁻CD8⁻ iNKT cells kill DAC-treated SK-MES-1 cell lines. SK-MES-1 cell lines were treated with PBS or DAC (50 nM, 100 nM and 1 μM) for 72 h. Media and treatments were replaced every 24 hours. MeOH was used as a vehicle control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing cell-surface CD107a expression by CD4⁻CD8⁻ iNKT cells after stimulation with medium (left) and αGalCer (right). **B)** Mean (± SEM) **f**requencies of CD4⁻CD8⁻ iNKT cells that expressed CD107a after co-culture with DAC-treated SK-MES-1 cells and stimulation with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated SK-MES-1 cells were compared to those exposed to SK-MES-1 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (*p < 0.05, **p < 0.01 and ***p < 0.001).

# VI.IV.V.   Combinatorial DAC and SAHA treatment induces CD1d in SK-MES-1 cell lines

Heavily methylated DNA correlates with chromatin that are in a transcriptionally repressive state due to hypomethylated histones (Eden *et al.*, 1998). The epigenetic processes of DNA methylation and histone deacetylation have been linked; studies have shown that the methyl-CpG-binding protein MeCP2 appears to be present in a complex with histone deacetylase activity (Nan *et al.*, 1998 and Jones *et al.*, 1998). MeCP2 interacts specifically with DNA methylation to facilitate transcriptional repression (Nan *et al.*, 1998) and this repression can be reversed by inhibiting histone deacetylase (Jones *et al.*, 1998). Cameron and colleagues showed that they could reactivate several hypermethylated genes by TSA and DAC in combination (Cameron *et al.*, 1999). SAHA is another HDACi that has a similar structure to TSA and affects the same histones. As SAHA is a FDA-approved drug it was decided to perform the following experiments with SAHA rather than TSA.

To assess whether combined DAC and SAHA treatment could induce sustained expression of CD1d in SK-MES-1 cells, SK-MES-1 cell lines were cultured in the presence of PBS or DAC at 100 nM, 500 nM and 1 µM for 72 hours (Figure 6.11). The media and treatments were replaced every 24 h. After 48 hours of treatment 5 µM SAHA was added to cells receiving DAC treatment for the final 24 hours. MeOH was used as a vehicle control. CD1d expression was analysed immediately (Figure 6.11C) or at day 4 post-treatment (Figure 6.11D). A statistically significant induction of CD1d was observed at day 4 after combined treatment with either 100 nM, 500 nM or 1 µM DAC and 5 µM SAHA ($p = 0.03$, 0.009 and 0.0002 respectively) compared with the vehicle control (Figure 6.11D). In addition, comparison of the effect of treatment on CD1d expression at day 4 compared to day 0 showed a statistically significant up-regulation of CD1d with all treatments ($p < 0.001$) at day 4 (Figure 6.11E).
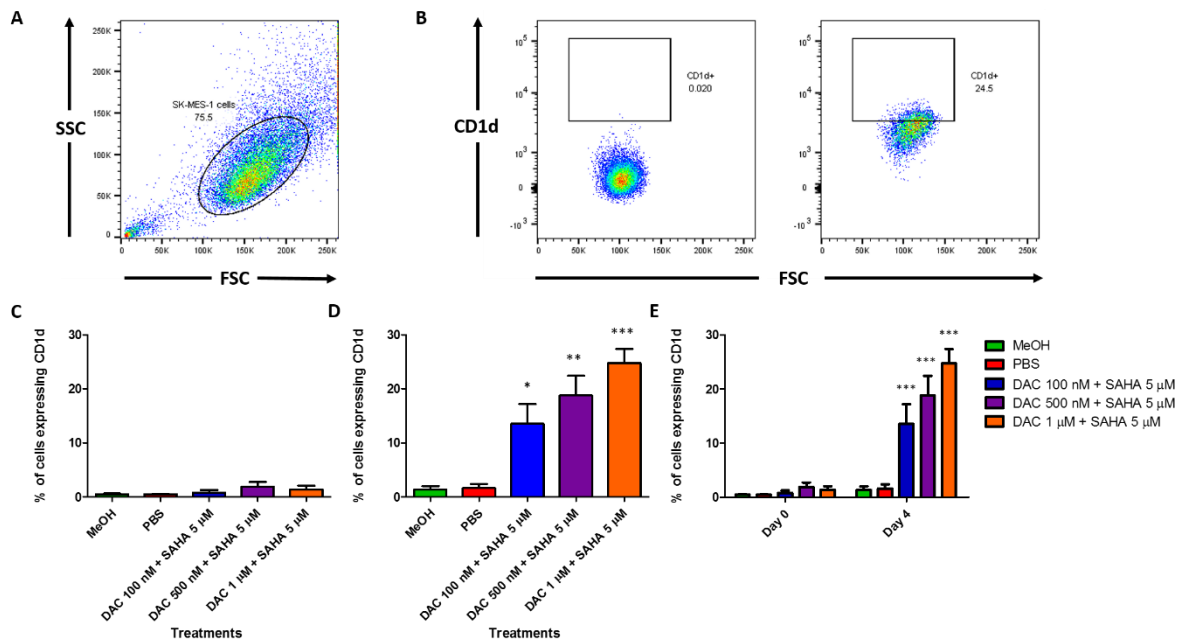
Figure 6.11: Combinatorial DAC and SAHA treatment induces expression of CD1d in SK-MES-1 cell lines. Cells were treated with PBS and DAC at either 100 nM, 500 nM or 1 µM for 72 hours. Media and DAC were replaced every 24 hours. After 48 hours 5 µM SAHA was added to the cells treated with DAC. MeOH was used as a negative control. Cells were analysed for expression of CD1d immediately after treatment and at Day 4 post treatment. **A)** Flow cytometric dot plot showing FSC and SSC properties of SK-MES-1 cells **B)** Flow cytometry dot plots showing CD1d expression by an FMO control (left) and gated SK-MES-1 cells (right). **C - D)** Mean (± SEM) frequencies of SK-MES-1 cells that expressed CD1d immediately following treatment **(C)** at day 4 following treatment **(D)**. A one-way ANOVA with Dunnett's post hoc test was used to determine significance. **E)** Mean (± SEM) frequencies of SK-MES-1 cells that expressed CD1d immediately and at day 4 following treatment. A two-way ANOVA with Bonferroni's post hoc test was used to determine significance of treatments at day 4 compared to day 0. Results are representative of three experiments. (*p = 0.03, **p = 0.009 and ***p < 0.001)

VI.IV.VI. Induction of CD1d in SK-MES-1 cells by combined DAC and SAHA treatment does not correlate with increased susceptibility to iNKT cell degranulation

To assess whether combined DAC and SAHA treatment can increase susceptibility of SK-MES-1 cells to cytoxicity by iNKT cells, SK-MES-1 cells were treated with PBS or DAC at 100 nM, 500 nM and 1 µM for 72 hours. The media and treatments were replaced every 24 hours. After 48 hours of treatment, 5 µM SAHA was added to the cells receiving DAC treatment. At three days post-treatment the cells were pulsed with glycolipids for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. Cell-surface expression of CD107a by iNKT cells was examined by flow cytometry (Figure 6.12). iNKT cells co-cultured with SK-MES-1 cells treated with 1 µM DAC and 5 µM SAHA showed a statistically significant decrease of CD107a when stimulated with αGalCer ($p < 0.05$) compared to iNKT cells co-cultured with vehicle control-treated SK-MES-1 cells. None of the treatments to SK-MES-1 cells resulted in significant up-regulation of CD107a by iNKT cells compared to untreated SK-MES-1 cells.

iNKT sub-populations were examined by flow cytometry to assess CD107a expression. One iNKT cell line used was homogeneous for CD4^{+} cells (Figure 6.13A), whereas most iNKT cell lines contained CD4^{+}, CD8^{+} and double-negative cells (Figure 6.13C). Statistical analysis of CD107a expression by CD8^{+} and CD4^{-}CD8^{-} populations was not possible as only two iNKT cell lines used contained these sub-populations. None of the iNKT cell subsets upregulated C107a upon co-culture with glycolipid-pulsed SK-MES-1 cells. CD107a up-regulation by CD4^{+} cells from the CD4^{+} iNKT cell line and within a mixed CD4^{+}, CD8^{+} and double-negative iNKT cell line co-cultured with DAC and SAHA-treated SK-MES-1 cells and stimulated with PMA and ionomycin is depicted in Figure 4.13B and D respectively. The iNKT cell populations homogeneous for CD4^{+}cells did not experience the same induction of CD107a when co-cultured with DAC and SAHA- treated
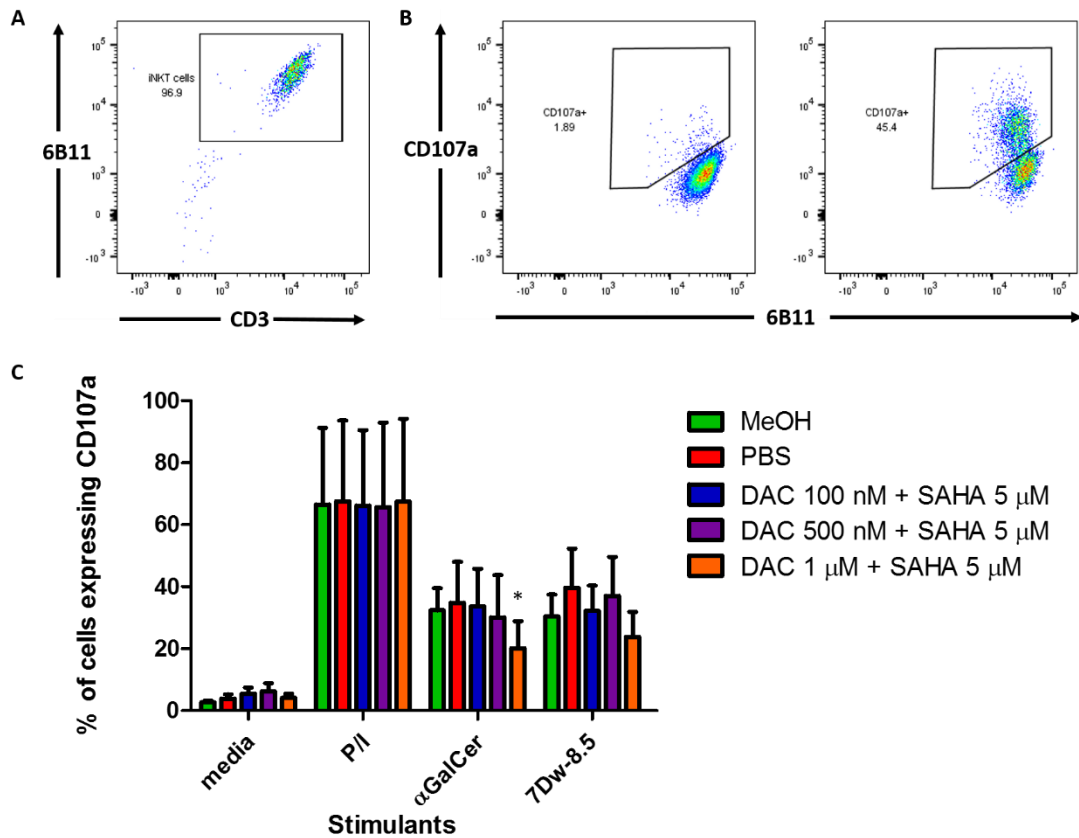
Figure 6.12: iNKT cells do not kill DAC and SAHA-treated SK-MES-1 cells. SK-MES-1 cell lines were treated with PBS and DAC (100 nM, 500 nM and 1 µM) at 24, 48 and 72 hours culture. At 72 hours, 5 µM SAHA was added to cells treated with DAC. MeOH treatment was used as a negative control. After a rest period of 3 days the cells were pulsed with either αGalCer or 7Dw8.5 for 24 hours, and then co-cultured with iNKT cells and mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing purity of iNKT cells as lymphocytes expressing CD3 and Vα24Jα18 T cell receptor, as detected by 6B11 antibody. **B)** Flow cytometry dot plot showing cell-surface CD107a expression by iNKT cells stimulated SK-MES-1 cells cultured in medium (left) and αGalCer (right). **C)** Mean (± SEM) frequencies of iNKT cells that expressed CD107a after co-culture with DAC- and SAHA-treated SK-MES-1 cells and stimulation with gylcolipids. The percentages of iNKT cells that expressed CD107a following each treatment were compared with those of iNKT cells treated with MeOH-treated SK-MES-1 and pulsed with the same glycolipids using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments. Results are representative of three experiments. (*p < 0.05).
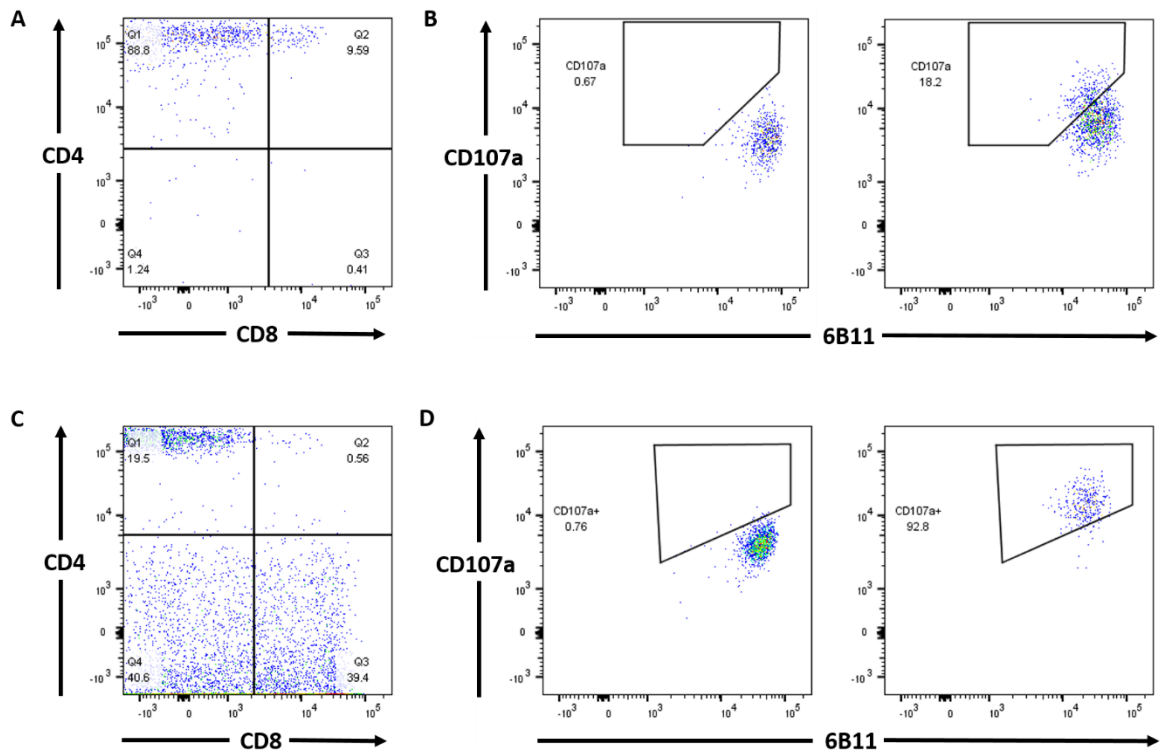
Figure 6.13: CD107a expression by CD4⁺ iNKT cells stimulated with PMA and ionomycin. iNKT cells were co-cultured with DAC and SAHA-treated SK-MES-1 cells (as described in the legend to Figure 6.12) and a mAb specific for CD107a, and stimulated with PMA and ionomycin. Following four hours of co-culture cells were stained with mAbs specific for CD4 and CD8, and analysed by flow cytometry. **A)** Flow cytometric dot plot showing CD4 and CD8 expression by an iNKT cell line which is homogeneous for CD4⁺. **B)** Cell-surface CD107a expression by the CD4⁺ iNKT cells shown in part A stimulated with PMA and ionomycin (left) and an FMO control (right). **C)** Flow cytometric dot plot showing CD4 and CD8 expression by an iNKT cell line which is heterogeneous for CD4⁺ and CD8⁺ cells. **D)** Cell-surface CD107a expression by CD4⁺ iNKT cells shown in part C stimulated with PMA and ionomycin (left) and an FMO control (right).

SK-MES-1 cells and stimulated with PMA and ionomycin compared with iNKT cell populations that were heterogeneous for CD4^{+}, CD8^{+} and double negative cells. These results suggest that CD8^{+} and double negative iNKT are critical for cytolytic degranulation by iNKT cells, and may be required for cytolytic degranulation by CD4^{+} iNKT cells.

VI.IV.VI.   High dose DAC treatment induces CD1d expression by A549 cells

Lung adenocarcinoma is the most commonly diagnosed histological subtype of NSCLC (Travis, 2011); nearly 40% of all lung cancers diagnosed in the United States are adenocarcinoma. Experiments described in Chapter V demonstrated that treatment of A549 cells with the DNMTi DAC led to an induction of CD1d expression at the mRNA level. In order to ascertain whether this induction could also occur at the protein level, A549 cells were treated with PBS or DAC at 100 nM, 1 μM and 5 μM for 72 hours (Figure 6.14). Following this treatment, cells were given a rest period of either 2, 5 or 7 days to see whether an increase of CD1d was maintained over a period of time. MeOH vehicle was used as a negative control. At each time point cells were analysed by flow cytometry for the presence of CD1d. At each time point there was a significant increase in CD1d expression by A549 cells treated with 5 μM DAC ($p < 0.0001$). At day 7 of recovery A549 cells treated with DAC at 1 μM also showed a significant increase in CD1d expression ($p = 0.02$). These results show that treatment with DAC can induce CD1d expression in A549 cells, and that this induction can be maintained over a number of days.
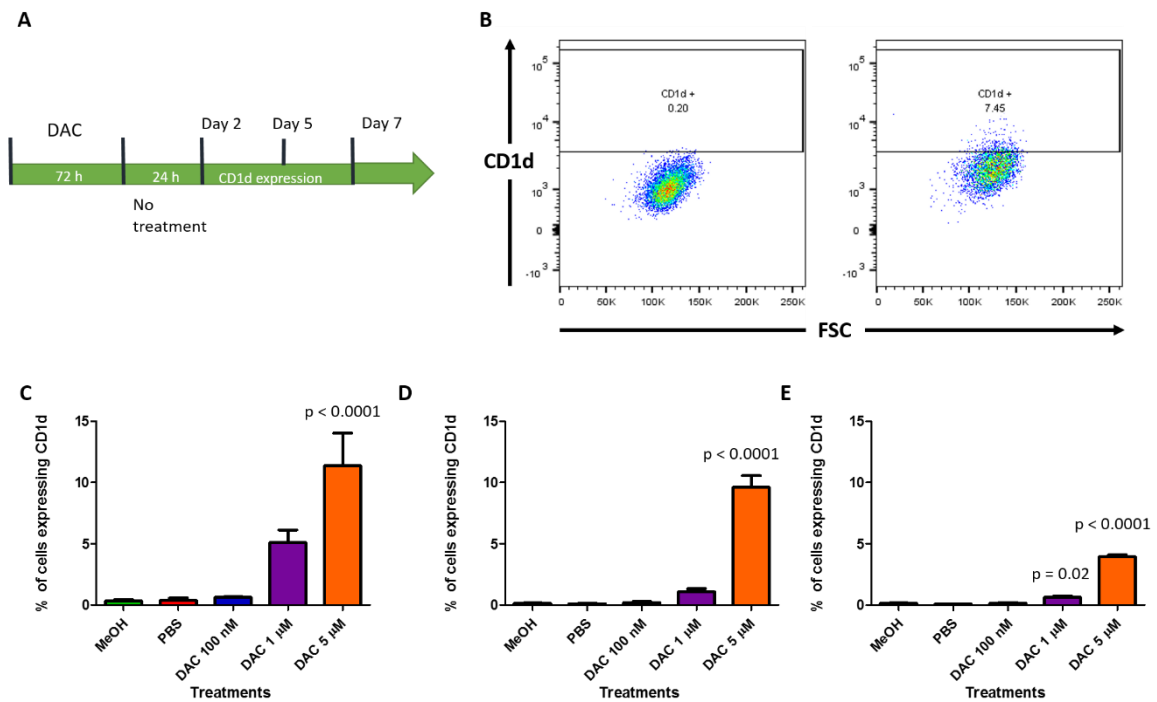
Figure 6.14: DAC induces up-regulation of CD1d in A549 cell lines at the protein level. A) A549 cells were given a one-off treatment of PBS or DAC at either 100 nM, 1 µM or 5 µM. MeOH was used as a vehicle control. After 72 hours the media were replaced. Cells were allowed a rest period of either 2 days, 5 days or 7 days, and then stained with a mAb specific for CD1d and analysed using flow cytometry. **B)** Flow cytometry dot plots showing CD1d expression in an FMO control (left) and A549 cells (right). **C- E)**. Mean (± SEM) percentages of A549 cells expressing CD1d at days 2 **(C)**, 5 **(D)**, and 7 **(E)**. A one-way ANOVA with Dunnett's post hoc test was used to determine whether any significant increase in CD1d expression resulted following DAC treatment when compared to the vehicle control. Results are representative of three experiments.

VI.IV.VII. Induction of CD1d expression by A549 cells using high dose DAC treatment increases their susceptibility to iNKT cell cytolytic degranulation

To assess whether the induction of CD1d by A549 cells by DAC treatment can in turn induce cytotoxicity of iNKT cells, A549 cells were treated with DAC at 100 nM, 1 µM and 5 µM as before. After 72 hours the cells were washed and cultured in media alone for 6 days. Figure 6.14 shows that CD1d induction was maintained for up to 7 days. Cells were pulsed for 24 hours with αGalCer or 7Dw-8.5, and then co-cultured with iNKT cells with a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. iNKT cells of greater than 99% purity were used for the co-cultures (Figure 6.15A). Following a four hour co-culture, cells were stained with mAbs specific for CD3 and 6B11, and assessed by flow cytometry (Figure 6.15B). iNKT cells co-cultured with A549 cells treated with 100 nM DAC showed a statistically significant increase of CD107a when stimulated with either αGalCer (p < 0.01) or 7Dw-8.5 (p < 0.05) (Figure 6.15C). iNKT cells co-cultured with A549 cells treated with 1 µM and 5 µM DAC showed a dose-dependent up-regulation of CD107a when stimulated with either αGalCer (p < 0.001) or 7Dw-8.5 (p < 0.001). These results demonstrate that high dose DAC treatment can increase CD1d expression in A549 cells, and that this increase correlates with increased susceptibility to iNKT cell cytolytic degranulation.

To determine whether specific iNKT sub-populations co-cultured with DAC-treated A549 cells show an increase in degranulation, CD107a expression by $CD4^+$ (Figure 6.16), $CD8^+$ (Figure 6.17) and $CD4^-CD8^-$ (Figure 6.18) cells was assessed by flow cytometry. A549 cells were treated with DAC at 100 nM, 1 µM and 5 µM as before. After 72 hours the cells were washed and cultured in medium alone for six days. Cells were then pulsed for 24 hours or αGalCer and 7Dw8.5, and co-cultured with iNKT cells with a mAb specific for CD107a. iNKT cells of greater than 99% purity
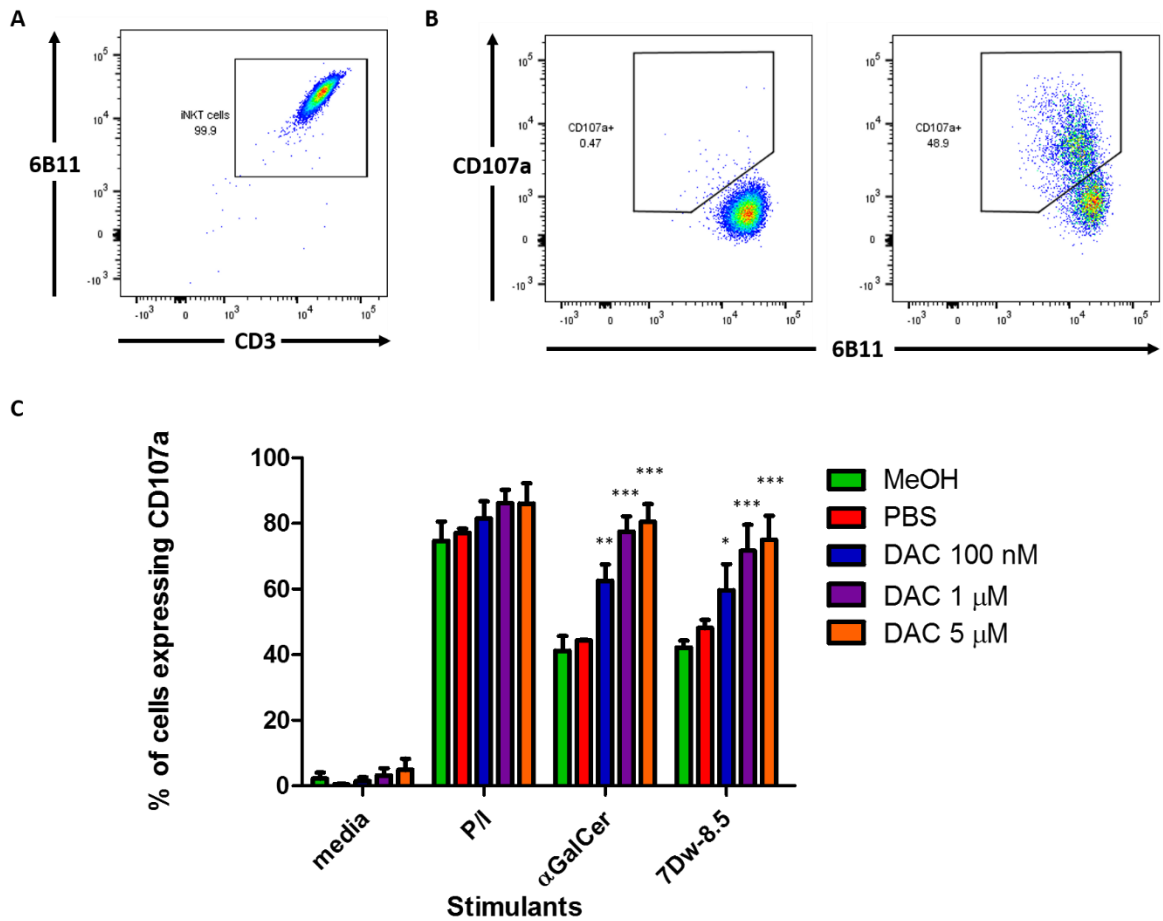
Figure 6.15: iNKT cells degranulate in response to DAC-treated A549 cell lines. A549 cell lines were treated with PBS or DAC (100 nM, 1 μM and 5 μM) for 72 h. MeOH was used as a negative control. After a rest period of 6 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing purity of iNKT cells as lymphocytes expressing CD3 and Vα24Jα18 T cell receptor, as detected by 6B11 antibody. **B)** Flow cytometry dot plot showing cell-surface CD107a expression by iNKT cells after exposure to vehicle (left) or DAC (right). **C)** Mean (± SEM) **f**requencies of iNKT cells that expressed CD107a after co-culture with DAC-treated A549 cells pulsed with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated SK-MES-1 cells were compared to those exposed to SK-MES-1 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments. (*p < 0.05, **p <0.01, ***p < 0.001).

were used in these experiments. Following a four hour co-culture, cells were stained with a panel of mAbs specific for CD4 and CD8, and assessed by flow cytometry. CD4$^+$ iNKT cells co-cultured with A549 cells treated with either 1 µM DAC (p < 0.01) or 5 µM DAC (p < 0.01) and pulsed with αGalCer showed a statistically significant up-regulation of CD107a (Figure 6.16C). CD8$^+$ cells co-cultured with A549 cells treated with either 100 nM, 1 µM or 5µM DAC and pulsed with either αGalCer (p < 0.001) or 7Dw-8.5 (p < 0.01, p < 0.001) showed a statistically significant dose-dependent induction of CD107a (Figure 6.17B). CD4$^-$CD8$^-$ cells co-cultured with A549 cells treated 100 nM – 5 µM DAC and stimulated with either αGalCer (p < 0.01, p < 0.010) or 7Dw-8.5 (p < 0.05, p < 0.001) also showed a statistically significant and dose dependent up-regulation of CD107a (Figure 6.18B). These results indicate that CD4$^+$ iNKT cells may kill DAC-treated A549 cells but that much greater frequencies of CD8$^+$ and double negative iNKT cells are capable of such cytotoxicity.

VI.IV.VIII. Transient low dose DAC treatment induces CD1d expression in A549 cells

In order to test whether low doses of DAC could induce and maintain CD1d expression at the protein level, A549 cells were treated with PBS or DAC at either 50 nM, 100 nM or 1 µM over 72 hours. Media and DAC were replaced every 24 hours. Cells were assessed for expression of CD1d by flow cytometry immediately following treatment and at four days post-treatment. A significant induction of CD1d expression was observed when A549 cells were treated with 1 µM DAC only (p = 0.0276) (Figure 6.19B), and strikingly this expression was maintained at day 4 post-treatment (p = 0.0007) (Figure 6.19C). These results show that transient low dose DAC treatment of A549 cells is accompanied by a prolonged induction of CD1d expression.

Figure 6.16: CD4$^+$ iNKT cells kill DAC-treated A549 cell lines. A549 cell lines were treated with PBS or DAC (100 nM, 1 μM and 5 μM) for 72 h. MeOH was used as a vehicle control. After a rest period of 6 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing frequencies of CD4$^+$ and CD8$^+$ within an iNKT cell line. **B)** Flow cytometry dot plot showing cell-surface CD107a expression by CD4$^+$ iNKT cells after stimulation with medium (left) and P/I (right). **C)** Mean (± SEM) frequencies of CD4$^+$ iNKT cells that expressed CD107a after co-culture with DAC-treated A549 cells and stimulation with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated A549 cells were compared to those exposed to A549 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (*p < 0.05, **p < 0.01).

Figure 6.17: CD8+ iNKT cells kill DAC-treated A549 cell lines. A549 cell lines were treated with PBS or DAC (100 nM, 1 μM and 5 μM) for 72 h. MeOH was used as a vehicle control. After a rest period of 6 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing cell-surface CD107a expression by CD8+ iNKT cells after stimulation with medium (left) and P/I (right). **B)** Mean (± SEM) frequencies of CD8+ iNKT cells that expressed CD107a after co-culture with DAC-treated A549 cells and pulsed with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated A549 cells were compared to those exposed to A549 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).
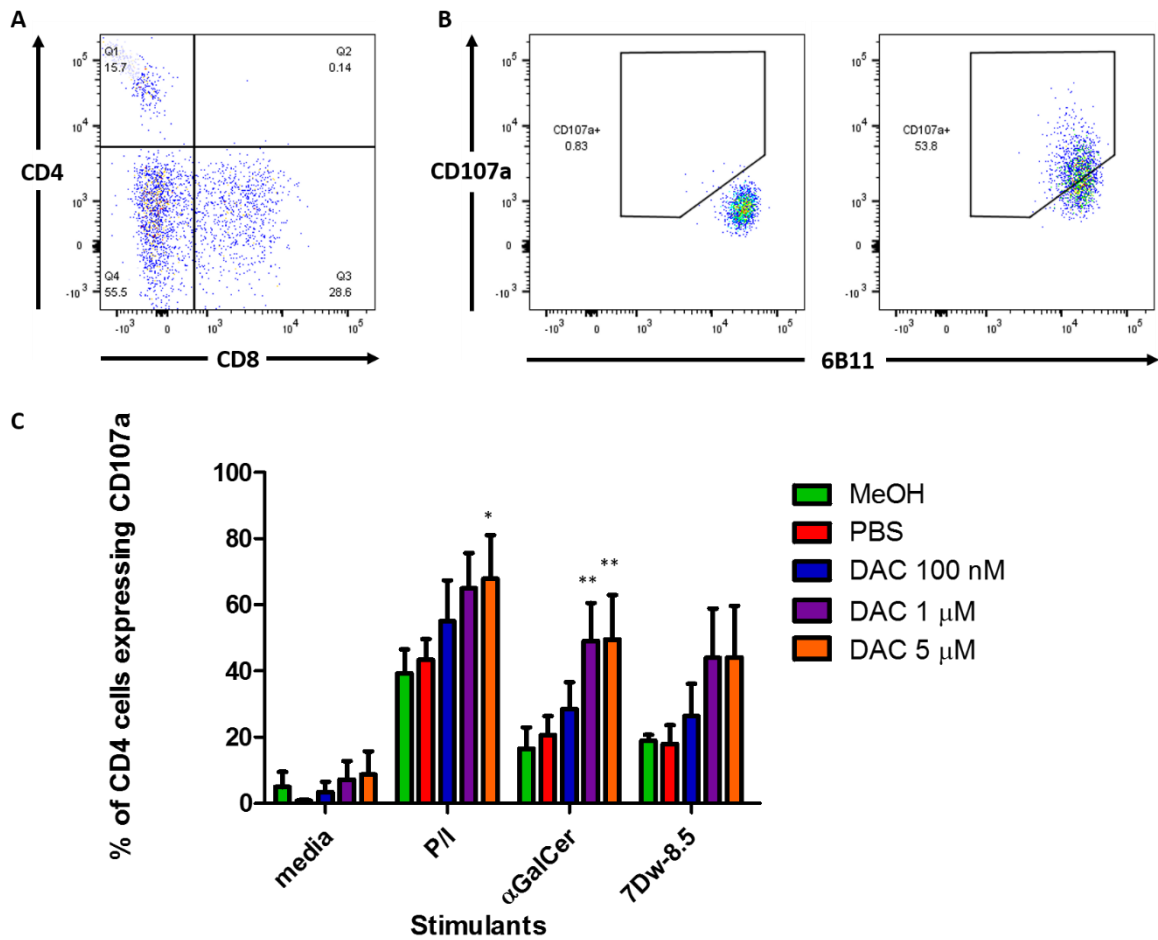
Figure 6.18: Figure 6.5: CD4⁻CD8⁻ iNKT cells kill DAC-treated A549 cell lines. A549 cell lines were treated with PBS or DAC (100 nM, 1 μM and 5 μM) for 72 h. MeOH was used as a vehicle control. After a rest period of 6 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing cell-surface CD107a expression by CD4⁻CD8⁻ iNKT cells after stimulation with medium (left) and αGalCer (right). **C)** Mean (± SEM) frequencies of CD4⁻CD8⁻ iNKT cells that expressed CD107a after co-culture with DAC-treated A549 cells and stimulation with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated A549 cells were compared to those exposed to A549 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
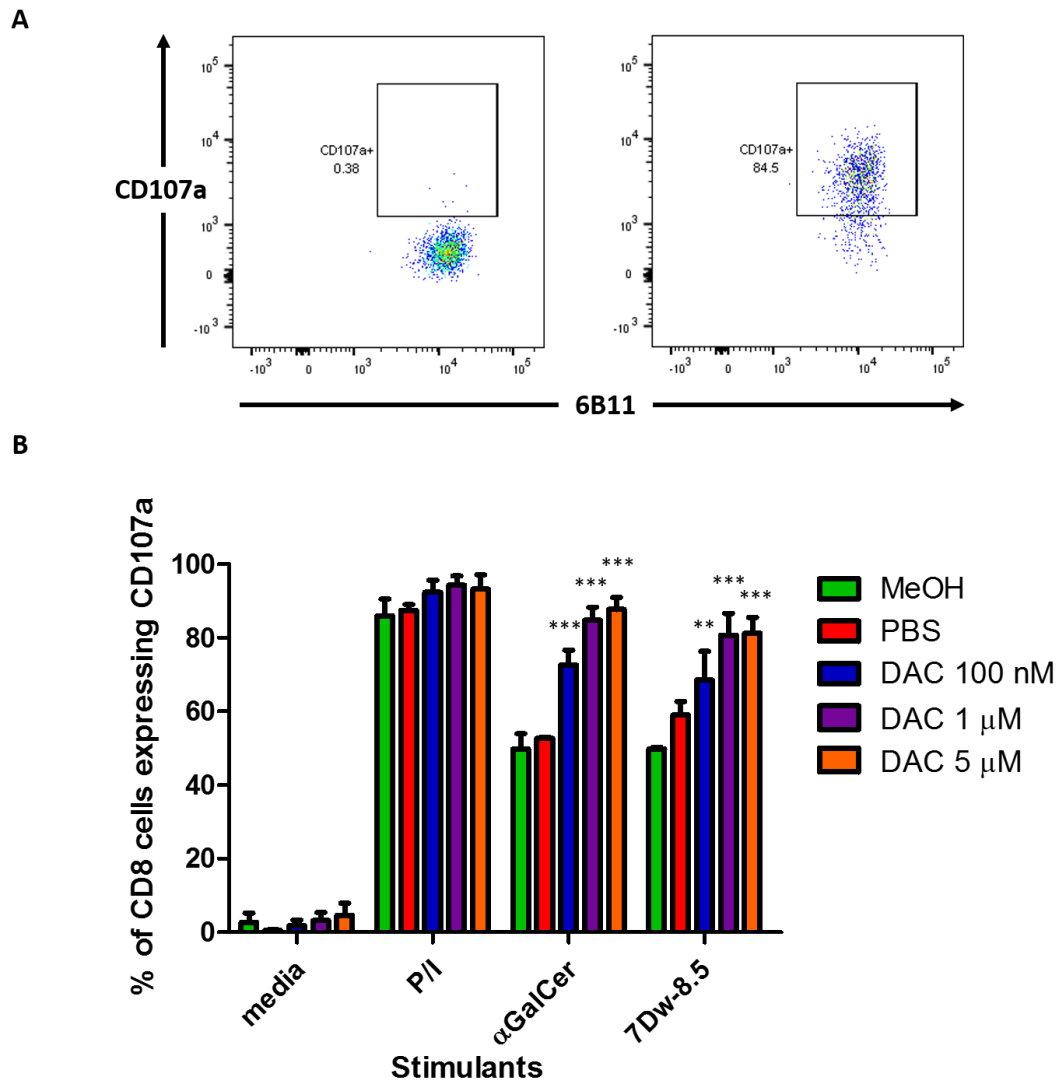
Figure 6.19: Figure 6.6: Transient low dose DAC treatment induces expression of CD1d in A549 cell lines.
A549 cells were treated with PBS and DAC at either 50 nM, 100 nM or 1 μM for 72 hours. Both media and DAC were replaced every 24 hours. MeOH was used as a vehicle control. After 72 hours cells were stained with anti-CD1d mAb and analysed immediately by flow cytometry. Cells were also cultured in medium for four hours and then stained with a mAb specific for CD1d and analysed by flow cytometry. **A)** Flow cytometry dot plots showing CD1d expression in an FMO control (left) and A549 cells (right). **B - C)** Mean (± SEM) frequencies of A549 cells that expressed CD1d immediately following treatment **(B)** and at day 4 following treatment **(C)** with various concentrations of DAC. A one-way ANOVA with Dunnett's post hoc test was used to determine significance. Results are representative of three experiments.

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VI.IV.IX.   Induction of CD1d by transient low dose DAC treatment in A549 cells increases their susceptibility to iNKT cell degranulation

To assess whether lower, less toxic concentrations of DAC can increase susceptibility of SK-MES-1 cells to cytoxicity by iNKT cells, A549 cells were treated with PBS or DAC at 50 nM, 100 nM and 1 μM for 72 h. The media and treatments were replaced every 24 h. At three days post-treatment the cells were pulsed with glycolipids for 24 hours and then co-cultured with iNKT cells (Figure 6.20A) and a mAb specific for CD107a. Cell-surface CD107a expression by the iNKT cells was examined by flow cytometry (Figure 6.20B). iNKT cells co-cultured with A549 cells treated with 50 nM – 1 μM DAC showed a statistically significant dose-dependent induction of CD107a when pulsed with αGalCer (p < 0.01 - p < 0.001) or 7Dw-8.5 (p < 0.05 - p < 0.001).

To determine which iNKT sub-populations degranulate in response to low dose DAC-treated A549 cells, CD107a expression by CD4$^+$ (Figure 6.21), CD8$^+$ (Figure 6.22) and CD4$^-$CD8$^-$ (Figure 6.23) cells was assessed by flow cytometry. A549 cells were treated with DAC at 50 nM, 100 nM and 1 μM as before. After 72 hours the cells were washed and cultured in medium alone for three days. Cells were pulsed for 24 hours with αGalCer or 7Dw8.5, and then co-cultured with iNKT cells. Following a four hour co-culture with a mAb specific for CD107a, cells were stained with mAbs specific for CD4 and CD8, and assessed by flow cytometry. CD4$^+$ iNKT cells co-cultured with A549 cells treated with 100 nM or 1 μM DAC and pulsed with αGalCer (p < 0.001) or 7Dw-8.5 (p < 0.001) showed a statistically significant up-regulation of CD107a (Figure 6.21C). CD8$^+$ cells co-cultured with A549 cells treated with 50 nM to 1 μM DAC and pulsed with αGalCer (p < 0.01) or 7Dw-8.5 (p < 0.05 – p < 0.001) showed a statistically significant and dose-dependent up-regulation of CD107a (Figure 6.22B).
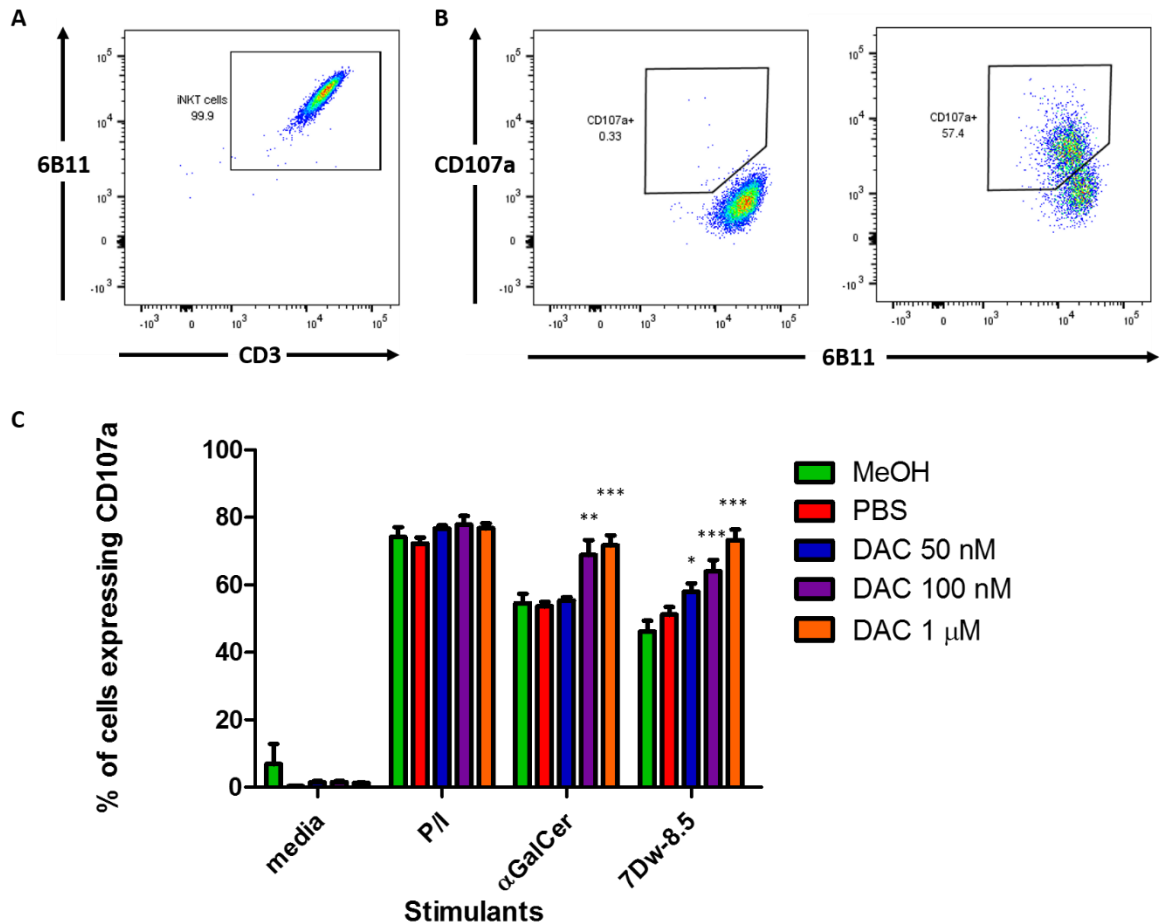
Figure 6.20: iNKT cells degranulate in response to low dose DAC-treated A549 cells. A549 cell lines were treated with PBS or DAC (50 nM, 100 nM and 1 μM) at 24, 48 and 72 hours. MeOH was used as a negative control. After a rest period of 3 days the cells were pulsed with either αGalCer or 7Dw8.5 for 24 hours, and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing purity of iNKT cells as lymphocytes expressing CD3 and Vα24Jα18 T cell receptor, as detected by 6B11 antibody. **B)** Flow cytometry dot plot showing cell-surface CD107a expression by iNKT cells after exposure to vehicle (left) or DAC (right). **C)** Mean (± SEM) frequencies of iNKT cells that expressed CD107a after co-culture with DAC-treated A549 cells pulsed with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated A549 cells were compared to those exposed to A549 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
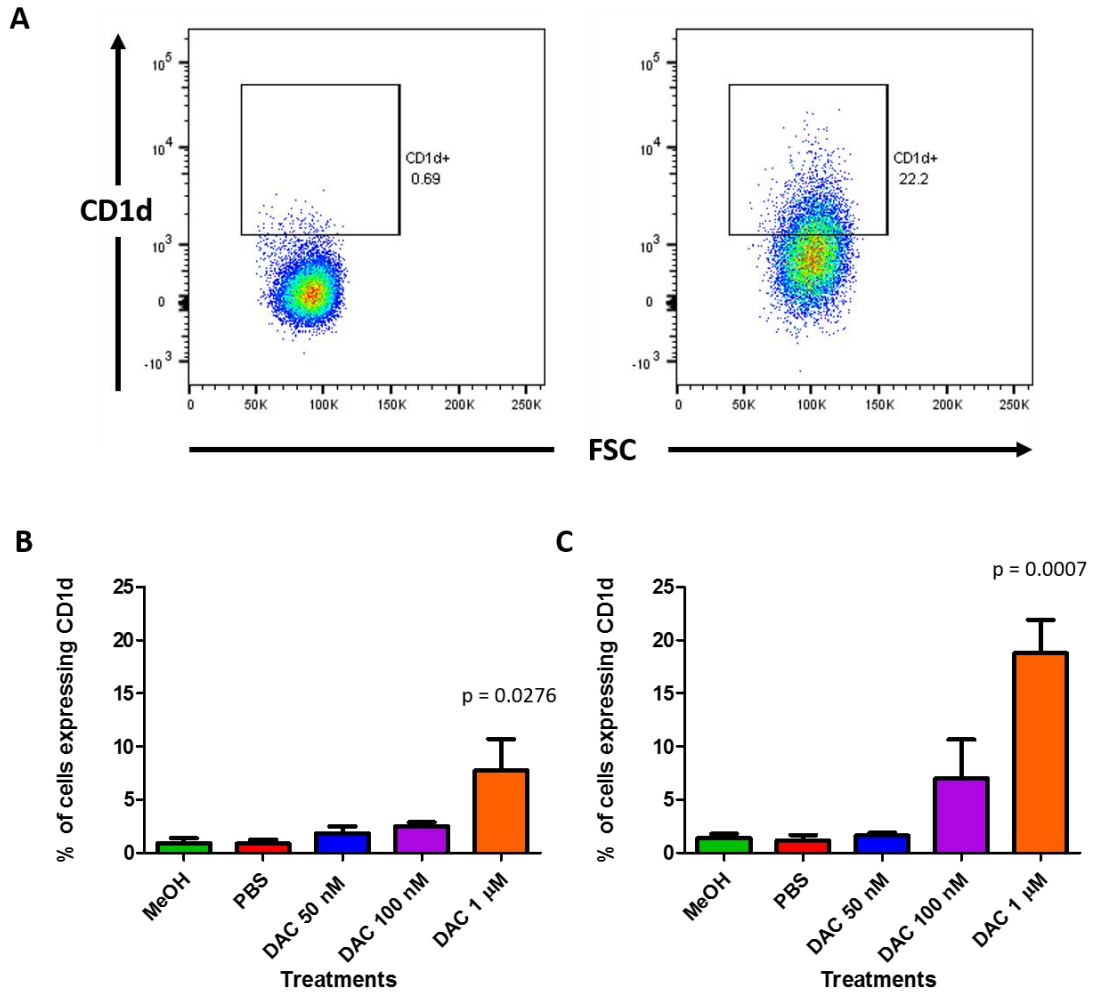
Figure 6.21: CD4⁺ iNKT cells kill DAC-treated A549 cell lines. A549 cell lines were treated with PBS or DAC (50 nM, 100 nM and 1 μM) for 72 h. Media and treatments were replaced every 24 hours. MeOH was used as a vehicle control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing frequencies of CD4⁺ and CD8⁺ within an iNKT cell line. **B)** Flow cytometry dot plot showing cell-surface CD107a expression by CD4⁺ iNKT cells after stimulation with medium (left) and P/I (right). **C)** Mean (± SEM) frequencies of CD4⁺ iNKT cells that expressed CD107a after co-culture with DAC-treated A549 cells and stimulation with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated A549 cells were compared to those exposed to A549 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (***p < 0.001).

Figure 6.22: CD8⁺ iNKT cells kill DAC-treated A549 cell lines. A549 cell lines were treated with PBS or DAC (50 nM, 100 nM and 1 μM) for 72 h. Media and treatments were replaced every 24 hours. MeOH was used as a vehicle control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing cell-surface CD107a expression by CD8⁺ iNKT cells after stimulation with medium (left) and αGalCer (right). **B)** Mean (± SEM) **f**requencies of CD8⁺ iNKT cells that expressed CD107a after co-culture with DAC-treated A549 cells and stimulation with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated A549 cells were compared to those exposed to A549 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 6.23: CD4⁻CD8⁻ iNKT cells kill DAC-treated A549 cell lines. A549 cell lines were treated with PBS or DAC (50 nM, 100 nM and 1 μM) for 72 h. Media and treatments were replaced every 24 hours. MeOH was used as a vehicle control. After a rest period of 4 days the cells were pulsed with either αGalCer or 7Dw-8.5 for 24 hours and then co-cultured with iNKT cells and a mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing cell-surface CD107a expression by CD4⁻CD8⁻ iNKT cells after stimulation with medium (left) and 7Dw-8.5 (right). **B)** Mean (± SEM) **f**requencies of CD4⁻CD8⁻ iNKT cells that expressed CD107a after co-culture with DAC-treated A549 cells and pulsed with gylcolipids. Percentages of iNKT cells that expressed CD107a in response to DAC-treated A549 cells were compared to those exposed to A549 cells treated with vehicle alone using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments (*p < 0.05, **p < 0.01 and ***p < 0.001).
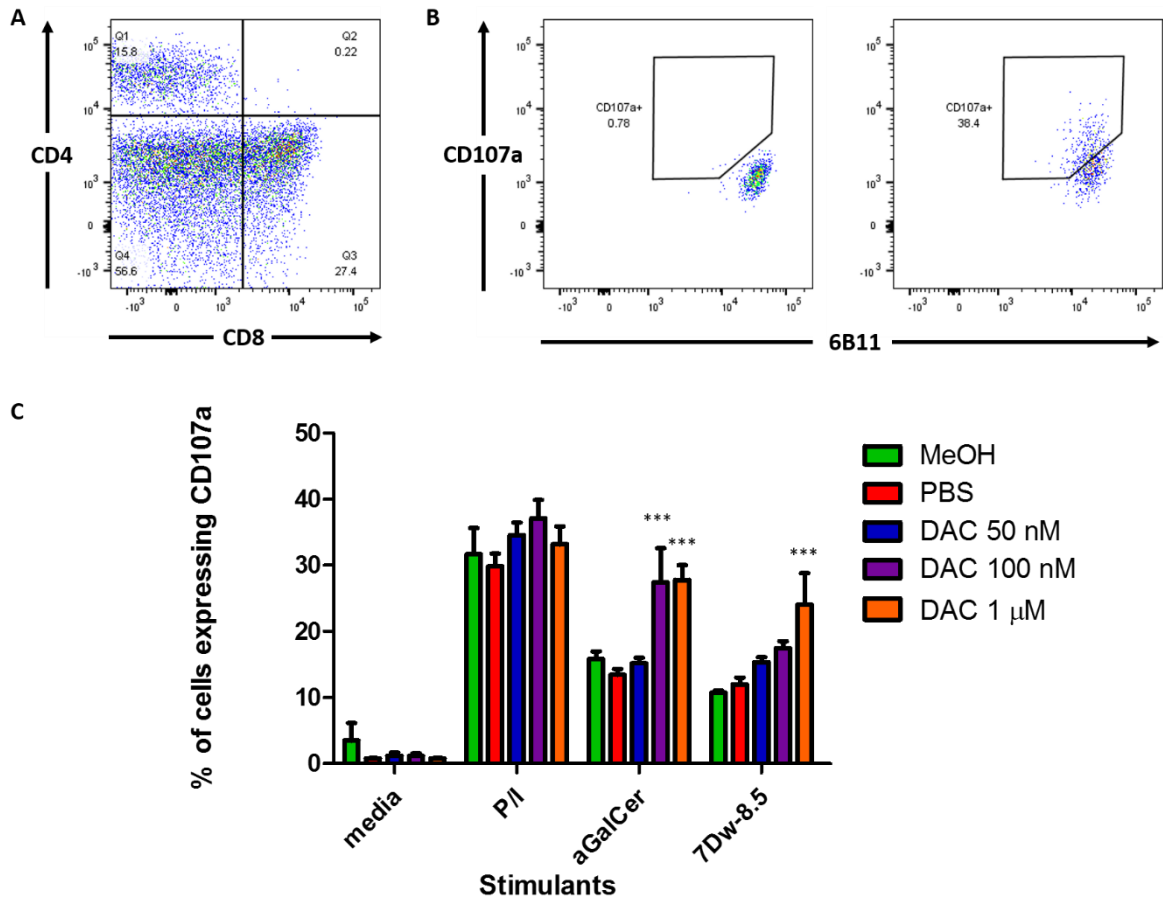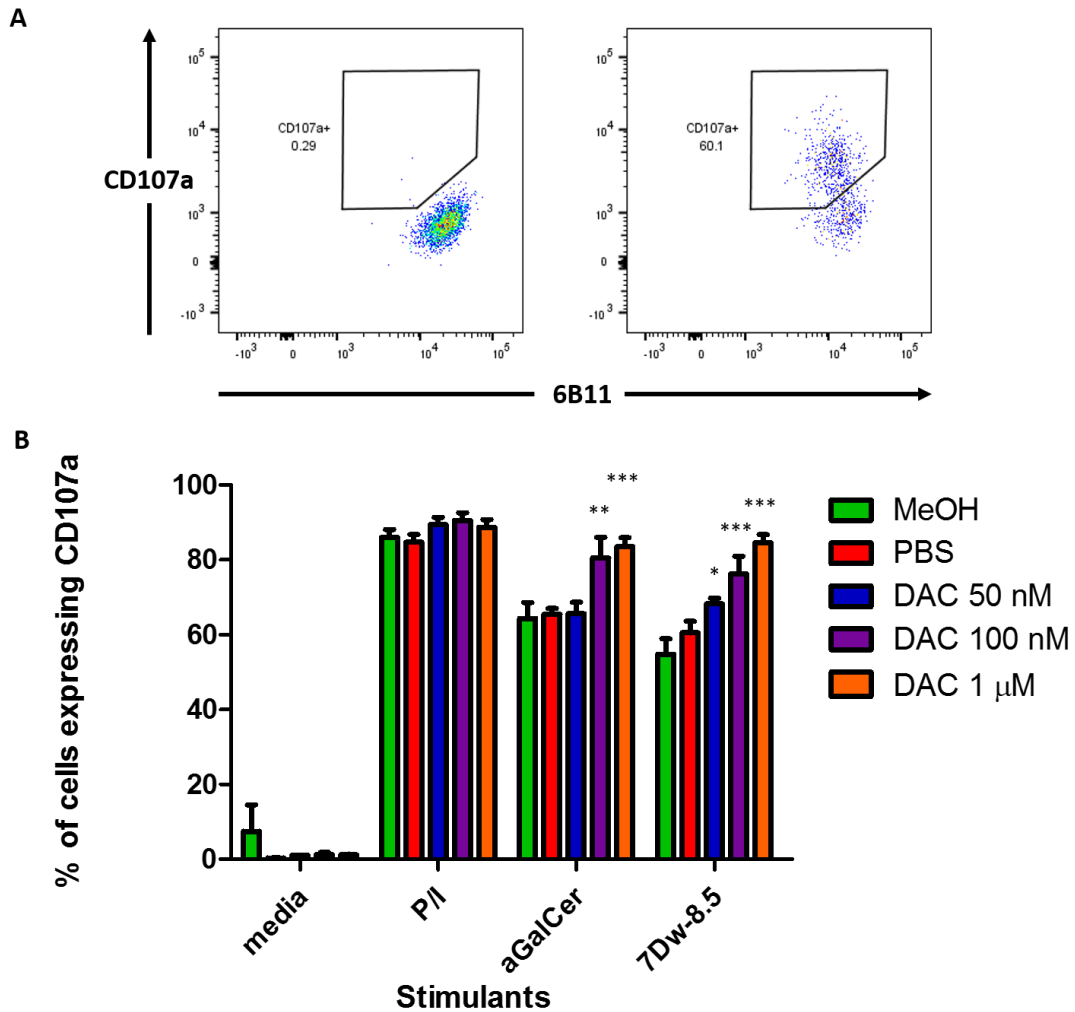
CD4$^-$CD8$^-$ cells co-cultured with A549 cells treated with 50 nM – 1 µM DAC and stimulated with αGalCer or 7Dw-8.5 showed a statistically significant and dose-dependent up-regulation of CD107a (p < 0.01 – p< 0.001) (Figure 6.23B).

VI.IV.X. Combinatorial DAC and SAHA treatment induces CD1d in A549 cell lines

To assess whether combined DAC and SAHA treatment could induce sustained expression of CD1d in A549 cells, A549 cell lines were cultured in the presence of PBS or DAC at 100 nM, 500 nM and 1 µM for 72 hours (Figure 6.24). The media and treatments were replaced every 24 h. After 48 hours of treatment 5 µM SAHA was added to cells receiving DAC treatment for the final 24 hours. CD1d expression was analysed immediately (Figure 6.24C) or at day 4 post-treatment (Figure 6.24D) by flow cytometry. A significant induction of CD1d expression by A549 cells was observed at day 4 after combined treatment with 500 nM or 1 µM DAC and 5 µM SAHA (p = 0.0102) compared with the vehicle control (Figure 6.24D). Comparison of the effect of treatment on CD1d expression at day 4 compared to day 0 showed significantly higher expression of CD1d at day 4 (p < 0.001) (Figure 6.24E).

VI.IV.XI. Induction of CD1d expression by A549 cells by combined DAC and SAHA treatment correlates with increased susceptibility to iNKT cell degranulation

To assess whether combined DAC and SAHA treatment can increase susceptibility of A549 cells to cytoxicity by iNKT cells, A549 cells were treated with PBS or DAC at 100 nM, 500 nM and 1 µM for 72 hours. The media and treatments were replaced every 24 hours. After 48 hours of treatment, 5 µM SAHA was added to the cells receiving DAC treatment. At three days post-treatment the cells were pulsed with glycolipids and then co-cultured with iNKT cells and a mAb
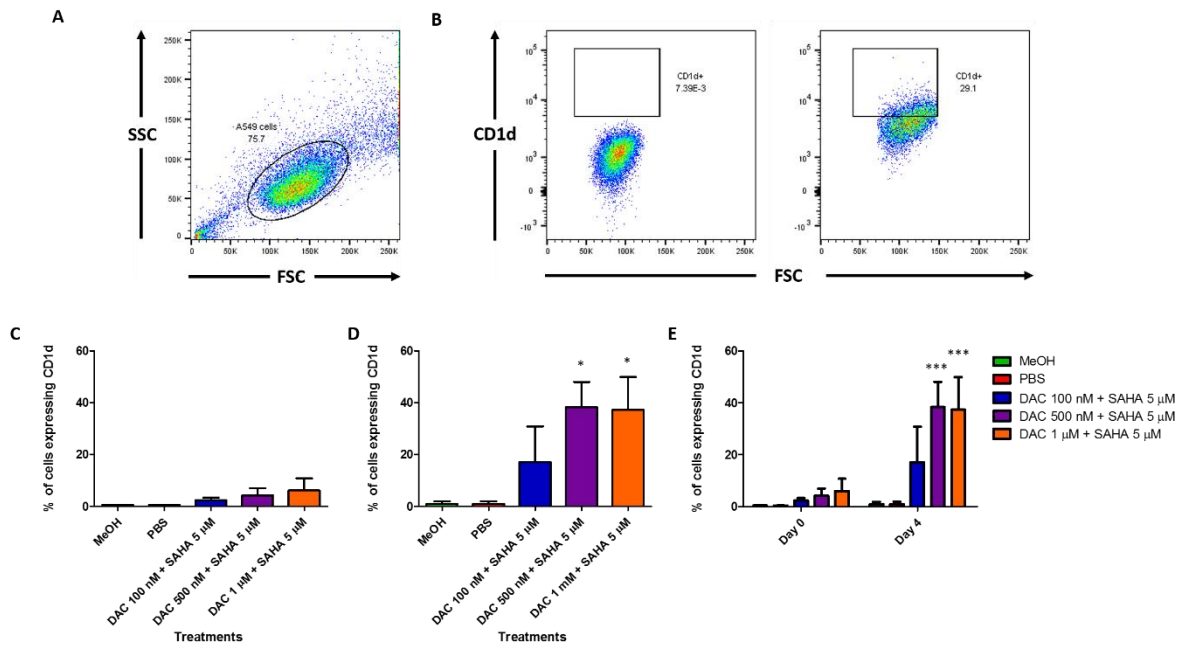
Figure 6.24: Combinatorial DAC and SAHA treatment induces expression of CD1d in A549 cell lines. Cells were treated with PBS and DAC at either 100 nM, 500 nM or 1 µM for 72 hours. Media and DAC were replaced every 24 hours. After 48 hours 5 µM SAHA was added to the cells treated with DAC. MeOH was used as a negative control. Cells were analysed for expression of CD1d immediately after treatment and at Day 4 post treatment. **A)** Flow cytometric dot plot showing FSC and SSC properties of A549 cells **B)** Flow cytometry dot plots showing CD1d expression by an FMO control (left) and gated A549 cells (right). **C - D)** Mean (± SEM) frequencies of A549 cells that expressed CD1d immediately following treatment **(C)** at day 4 following treatment **(D)**. A one-way ANOVA with Dunnett's post hoc test was used to determine significance. **E)** Mean (± SEM) frequencies of A549 cells that expressed CD1d immediately and at day 4 following treatment. A two-way ANOVA with Bonferroni's post hoc test was used to determine significance of treatments at day 4 compared to day 0. Results are representative of three experiments. (*$p < 0.05$, ***$p < 0.001$).
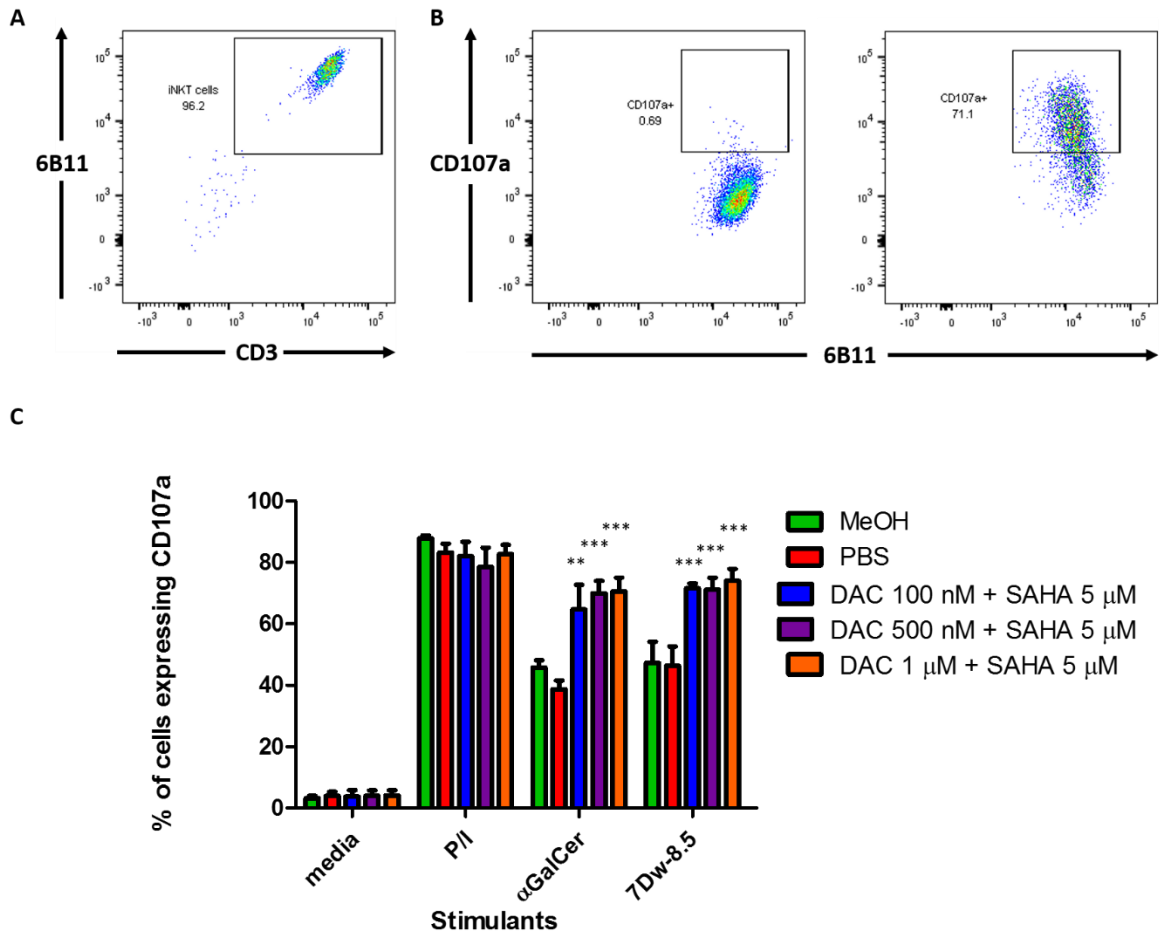
Figure 25: iNKT cells do not kill DAC and SAHA-treated A549 cells. A549 cell lines were treated with PBS and DAC (100 nM, 500 nM and 1 µM) at 24, 48 and 72 hours culture. At 72 hours, 5 µM SAHA was added to cells treated with DAC. MeOH treatment was used as a negative control. After a rest period of 3 days the cells were pulsed with either αGalCer or 7Dw8.5 for 24 hours, and then co-cultured with iNKT cells and mAb specific for CD107a. PMA and ionomycin (P/I) treatment was used as a positive control. **A)** Flow cytometry dot plot showing purity of iNKT cells as lymphocytes expressing CD3 and Vα24Jα18 T cell receptor, as detected by 6B11 antibody. **B)** Flow cytometry dot plot showing cell-surface CD107a expression by iNKT cells stimulated with media (left) and αGalCer (right). **C)** Mean (± SEM) frequencies of iNKT cells that expressed CD107a after co-culture with DAC- and SAHA-treated A549 cells and pulsed with gylcolipids. The percentages of iNKT cells that expressed CD107a following each treatment were compared with those of iNKT cells treated with MeOH-treated A549 cells and pulsed with the same glycolipids using a two way ANOVA with post hoc Bonferroni's multiple comparison test. Results are representative of three experiments. Results are representative of three experiments. (*$p < 0.01$, **$p < 0.001$).

specific for CD107a (Figure 6.25). Cell-surface expression of CD107a by iNKT cells was examined by flow cytometry (Figure 6.25B). iNKT cells co-cultured with A549 cells treated with 100 nM to 1 μM DAC and 5 μM SAHA show a statistically significant induction of CD107a when stimulated with αGalCer ($p < 0.001$) and 7Dw-8.5 ($p < 0.001$) (Figure 6.25C). Thus combinatorial treatment of A549 cells with DAC and SAHA may prime them for CD1d expression, and lysis by iNKT cells.

VI.V. Discussion

Cancer is one of the leading causes of death worldwide and as such there is a drive to identify novel therapeutics targets that can combat the disease more effectively. Due to the highly heterogeneous nature of cancer, even within tumour types (Levenson, 2010), it is important to study the alterations that are present within each cancer type in the hopes of pursuing personalised treatments in the future. Aberrant epigenetic alterations have been identified in all cancer types studied (Feinberg *et al.,* 2006), and because of their reversible nature they make an exciting target for therapeutics.

In recent years immunotherapy has re-emerged as an important modality in treating cancer. While clinical trials for immunotherapies have been plagued with a lack of success in the past, the approval for Ipilimumab (anti-CTLA-4) against melanoma and Opdivo (anti-PD1) for squamous cell carcinoma are success stories that delineate the very real possibility of immunotherapies as adjunct therapies for lung cancer.

Chapter 5 demonstrated the potential of using epigenetic targeting therapies to increase CD1d expression at the mRNA level in NSCLC cell lines. To test whether increased CD1d expression by NSCLC cell lines could lead to increased susceptibility to iNKT cytolytic degranulation, a SK-MES-1 cell line was transfected with CD1d. In co-culture experiments with iNKT cells, this transfected cell line showed increased susceptibility to iNKT cell-mediated cytolytic degranulation than its parent SK-MES-1 cell line.

The aim of this chapter were to assess whether DNMTi and HDACi could increase CD1d expression in NSCLC cell lines at the protein level, and whether such increases correlates with increased susceptibility to iNKT cell-mediated cytolytic degranulation.

Lung squamous carcinoma (SK-MES-1) cells were treated with three different concentrations of DAC, a FDA-approved DNMTi for 72 hours. Cells were assessed by flow cytometry at days 2, 5 and 7 of recovery for expression of CD1d. SK-MES-1 cells showed a significant increase of CD1d

expression when treated with either 1 μM or 5 μM DAC, and allowed 5 days of recovery. This indicates that CD1d expression can be induced and maintained in SK-MES-1 by a one-off high dose DAC treatment.

This increase in CD1d expression correlated with increased susceptibility to iNKT cell-mediated cytolytic degranulation. Cytolytic degranulation is a cellular process where apoptosis-inducing cytotoxic molecules are released from secretory vesicles within the cells. This mechanism is used by various cells of the immune system including T cells, NK cells and iNKT cells. Lysosomal-associated membrane protein -1 (LAMP-1 or CD107a) is a marker of degranulation following T or NK cell stimulation (Betts *et al.*, 2003). Under normal conditions CD107a resides in vesicles within cells. When these cells come into contact with target cells CD107a is up-regulated on the cell surface. Our lab has previously shown that CD107a expression at the iNKT cell surface correlates well with the induction of target cell death as measured by chromium release assays or flow cytometric assays of apoptosis (Hogan *et al.*; 2011).

DAC or PBS-treated SK-MES-1 cells were pulsed with either αGalCer or 7Dw-8.5 and co-cultured with iNKT cells. After four hours a significant increase in cell-surface CD107a expression was observed when iNKT cells were co-cultured with SK-MES-1 cells treated with 1 μM DAC and pulsed with either αGalCer or 7Dw-8.5 compared to when iNKT cells were cultured with PBS-treated SK-MES-1 pulsed with the glycolipids. CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells ,but not CD4$^+$ iNKT cells, showed a significant up-regulation of CD107a when co-cultured with SK-MES-1 cells treated with 1 μM DAC and stimulated with either αGalCer or 7Dw-8.5. These results show that CD1d expression by SK-MES-1 cells is epigenetically regulated and treatment of these cells with high dose DAC results in CD1d up-regulation making the cells susceptible to lysis by CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells.

High dose DAC treatment is associated with extreme toxicities so we next tested if transient, low dose DAC treatments similar to those used in humans could also induce and maintain CD1d

expression in SK-MES-1 cells. Cells were treated with PBS and DAC at 50 nM, 100 nM and 1 µM for 72 hours. After 4 days, a significant increase in CD1d expression was observed when SK-MES-1 cells were treated with 1 µM DAC when compared to the vehicle control. This result shows that CD1d expression can be induced and maintained in SK-MES-1 cells following treatment with low dose DAC.

To assess whether this induction of CD1d expression is accompanied by increased iNKT cell-mediated cytolytic degranulation, iNKT cells were co-cultured with αGalCer or 7Dw-8.5-pulsed SK-MES-1 cells treated with low-dose DAC. iNKT cells showed a significant increase in CD107a when co-cultured with SK-MES-1 cells treated with all concentrations of DAC and stimulated with either glycolipid. Analysis of iNKT cell sub-populations indicated that CD4$^+$, CD8$^+$ and CD4$^-$ CD8$^-$ iNKT cells underwent significant degranulation when co-cultured with DAC-treated SK-MES-1 cells pulsed with either αGalCer or 7Dw-8.5. However, the frequencies of degranulating CD8$^+$ and double negative iNKT cells were higher than those of CD4$^+$ iNKT cells. These results show that low-dose DAC treatment is accompanied by a significant induction of CD1d that correlates with increased susceptibility to iNKT-cell-mediated cytolytic degranulation by all subsets of iNKT cells.

Studies in recent years have increasingly shown how the interaction between DNA methylation and histone modifications result in gene silencing (Esteller, 2008). Cameron and colleagues showed that they could synergistically reactivate several hypermethylated genes by TSA and DAC in combination (Cameron *et al.*, 1999). We found that combined treatment of DAC and SAHA resulted in significant induction of CD1d expression by SK-MES-1 cells at day 4 for all concentrations for DAC and SAHA tested. These results indicate that combinatorial DAC and SAHA treatment can induce and maintain CD1d expression in SK-MES-1 cells.

The induction of CD1d expression by combined treatment of SK-MES-1 cells with DAC and SAHA did not correlate to increased susceptibility with iNKT cell-mediated cytolytic degranulation. SK-

MES-1 cells were treated as before, allowed a three day recovery period, pulsed for 24 hours with either αGalCer or 7Dw-8.5, and co-cultured with iNKT cells which were subsequently analysed by flow cytometry for the presence of cell-surface CD107a. No significant up-regulation of CD107a was observed when iNKT cells are co-cultured with SK-MES-1 cells treated with DAC and SAHA, and pulsed with glycolipids. Since one of the experiments was performed with an iNKT cell population that only contained CD4+ cells which showed less potent degranulation than CD8- and double negative iNKT cells, significant degranulation by total iNKT cells was not observed for any treatments. This indicates that CD8+ and CD4-CD8- iNKT cells are critical for iNKT cell-mediated cytolytic degranulation.

Lung adenocarcinoma is the most commonly diagnosed histological subtype of NSCLC (Travis, 2011); nearly 40% of all lung cancers diagnosed in the United States are adenocarcinoma. We found that high dose DAC treatment induced CD1d expression by the lung adenocarcinoma lines; this induction was maintained for up to 5 days. This induction of CD1d expression was associated with a significant increase in CD107a expression when iNKT cells were co-cultured with A549 cells treated with all concentrations of DAC and stimulated with either αGalCer or 7Dw-8.5. Analysis of iNKT cell sub-populations revealed that CD4+ CD8+ and CD4-CD8- iNKT cells degranulated when co-cultured with A549 cells treated with DAC and pulsed with αGalCer or 7Dw-8.5. These results indicate that high dose DAC treatment can induce CD1d expression in A549 cells, and that this induction correlates with increased susceptibility to iNKT cell-mediated cytolytic degranulation, particularly by CD8+ and CD4-CD8- iNKT cells.

We also investigated if low dose DAC treatments could also induce and maintain CD1d expression in A549 cells. We found that 1 μM DAC upregulated CD1d expression indicating that CD1d expression can be induced and maintained in A549 cells following treatment with low dose DAC. This induction of CD1d expression was accompanied by increased iNKT cell-mediated cytolytic degranulation against αGalCer and 7Dw-8.5-pulsed A549 cells. Again CD4+, CD8+ and

CD4$^-$CD8$^-$ iNKT cells exhibited cytolytic degranulation in response to A549 cells treated with low dose DAC and pulsed with αGalCer or 7Dw-8.5, but CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells degranulated most potently. These results show that low-dose DAC treatment is accompanied by a significant induction of CD1d that correlates with increased susceptibility to CD8$^+$ and CD4$^-$CD8$^-$ iNKT cell-mediated cytolytic degranulation.

We also assessed if combined treatment of DAC and SAHA could induce CD1d expression by A549 cells, making them targets of iNKT cell lysis. We found that 500 nM DAC and 5 μM SAHA were sufficient to induce CD1d expression at day 4, and this treatment made A549 cells susceptible to iNKT cell mediated cytotoxicity.

In conclusion, CD1d expression can be up-regulated in NSCLC cells following treatment with epigenetic targeting therapies. This induction of CD1d correlates with increased susceptibility to iNKT cell-mediated cytolytic degranulation. CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells are most frequently degranulated but CD4$^+$ were also capable of degranulation. While these results are strictly pre-clinical work, they do demonstrate the combined potential of epigenetic targeting therapies and adoptive transfer of iNKT cells at treating NSCLC. These experiments need to be repeated with primary human NSCLC cells, and since iNKT cells are depleted in blood and BAL of NSCLC patients, it is likely that future immunotherapies involving epigenetic up-regulation of CD1d might need to be carried out in combination with adoptive iNKT cell transfer. Since CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells most potently degranulate, sorting and expanding CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells in the absence of CD4$^+$ iNKT cells might be more beneficial for immunotherapies. Our lab previously showed that CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells most frequently expressed the NK cell markers CD56, CD161 and NKG2D, and most potently killing CD1d-expressing cell lines and primary leukemia cells (O'Reilly et al., 2011). In conclusion, these results demonstrate new techniques to increasing CD1d expression in NSCLC cells lines making them more susceptible to iNKT cell lysis.

VII. General Discussion

Cancer is one of the leading causes of death worldwide, accounting for 14.1 million new cases, as well as 8.2 million deaths in 2012 (Torre *et al.*, 2015). Lung cancer accounts for about 19.4% of cancer mortalities, and is the most common form of cancer-related death globally (Siegel *et al.*, 2014). An estimated 1.8 million new cases of lung cancer were diagnosed in 2012, accounting for approximately 13% of total cancer diagnoses (Torre *et* al., 2015). For newly diagnosed patients the five year net survival is 15.7%.

NSCLC accounts for approximately 85% of all lung cancer cases, and comprises of adenocarcinoma, squamous cell carcinoma and large cell carcinoma, of which adenocarcinoma and squamous carcinoma are most commonly diagnosed. 40% of new NSCLC patients are diagnosed with advanced disease (Goldstraw *et al.*, 2007). Nucleotide sequencing studies have revealed that lung cancer is a molecularly heterogeneous disease and the mutational landscape of each histological subtype is distinct (TCGA, 2014, TCGA, 2012, Bignell *et al.*, 2010 and Weir *et al.*, 2007). Elucidation of mutations in NSCLC have led to the development of inhibitors of oncogenes such as tyrosine kinas inhibitors, gefitinib and erlotinib (Thatcher *et al.*, 2005 and Shepherd *et al.*, 2005), which have shown dramatic results in specific NSCLC patient sub-populations. However, the majority of NSCLC patients have no currently identifiable genetic mutations.

Mortality rates for NSCLC patients remain high because of difficulties in early detection and resistance to current therapeutics. In advanced NSCLC cases overall survival is only marginally improved by standard platinum-based chemotherapy, which causes substantial morbidity. Furthermore, many tumours become resistant to this type of therapy. In light of this there has been a drive to develop novel therapeutics to overcome this platinum-based resistance.

Tumours can become densely infiltrated by cells of the innate and adaptive immune systems which can play both tumour-promoting and anti-tumour roles. Macrophages, mast cells, and lymphocytes can promote tumour angiogenesis, proliferation, invasion and metastasis by releasing growth factors, survival factors, proangiogenic factors, etc., to the tumour microenvironment (Hanahan and Weinberg, 2011, DeNardo *et al*., 2010, Grivennokov *et al*., 2010 and Qian and Pollard, 2010). On the other hand NK cells, CD8^{+} CTLs, iNKT cells and γδT cells can recognise and kill tumour cells and release cytokines, such as IFN-γ, that stimulate other anti-tumour responses.

Lymphocyte populations are altered in NSCLC; the frequencies of T and B cells are decreased in the peripheral smears of patients with advanced lung cancer (Sato *et al.*, 1989) and absolute lymphocyte counts are decreased in the tumour microenvironment of advanced lung cancer (Wesselius *et al*., 1987). A higher CD4^{+} T cell count is associated with a poorer prognosis in lung cancer (Perterson *et al*., 2006), while a higher CD8^{+} T cell count correlated with improved prognosis (Dieu-Nosjean *et al*., 2008). In addition, NK cell tumour infiltration is associated with improved prognosis in NSCLC carcinomas (Platonova *et* al., 2011 and Carrega *et al*., 2008).

The first aim of the present study was to enumerate and phenotypically characterise lymphocyte populations in BAL and peripheral blood samples from NSCLC patients and to compare them to those of control subjects. T cell, B cell, NK cell and γδT cell frequencies were found to be similar in BAL samples from NSCLC patients and non-cancer control patients. Peripheral blood analysis of NSCLC patients found that total T cell frequencies were higher in NSCLC patients than healthy controls, although there was no difference in absolute numbers of T cells. Analysis of T cell subpopulations did not reveal any differences between NSCLC patients and controls. NK and Vδ1 cell frequencies were depleted from the peripheral blood of NSCLC patients, but absolute numbers were similar in the two populations. iNKT cells were the only lymphocyte population tested that was found to be significantly depleted from both BAL and

blood of NSCLC patients compared to controls. This finding led us to hypothesize that iNKT cells represent an important component of anti-tumour immunity against NSCLC.

iNKT cells are a heterogeneous population of T lymphocytes that recognise the polymorphic MHC class I like molecule CD1d. They are characterized by their expression of an invariant T cell receptor (TCR), Vα24-Jα18/Vβ11 in humans, which recognises glycolipid antigens presented by CD1d. iNKT cells were first shown to be necessary for tumour elimination in a mouse murine model (Cui *et al*., 1997). Mice with iNKT cell deficiencies were unable to reject tumours in an IL-12-mediated fashion. iNKT cells kill tumour cells following presentation of αGalCer by dendritic cells (Kawano *et al*., 1998). Cytotoxic activity against B cell melanoma was observed when mice with iNKT cells were injected with αGalCer. Adoptive transfer of αGalCer-pulsed dendritic cells in a murine model showed that targeted delivery of αGalCer triggered optimal stimulation of iNKT cells, which was maintained after the challenge had dissipated (Macho-Fernandez *et al*., 2014).

Several phase I trials of the anti-tumour activity of αGalCer in humans have shown little therapeutic benefit (Exley and Nakayama, 2011). αGalCer was first tested in humans in a phase I trial in patients with refractory solid tumours. There was minimal toxicity but disease stabilisation occurred in only 7 out of 24 patients. A phase I study of αGalCer-pulsed dendritic cells in patients with advanced or recurrent NSCLC saw no serious adverse effects but no patient achieved a partial or complete response (Ishikawa *et al*., 2005). A phase I clinical trial of intra-arterial infusion of *in vitro* expanded iNKT cells with injection of αGalCer-pulsed APCs in 8 patients with recurrent head and neck carcinoma resulted in a transient partial response in 3 patients and disease stabilisation in 4 patients (Kunii *et al*., 2009). It is thought that such discrepancies between mouse and human trials are the result of dramatic differences in iNKT abundancies between the two species, and as such an inherent flaw with conventional mouse models.

Another reason for the low clinical efficacies of the iNKT cell-based immunotherapies tested in human to date may be that tumour cells frequently do not express CD1d. iNKT cells can directly recognise and kill CD1d-bearing tumour cells. Some tumour types such as prostate cancers, myelomonocytic leukaemia and some neurologic tumours, express CD1d and as such can be targeted for NKT-mediated cell killing (Metelitsa *et al.*, 2003). However, most human and mouse solid tumours are CD1d negative. Over the last number of years increasing evidence has suggested that cancer cells are capable of downregulating MHC class I molecules in an attempt to evade immunosurveillance (Algarra *et al.*, 2004 and Jäger *et al.*, 2001). We hypothesized that NSCLC cells similarly downregulate CD1d expression to evade the actions of iNKT cells. Such downregulation of CD1d expression could be the result of epigenetic modifications that take place in cancer cells.

Epigenetics is defined as the sum of all stable and heritable changes to gene expression that does not occur as a result of changes to DNA sequences (Probst *et al.*, 2009). Chromatin structure controls DNA organisation and allows for the transcriptional repression or activation of genes (Sharma *et al.*, 2010). DNA methylation works as a gene silencing mechanism. Hypermethylation of CpG islands is present in almost all types of human cancers and is associated with transcriptional silencing of genes (Jones and Baylin, 2002). Aberrant hypomethylation can cause activation of proto-oncogenes. It has been shown that DNMTs are up-regulated and associated with poor prognosis in lung cancer (Lin *et al.*, 2007). In addition, abnormal gene silencing in NSCLC by DNA CpG hypermethylation has been linked to platinum-based resistance.

HPTMs are also involved in gene regulation and cellular function. The N-terminal tails of histone proteins can undergo a variety of modifications including acetylation, phosphorylation, ubiquitination, methylation and sumoylation on specific residues (Kouzarides 2007). HPTMs can cause either gene silencing or induction by changing the accessibility of transcription factors to

the chromatin (Sharma *et al.*, 2010). Aberrant histone modifications have been implicated in many different types of cancer, including NSCLC, where they have both a predictive and prognostic value (Barlési *et al.*, 2007). HDACs regulate gene expression by removing acetyl groups from ε-N-acetyl lysine amino acids on histones, and are associated with abnormal gene expression in NSCLC. Lysine acetylation is usually associated with gene activation (Sharma *et al.*, 2010), whereas the loss of histone acetylation causes repression of gene expression. Overexpression of HDACs has been observed in a variety of cancers (Halkidou *et al.*, 2004).

We analysed A549 and SK-MES-1 cell lines for expression of CD1d by flow cytometry, and found that these cells are CD1d-negative. We hypothesized that over-expression of CD1d in an NSCLC cell line would make them more susceptible to killing by iNKT cells. To test this hypothesis we stably transfected a SK-MES-1 cell line with CD1d and verified its expression by flow cytometry. The CD1d-transfected cell line was sorted to ensure a homogeneous CD1d$^+$ population. iNKT cells were isolated from PMBCs and expanded *in vitro*. Lines of expanded iNKT cells were co-cultured with the CD1d-transfected SK-MES-1 cell line and their parent SK-MES-1 cell line. SK-MES-1 cells over-expressing CD1d showed an increased susceptibility to iNKT cell-mediated cytolytic degranulation than their parent cells.

Treatment with HDACi TSA and SAHA were shown to induce CD1d expression in A549 cells (Yang *et al.*, 2012). We hypothesized that CD1d expression is epigenetically regulated and that treatment with DNMTi and HDACi could induce CD1d expression in NSCLC cancer cell lines. A549 and SK-MES-1 cells were treated with HDACi TSA and SAHA, and CD1d was found to be significantly increased at the mRNA level; this significant increase in expression was maintained following a recovery period in SAHA-treated A549 and SK-MES-1 cell lines. To see whether a similar induction of CD1d expression was possible using DNMTi, A549 and SK-MES-1 cells were treated with DAC and GEM. Both cell lines showed an increase in CD1d expression at the mRNA following DNMTi treatment. To assess whether the changes in CD1d expression were due to

direct conformational changes at the CD1d promoter, a ChIP analysis was performed on A549 cells treated with TSA. The analysis showed that HDAC inhibition causes a conformational change in the chromatin landscape, allowing HPTMs to occur at the promoter region of the *CD1d* gene.

We hypothesized that DAC, alone or in combination with SAHA, could be used to induce CD1d expression at the protein level in A549 and SK-MES-1 cell lines, and that this induction would correlate with increased iNKT cell-mediated cytolytic degranulation. In a series of preclinical experiments, A549 and SK-MES-1 cells were treated with a once-off high dose DAC treatment which resulted in a significant increase in CD1d expression that was maintained up to 7 days post-treatment. This increase in CD1d expression was associated with an increase in cytolytic degranulation by iNKT cells when co-cultured with DAC-treated A549 and SK-MES-1 cells.

High dose DAC treatment is associated with extreme toxicities (Abele *et al.*, 1987) whereas transient DAC treatment have been shown to sustain long-term anti-tumour effects and to maintain its targeting of DNA methylation processes and alterations of gene expression (Tsai *et al.*, 2012). A549 and SK-MES-1 treated with a series of low dose DAC treatments were found to induce and maintain CD1d expression for up to four days. This increase in CD1d expression was associated with increased cytolytic degranulation with iNKT cells when co-cultured with DAC-treated A549 and SK-MES-1 cells.

The epigenetic processes of DNA methylation and histone deacetylation are linked, and Cameron and colleagues found that they could synergistically reactivate several hypermethylated genes using TSA and DAC in combination (Cameron *et al.*, 1999). A549 and SK-MES-1 cells were treated with a combinatorial DAC and SAHA treatment which resulted in the increase and maintenance of CD1d expression for up to four days. This increase in CD1d expression was associated with increased iNKT cell mediated cytolytic degranulation against A549 cell lines but not SK-MES-1.

Future work would include enumerating iNKT cell in a larger cohort of patients and controls, as some of these analyses were restricted by the small sample size. It would also be interesting to enumerate and phenotypically characterise CD4$^+$, CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells from NSCLC patients. While this study showed that all iNKT subpopulations tested degranulated in response to co-culture with DAC-treated NSCLC cell lines previous work from our lab has suggested that CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells mediate anti-tumour immunity while CD4$^+$ may play a pro-tumorigenic role (O'Reilly et $al.$, 2011). Functional analysis of iNKT cells from NSCLC patients would give us a greater understanding of iNKT deficiencies in NSCLC; this may require ex $vivo$ expansion. Other work would include examining CD1d expression in primary NSCLC tumours, and assessing the ability of DAC and SAHA to induce CD1d expression by these cells in $vitro$, and whether this treatment resulted in increased susceptibility to iNKT cell-mediated killing. These experiments are required before these treatments can be tested in the clinic. Since we have shown that iNKT cells are depleted in the blood and BAL of NSCLC patients, it is likely that therapies involving CD1d up-regulation by tumour cells would need to be carried out in combination with adoptive iNKT cell therapy.

Therapeutic strategies involving combinations of iNKT cells, DCs, glycolipids and/or epigenetic modifying agents provide additional ways to complement current chemotherapies for the development of personalised medicines to target the diversity of cancers. The epigenetic agents used in this study are FDA approved drugs that are indicated in the treatment of acute myeloid leukaemia and cutaneous T cell lymphoma. The feasibility of using adoptive immunotherapy using iNKTs with or without αGalCer-pulsed DCs has been shown to have minimal toxicity in cancer patients. By combining these therapies we have suggested a novel method of overcoming the shortfalls of immunotherapies against NSCLC that have previously been tested and found to have therapeutic benefit in other patient populations.

iNKT cell-based therapies have shown limited success to date, possibly because they were carried out in patients with advanced disease, who have had prior treatments that could inhibit iNKT cell activities. In addition this study has shown that iNKT cells are depleted in NSCLC patients and that NSCLC cell lines are CD1d-negative, which we believe could contribute to lung tumorigenesis. Our group has previously shown that CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells are the main effectors of iNKT cell-mediated cytolysis, and that CD4$^+$ iNKT cells may have a pro-tumorigenic role (O'Reilly *et al.*, 2011). While this study found that all iNKT subsets tested were capable of degranulating upon co-culture with CD1d-expressing NSCLC cells, the cytolytic activities of CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells were superior. These are important considerations for future iNKT cell-based immunotherapies, and future trials using sorted CD8$^+$ and CD4$^-$CD8$^-$ iNKT cells could help overcome some of the present limitations of iNKT cell-based immunotherapies.

Other type 2 NKT ells expressing αβ or γδ TCRS are known to recognise CD1d, therefore, our proposed strategy of immunotherapy involving CD1d induction may ultimately result in the recruitment of other cells. In addition, it is possible that epigenetic modifying agents can induce expression of other CD1d isotypes, CD1a, b and c, which could potentially lead to additional immune activation and anti-tumour activity. These are exciting avenues to explore when moving forward with this combinatorial therapy in the future and could perhaps result in synergistic anti-tumour responses in lymphocytes that recognise CD1d. Furthermore, therapies involving the induction of CD1d could ultimately be applied to other diseases that require boosting of immune responses and perhaps inhibition of immune responses.

In conclusion, this study illustrates the feasibility of using epigenetic targeting therapies to induce CD1d expression in NSCLC cell lines. Treatment with DAC, alone or in combination with SAHA, at doses equivalent to those given therapeutically, could induce CD1d expression in NSCLC cell lines making them more susceptible to iNKT cell-mediated cytolytic degranulation.

While these results are strictly preclinical, they show a novel method of stimulating iNKT

cytotoxicity against NSCLC.

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