Therapeutic potential of PI3K signalling in lung squamous cell carcinoma

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Declaration

I, Zoe Whiteman, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
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Abstract

Lung cancer is the leading cause of cancer-related death worldwide. Lung squamous cell carcinoma (LUSC), a lung cancer subtype, develops through multiple pre-invasive disease grades that progress to invasive LUSC. This presents opportunities for interventional therapies to halt LUSC development at pre-invasive stages, to improve patient prognosis.

Here, I investigated the therapeutic potential of targeting phosphoinositide 3-kinase (PI3K) signalling during LUSC development. I used both in vitro and in vivo approaches to examine disease-intrinsic and microenvironment features that may influence disease fate.

I optimised strategies to develop a human in vitro model to study the common genetic changes associated with dysregulated PI3Kα signalling in LUSC. Such a model will enable mechanistic insight into the prevalence of these genomic changes.

In the N-nitroso-tris-chloroethylurea (NTCU)-induced LUSC murine pre-clinical model, which histologically recapitulates both pre-invasive and invasive LUSC, I evaluated changes in PI3Kα signalling and T-lymphocyte populations, in situ. Using this model, I also assessed PI3Kα and PI3Kδ inhibitors as interventional LUSC therapies.

I demonstrate that PI3Kα signalling may have a heterogeneous role in NTCU-induced disease, and that the lung T-cell immune composition undergoes dynamic changes during LUSC development. CD4+FOXP3+ regulatory T-cells accumulate in proximity to the bronchial tree, including areas of pre-invasive and invasive disease, likely promoting an immunosuppressive environment that facilitates disease progression.
Modulating PI3Kα signalling, by BYL719 treatment, had no effect on NTCU-induced disease development. However, the PI3Kδ inhibitor PI-3065, a small molecule that impairs regulatory T-cell maintenance and functionality, reduced NTCU-induced tumour incidence and size. This was associated with fewer regulatory T-cells in proximity to disease, and an increased cytotoxic tumour immune microenvironment.

This study supports recent work describing the importance of the immune microenvironment in determining pre-invasive disease fate. My work also highlights regulatory T-cell-targeted immunomodulatory treatments, such as PI3Kδ inhibition, as important interventional lung cancer therapeutic candidates.
**Impact Statement**

Lung cancer causes 1.8 million deaths annually worldwide. The high mortality rate of lung cancer is predominately attributed to advanced stage at diagnosis. Lung squamous cell carcinoma (LUSC) is a major subtype of lung cancer that develops stepwise through a series of pre-invasive grades. With the success of recent lung cancer screening trials pre-invasive disease, which is associated with very few patient symptoms, can be more robustly identified. However, pre-invasive disease has the potential to either regress, remain stable or to progress to invasive LUSC. Therefore, patients with pre-invasive disease are often not treated and instead monitored until invasive LUSC develops.

This presents the opportunity to interventionally treat these patients to prevent invasive LUSC development and minimise patient suffering and reduce lung cancer morbidity. However, interventional strategies have so far been clinically unsuccessful, in both pre-clinical settings and human clinical trials.

To properly evaluate efficacious interventional therapeutics for LUSC, suitable pre-clinical models are required. My work describes optimised strategies to develop an *in vitro* model of early lung carcinogenesis, and the evaluation and use of an *in vivo* autochthonous preclinical model to assess the interventional therapeutic potential of phosphoinositide 3-kinase (PI3K) targeted inhibitors during LUSC progression.

I have optimised a gene-editing strategy of an immortalised human bronchial epithelial cell-line and described a system in which this could be used to study common LUSC genomic PI3K alterations. This strategy will allow identification of phenotypic changes associated with PI3K dysregulation to define its role in the molecular pathogenesis of LUSC and ultimately assess whether targeting PI3Kα may be of therapeutic value. This strategy could be applied to other
molecular features of LUSC to identify additional interventional therapeutic candidates.

My *in vivo* studies have centred around the use of the N-nitroso-tris-chloroethylurea (NTCU)-induced LUSC murine model, which histologically recapitulates both pre-invasive and invasive LUSC. I conclude that this model recapitulates features of the T-cell immune microenvironment of human LUSC and heterogeneous disease PI3Kα signalling. This further demonstrates the relevance of the NTCU model as a preclinical model of LUSC.

I demonstrate how interventional therapies can be evaluated using the NTCU model with both pre-invasive and invasive disease quantification. Intervenotional PI3Kα inhibition showed no disease modulation in this context. In contrast, interventional PI3Kδ inhibition significantly lowered tumour incidence and size. This was accompanied by lower numbers of disease-associated regulatory T-cells and an enrichment of cytotoxic T-cells in the tumour microenvironment.

My work highlights PI3Kδ inhibition as a promising interventional treatment for LUSC by modulating the T-cell lung microenvironment. It opens an important area of future study to evaluate the full potential of PI3Kδ inhibition on overall survival. This could eventually lead to the assessment of PI3Kδ inhibitors in interventional LUSC clinical trials. If successful PI3Kδ inhibitors could prevent patient suffering and decrease the global burden of LUSC cases.

This work adds to the emerging potential of PI3Kδ inhibitors in the treatment of solid tumours and highlights PI3Kδ inhibitors to be investigated in cancer chemoprevention contexts.
Table of Contents

1. Introduction .................................................................................................................. 12

1.1. Lung structure and function ....................................................................................... 12
    1.1.1. Epithelium and alveolar structure ........................................................................ 12

1.2. Lung Cancer ................................................................................................................. 14
    1.2.1. Lung cancer subclassification .............................................................................. 14
    1.2.2. Risk factors .......................................................................................................... 16

1.3. Pathogenesis of lung squamous cell carcinoma ......................................................... 17
    1.3.1. Histology of LUSC development ......................................................................... 17
    1.3.2. Molecular profile throughout LUSC development ............................................. 18
    1.3.3. The immune compartment throughout LUSC development ............................. 20

1.4. Treatments for NSCLC ............................................................................................... 28
    1.4.1. Targeted therapies ............................................................................................... 29
    1.4.2. Immune checkpoint inhibition ............................................................................. 31
    1.4.3. The use of interventional treatments to interrupt LUSC development ................ 33

1.5. Phosphoinositide 3-kinase signalling ......................................................................... 36
    1.5.1. PI3K- an overview .............................................................................................. 36
    1.5.2. PI3K dysregulation in cancer .............................................................................. 43

1.6. Hypothesis .................................................................................................................. 47

1.7. Aims ............................................................................................................................ 47

2. Methodology .................................................................................................................. 48

2.1. Cell Culture ................................................................................................................. 48
    2.1.1. Tissue culture plastic and coating ......................................................................... 48
    2.1.2. Media and Reagents ............................................................................................ 48
    2.1.3. Cell lines .............................................................................................................. 49
    2.1.4. Establishing human basal epithelial cell primary cultures ............................... 50
    2.1.5. Cryopreservation of cells .................................................................................... 52

2.2. Modulation of gene expression in vitro ...................................................................... 53
    2.2.1. Transfection ........................................................................................................ 53
    2.2.2. shRNA knockdown of TP53 in HBEC-3KT cells .............................................. 54

2.3. Molecular Biology ..................................................................................................... 56
    2.3.1. Cloning ............................................................................................................... 56
    2.3.2. PCR ..................................................................................................................... 57
    2.3.3. Western Blotting .................................................................................................. 61
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.4. Quantitative PCR</td>
<td>62</td>
</tr>
<tr>
<td>2.4. <em>in vivo</em> experiments</td>
<td>64</td>
</tr>
<tr>
<td>2.4.1. Animal study size calculations</td>
<td>64</td>
</tr>
<tr>
<td>2.4.2. NTCU treatment</td>
<td>64</td>
</tr>
<tr>
<td>2.4.3. Inhibitor administration</td>
<td>65</td>
</tr>
<tr>
<td>2.4.4. Tissue collection</td>
<td>65</td>
</tr>
<tr>
<td>2.5. Cell Analysis</td>
<td>68</td>
</tr>
<tr>
<td>2.5.1. Flow Cytometry</td>
<td>68</td>
</tr>
<tr>
<td>2.5.2. Fluorescence-activated cell sorting</td>
<td>69</td>
</tr>
<tr>
<td>2.5.3. In situ cell analysis</td>
<td>73</td>
</tr>
<tr>
<td>2.6. Assessment of inhibitor administration effect</td>
<td>78</td>
</tr>
<tr>
<td>2.6.1. Assessment of the systemic effects of inhibitors</td>
<td>78</td>
</tr>
<tr>
<td>2.6.2. Assessment of NTCU-induced disease</td>
<td>78</td>
</tr>
<tr>
<td>2.7. Single cell RNA sequencing</td>
<td>81</td>
</tr>
<tr>
<td>2.8. Statistical analysis</td>
<td>81</td>
</tr>
<tr>
<td>3. <em>Modelling</em> LUSC-associated PI3K dysregulation in human bronchial epithelial cells</td>
<td>82</td>
</tr>
<tr>
<td>3.1. Background</td>
<td>82</td>
</tr>
<tr>
<td>3.2. Aims</td>
<td>85</td>
</tr>
<tr>
<td>3.3. Results</td>
<td>86</td>
</tr>
<tr>
<td>3.3.1. PI3K pathway changes in LUSC samples</td>
<td>86</td>
</tr>
<tr>
<td>3.3.2. CRISPR/Cas9n gene editing strategy in HBECs</td>
<td>90</td>
</tr>
<tr>
<td>3.3.3. Optimisation of the transfection of primary HBECs</td>
<td>94</td>
</tr>
<tr>
<td>3.3.4. Optimisation of the transfection of immortalised HBECs</td>
<td>99</td>
</tr>
<tr>
<td>3.4. Discussion</td>
<td>103</td>
</tr>
<tr>
<td>3.5. Summary</td>
<td>106</td>
</tr>
<tr>
<td>4. <em>Assessment of the involvement of the PI3K pathway and immune microenvironment in NTCU-induced lung squamous cell carcinoma.</em></td>
<td>107</td>
</tr>
<tr>
<td>4.1. Background</td>
<td>107</td>
</tr>
<tr>
<td>4.2. Aims</td>
<td>111</td>
</tr>
<tr>
<td>4.3. Results</td>
<td>112</td>
</tr>
<tr>
<td>4.3.1. PI3K signalling in NTCU-induced disease</td>
<td>114</td>
</tr>
</tbody>
</table>
4.3.2. Longitudinal analysis of the immune microenvironment of NTCU-induced disease ................................................................. 118
4.3.3. scRNAseq of epithelial and immune populations following NTCU treatment... 134

4.4. Discussion .................................................................................. 136
4.5. Summary ................................................................................... 140

5. Pharmacological inhibition of PI3Kα and PI3Kδ as intervention therapies to NTCU-induced lung squamous cell carcinoma .......... 141

5.1. Background .............................................................................. 141
5.2. Aims ......................................................................................... 144
5.3. Results ..................................................................................... 145
  5.3.1. PI3K inhibitors as interventional therapies ................................. 145
  5.3.2. Weight change associated with NTCU and inhibitor treatment ........ 146
  5.3.3. Diet consumption .................................................................. 148
  5.3.4. Side-effects and adverse reactions ........................................... 149
  5.3.5. Pharmacologically active inhibitor administrations ..................... 152
  5.3.6. NTCU-induced disease modulation by interventional PI3K targeted therapies 158
  5.3.7. Hyperactivation of the insulin pathway ...................................... 166

5.4. Discussion ................................................................................. 168
5.5. Summary ................................................................................... 171

6. Assessing immune modulation by PI3Kδ inhibition in NTCU-induced lung carcinogenesis ......................................................... 172

6.1. Background .............................................................................. 172
6.2. Aims ......................................................................................... 175
6.3. Results ..................................................................................... 176
  6.3.1. Systemic spleen changes .......................................................... 176
  6.3.2. T-cell localisation in the lung after PI-3065 treatment .................... 182

6.4. Discussion ................................................................................. 195
6.5. Summary ................................................................................... 199

7. Discussion...................................................................................... 200
  7.1.1. Summary ............................................................................... 200
  7.1.2. Immediate future directions ..................................................... 203
  7.1.3. Considerations for the application of PI3Kδ inhibitors as interventional therapies for LUSC development .................................................. 204
8. Appendix .............................................................................................................. 207
9. Bibliography........................................................................................................ 211
1. Introduction

1.1. Lung structure and function

The main purpose of the lungs is to oxygenate blood for the supply of all body tissues. The lungs are composed of multiple lobes. Human lungs are composed of five lobes asymmetrically distributed, with three lobes on the right and two on the left. In contrast, in mice, the five lobes are distributed with four on the right and one on the left.

Lung tissue structures can be separated into either airway or parenchyma. Airways consist of the bronchus which extend distally from the trachea and bifurcates into the right and left halves respectively. As the bronchi extend distally within the lung, airway diameter narrows and airways further branch into bronchioles and terminate in alveoli. The parenchyma is composed of the bronchioles, alveolar ducts, alveoli and is responsible for gas exchange (Figure 1.1) (Chaudhry and Bordoni 2022).

1.1.1. Epithelium and alveolar structure

In the human lung the bronchial airway is lined by a pseudostratified epithelium. This epithelium is made up of multiple cell types, each of which facilitate a particular function. These include basal cells which are tightly attached to the basement membrane and act as progenitors to regenerate all epithelial cell types during normal homeostasis and injury. Ciliated cells, mucinous producing goblet cells and club cells line the airways to lubricate, trap and clear debris to maintain effective gas exchange function. Neuroendocrine cells are distributed throughout the airway with concentrations at airway branch points. These concentrations are termed neuroepithelial bodies and function in oxygen sensing. The composition of the pseudostratified epithelium varies with bronchial tree hierarchy (Figure 1.1) (Rock, Randell and Hogan 2010, Hogan et al. 2014).
In contrast, the bronchial airways in mice are lined with a simple columnar epithelium with pseudostratified epithelium including basal cells restricted to the trachea and extrapulmonary regions. The simple columnar epithelium in mice lack basal cells but include ciliated, club and neuroendocrine cells (Rock et al. 2010).

In mice the transition from bronchioles into alveoli is well characterised and termed the bronchiolar alveolar junction. However, this transition of terminal bronchioles into alveoli in humans is poorly characterised but includes a simple cuboidal epithelium. The alveoli, the site of gas exchange, are made up of two cell types in both mice and humans, AT1 and AT2 cells (Hogan et al. 2014).

Figure 1.1 - Lung anatomical structure of human and mouse lungs. Key structures labelled with depiction of pseudostratified epithelium found in proximal human lung. Figure created using BioRender.com.
1.2. Lung Cancer

Cancer ranks as one of the leading causes of death worldwide. With world cancer burden expected to rise by 47% from 2020 to 2040, cancer mortality is only set to increase. Worldwide, lung cancer represents 11% of all cancer diagnoses and is the most common cause of cancer related death; attributed with 18% of all cancer deaths. This equates to a staggering, 1.8 million deaths per year (Sung et al. 2021).

The high mortality rate of lung cancer is predominately attributed to late-stage diagnosis. Five-year survival of lung cancer patients dramatically decreases with stage of disease at diagnoses. The 5-year survival rate of lung cancer patients with the earliest, stage I disease is 60.9%. This falls to just 3.9% in patients diagnosed with most advanced, stage IV lung cancer. In 2019, 42.4% of lung cancers diagnosed in England were stage IV (CRUK 2022a). The resulting overall, 5-year survival rates for lung cancer are low at 10-20%, in most countries (2010-2014) (Sung et al. 2021).

This demonstrates that if lung cancers are diagnosed at earlier stages and further progression can be prevented, there can be profound reductions in the mortality rate of lung cancer patients. To achieve this, methods to aid early-detection of lung cancer and new treatments to intercept lung cancer development will be essential.

1.2.1. Lung cancer subclassification

Broadly, lung cancer can be subdivided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Each have varying histology, treatment and prognosis.

SCLC accounts for 15% of lung cancer (CRUK 2022c), is predominately associated with tobacco smoking and can be identified histologically by small
cells with very little cytoplasm (Kalemkerian et al. 2011). SCLC is a fast-growing cancer that occurs centrally within the lung and originates from lung neuroendocrine cells. SCLC tumours are often diagnosed at advanced stage and therefore often inoperable. In the absence of surgery, treatment utilises chemotherapy and radiotherapy (Kalemkerian et al. 2011). SCLC tumours are fast growing and metastasise early, which further significantly limits patient prognosis (CRUK 2022c). Only 5% of patients diagnosed with extensive-stage SCLC survive 2 years post diagnosis (Kalemkerian et al. 2011).

NSCLC accounts for the majority of lung cancer diagnoses and can be further subdivided into adenocarcinoma, squamous cell carcinoma and large cell carcinoma (Herbst, Morgensztern and Boshoff 2018).

Adenocarcinoma accounts for 40% of lung cancers and originates from the mucinous producing bronchial glands of the lung, alveolar type II cells and hence arises peripherally (Zappa and Mousa 2016). Lung adenocarcinomas (LUAD) are the most common lung cancer subtype in non-smokers (Dubin and Griffin 2020). Tumours can be identified by staining of Napsin A (Nap-A) and thyroid transcription factor 1 (TTF1) and can be further subclassified histologically into four groups: acinar, papillary, lepidic and micropapillary (Inamura 2017).

Lung squamous cell carcinoma (LUSC) accounts for 30% of lung cancer diagnoses and is most often seen in patients with a history of tobacco smoking (Perez-Moreno et al. 2012). LUSC originates from the squamous bronchial epithelium and often occurs within the central region of the lung (Perez-Moreno et al. 2012). LUSC tumours are histologically identified by staining for p40, p63 and keratin 5 (KRT5)(Inamura 2017, Affandi et al. 2018). LUSC tumours can be separated into three types, keratinising, non-keratinising and basaloid (Inamura 2017). Before tumour establishment, LUSC develops through early pre-malignant stages, which will be discussed in detail in further sections.
Large cell carcinoma (LCC) is the rarest lung cancer subtype, making up only 5% of lung cancers. LCC tumours often arise centrally within the lung and are characteristically composed of large round undifferentiated cells. Tumour diagnosis is characterised by a lack of both adenocarcinoma and squamous differentiation markers, so advances in molecular subtyping has reduced LCC diagnoses (Zappa and Mousa 2016).

### 1.2.2. Risk factors

The major risk factor associated with lung cancer is smoking, with 71% of lung cancer cases in the UK associated with direct patient smoking and 1% associated with passive smoking (CRUK 2022b). In addition, 13% of lung cancer cases in the UK have been linked to occupational exposures, which include exposure to asbestos, silica, radon and diesel engine exhaust (Brown et al. 2018). A further 8% have been associated with air pollution, and 5% with exposure to ionising radiation (Brown et al. 2018). On top of environmental exposures, an increased lung cancer risk has also been associated with genetics. Individuals with a sibling who has had lung cancer have an 82% increase in lifetime lung cancer risk (Coté et al. 2012). Many institutional and national measures have been implemented to limit lung cancer risk; these are predominately aimed at limiting environmental exposure.
1.3. Pathogenesis of lung squamous cell carcinoma

Despite the common advanced diagnosis of LUSC, LUSC has a pathologically identifiable pre-malignant phase. Patients with pre-invasive stages of LUSC are most often asymptomatic so this stage of LUSC often goes undiagnosed. However, pre-invasive lesions can be identified, if patients present in clinic, with the use of autofluorescence bronchoscopy. Autofluorescence bronchoscopy exploits the observation that abnormal tissue shows reduced fluorescence when excited with blue light (wavelength 380-460nm) (Zhu et al. 2012).

1.3.1. Histology of LUSC development

LUSC develops stepwise through a series of pre-invasive stages (Figure 1.2) (Thakrar et al. 2017). The World Health Organisation (WHO) has classified these LUSC development stages into 9 categories, ranging from normal epithelium through, inflammation, hyperplasia, squamous metaplasia, mild dysplasia, moderate dysplasia, severe dysplasia to carcinoma in situ (CIS) and invasive LUSC (Daniels and Sutedja 2013). Throughout these stages epithelial morphology changes towards increasing disorder. Initial stages display changes only in the basal layer of the epithelium, with enlarged nuclei and loss of nuclear orientation. With progression, these changes, along with cell disorganisation and loss of polarity, affect the entirety of the epithelium. Invasive LUSC requires eventual breaking of the basement membrane and invasion of cells into the parenchyma (Daniels and Sutedja 2013).

Figure 1.2 - LUSC progresses step-wise through pre-invasive disease stages that have distinct morphological changes. H&E stained characteristic examples of LUSC pre-invasive grades and invasive squamous cell carcinoma. Figure adapted from Thakrar et al 2017.
Longitudinal studies have demonstrated the stepwise nature of these pre-invasive grades, with high-grade lesions more likely to progress to invasive cancer than low grade lesions. The presence of persistent high-grade lesions also increase the risk of LUSC (Merrick et al. 2016). However, it has been repeatedly demonstrated that this stepwise development is not unidirectional, and that even high-grade pre-invasive lesions are capable of regression to early pre-invasive grades or complete regression to normal epithelium (Daniels and Sutedja 2013).

1.3.2. Molecular profile throughout LUSC development

There have been many studies investigating the molecular changes throughout LUSC development. Understanding the temporal molecular alterations of the disease could lead to the development of successful targeted therapies.

Commonly changed pathways in LUSC include squamous differentiation via sex determining region Y-box 2 (SOX2), cell cycle regulation by cyclin dependent kinase inhibitor 2A (CDKN2A), intracellular signalling by phosphoinositide 3-kinase /protein kinase B (PI3K/AKT) and cellular stress regulation with kelch like ECH associated protein 1 (KEAP1). These pathways are altered either genomically, transcriptomically or epigenetically, and often cooperate to mediate LUSC development and maintenance (Gómez-López, Whiteman and Janes 2021).

It has been demonstrated that many of the molecular changes characteristic of LUSC occur stepwise throughout pre-invasive disease stages. For instance, amplification of distal 3q, including SOX2, arises from low grade, moderate dysplasia onwards (van Boerdonk et al. 2011, McCaughan et al. 2010). Genetic tumour protein 53 (TP53) changes, including mutations and loss of heterozygosity (LOH) arise even earlier, from metaplasia onwards (Sundaresan et al. 1992). Whereas, 5q LOH is seen only in high-grade lesions from severe dysplasia (Foster et al. 2005). Interestingly, it has been
demonstrated that some alterations, including telomerase overexpression and 3p LOH, are even present in normal epithelium in smokers (Mao et al. 1997, Lantuejoul et al. 2005). In fact, a recent study has identified common cancer driver genes in phenotypically normal bronchial cells from smokers, including notch receptor 1 (NOTCH1), TP53, FAT Atypical Cadherin 1 (FAT1) and Phosphatase and tensin homolog (PTEN) (Yoshida et al. 2020).

A recent study from the Janes group characterised the genomic, transcriptomic and epigenomic profiles of CIS lesions (Teixeira et al. 2019). CIS lesions have a similar mutational burden and copy number alterations as invasive LUSC (Teixeira et al. 2019). CIS lesions showed strong tobacco smoke mutational signatures, similar to that seen in LUSC (Teixeira et al. 2019). Similar driver mutations were seen in CIS lesions to LUSC and included alterations in TP53, SOX2, CDKN2A and AKT2. CIS lesions displayed the most common genomic alteration in LUSC, amplification of distal 3q (Mendez and Ramirez 2013) and additional common losses in 3p and 5q (Teixeira et al. 2019).

These CIS lesions were longitudinally tracked to determine progression rate and to compare the molecular profiles of progressive vs regressive CIS lesions. CIS lesions were shown to progress in only 50% of cases, regress in 30% and display stable disease in the remaining 20% of cases. Progressive CIS lesions had a higher somatic mutation burden and more driver mutations than regressive lesions. However, no driver mutation perfectly discriminated between progressive and regressive lesions.

Both gene expression and methylation profiles significantly differed between progressive and regressive CIS lesions (Teixeira et al. 2019). Differentially expressed genes included KEAP1, Suppressor of Mothers against Decapentaplegic (SMAD) and NK2 homeobox 1 (NKX2-1). Differential methylated positions were found in driver genes including NKX2-1, NOTCH1 and CDKN2A. Comparing molecular profiles of progressive CIS with
regressive CI demonstrate intrinsic differences, which are likely to contribute to determination of lesion fate.

1.3.3. The immune compartment throughout LUSC development

The lung is composed of immune cells that represent both the innate and adaptive immune compartments. These include macrophages, neutrophils, myeloid-derived suppressor cells, natural killer (NK) cells and dendritic cells of the innate compartment, and T- and B-cells of the adaptive immune response (Ardain, Marakalala and Leslie 2020). These immune cells form the first line of defence against inhaled pathogens. Lung pathologies, including cancer, alter the immune compartment composition and its distribution.

It is well established that the tumour immune microenvironment is influential in both the development and maintenance of tumours. This influence is heavily cancer type dependent, but NSCLC tumours are thought to be immune ‘hot’ tumours, with signs of inflammation and immune cell infiltration (Binnewies et al. 2018).

In addition to the immune cells, the tumour microenvironment is also made of the tumour-associated stromal cells. These further mediate tumour-immune interactions and include endothelial cells and fibroblasts. Dissecting the pro-oncogenic influence of the tumour immune microenvironment throughout the development of LUSC can identify new therapeutic opportunities.

1.3.3.1. Immune profile of pre-invasive LUSC

The stepwise development of LUSC occurs in a complex microenvironment that includes a dynamic immune compartment. Recent studies have utilised the growing accessibility of cohorts of pre-invasive disease specimens to better understand the co-evolution of LUSC through progression of pre-invasive disease and the immune microenvironment.
A study by Masceaux et al. used gene-expression profiling and multispectral imaging of biopsies representing histological stages of pre-invasive disease and invasive LUSC to delineate the associated changes in the immune compartment. When compared to normal epithelium low grade lesions downregulate genes that negatively regulate the immune system, including tumour necrosis factor ligand superfamily member 14 (TNFRSF14), Cluster of differentiation 200 (CD200), Cluster of differentiation (CD59), transforming growth factor β 3 (TGFB3) and human leukocyte antigen G (HLA-G) (Mascaux et al. 2019). Low grade lesions displayed activation of mast cells, neutrophils and increased antigen processing and presentation. This demonstrates that the immune compartment is activated at the earliest stages of LUSC disease (Angelova, Mascaux and Galon 2021).

High-grade lesions displayed enhanced immune activation, with increased innate myeloid-derived cells, neutrophils, dendritic cells and M1 macrophages (Mascaux et al. 2019). More adaptive immune cells, including T-cells and T-follicular helper cells, were also seen. High-grade lesions also displayed higher immune activation, indicated by increased tumour necrosis factor ligand superfamily member 9 (TNSF9) expression. In addition to immune activation immunosuppressive mechanisms were observed in high grade lesions. This was evident as enhanced expression of immune checkpoints including programmed cell death ligand 1 (PD-L1), cytotoxic T-lymphocyte associated protein 4 (CTLA4) and indoleamine 2,3-dioxygenase 1 (IDO1). Thus, demonstrating that immune checkpoint mechanisms of immunosuppression evolve before progression to invasion (Mascaux et al. 2019).

This study has increased our understanding of the dynamic changes in the immune environment that occur throughout LUSC development, highlighting that immune changes occur from the earliest stages. However, the progression of pre-invasive disease is dynamic and non-unilateral, therefore
other studies have investigated the role of the immune compartment in lesion fate, using longitudinal lesion tracking.

Immunosurveillance and immune infiltration have been repeatedly demonstrated as a determining factor in the fate of pre-invasive LUSC lesions. Impairment of immunosurveillance in progressive high-grade lesions have been demonstrated by multiple mechanisms (Figure 1.3).

Antigen processing and presentation have been shown to be significantly decreased in both persistent high-grade lesions (Beane et al. 2019) and progressive CIS lesions (Pennycuick et al. 2020). When compared to regressive, progressive CIS lesions carried more mutations and copy number alterations in genes involved in antigen presenting and processing mechanisms. A subset of these altered genes were found to be positively selected, indicating their importance in determining lesion fate (Pennycuick et al. 2020). This impaired antigen processing/presenting results in reduced activation of the adaptive immune response.

Reduced immunosurveillance in progressive/persistent lesions is also favoured by reduced interferon signalling (Beane et al. 2019) and associated with downregulation of other immune cell activators, such as TNFSF9 (Pennycuick et al. 2020). TNFSF9 promotes cytotoxic T-cell and NK activation (Hashimoto 2021) so therefore, may impair the cytotoxic response to CIS lesion progression.

Cytokine and chemokine expression have been shown to differ between progressive and regressive CIS lesions. Regressive CIS lesions express higher levels of proinflammatory cytokines, e.g. interleukin 2 (IL2) and tumour necrosis factor (TNF) (Pennycuick et al. 2019), which likely promote immunosurveillance by activation of T-lymphocytes and could play a part in regression. On the other hand, C-C Motif Chemokine Ligand 27 (CCL27) is
upregulated in progressive CIS lesions (Pennycuick et al. 2020). This presents a potential mechanism of immune escape via PI3K signalling, as seen in a preclinical model of melanoma (Murakami et al. 2003). This is further supported by CCL27 expression correlating with PIK3CA and AKT1 expression in progressive CIS lesions. It has been suggested that the lack of CCL27 expression in both normal epithelium and LUSC could highlight this as a target for interventional therapy (Pennycuick et al. 2020).

Although not a determinant factor in lesion fate, a subset of progressive CIS lesions expressed the immune checkpoint PD-L1 (Pennycuick et al. 2020). This further demonstrates that immune checkpoint immunosuppressive mechanisms are present before invasion highlighting these as an exploitable therapeutic mechanism.

These impaired immunosurveillance mechanisms result in reduced immune infiltration in progressive CIS lesions. Regressive CIS lesions had more immune infiltration of overall lymphocytes and CD8+ cytotoxic T-cells than CIS lesions that progressed to cancer (Pennycuick et al. 2020). Infiltration of CD68+ M1 macrophage and CD4+ T-cells were significantly increased in progressive/persistent high-grade lesions whereas M2 CD68+ CD163+ macrophages and CD8+ T-cells were decreased (Beane et al. 2019). Progressive lesions had higher levels of macrophage-produced CXCL8 (Pennycuick et al. 2020). CXCL8 has been implicated in favouring tumour progression by promoting tumour cell survival and inhibiting anti-tumour immunity (Xiong et al. 2022). This suggests a role for immunosuppressive M1 macrophages in progression of pre-invasive lesions.
Figure 1.3 - Summary of the potential mechanisms of impaired immunosurveillance demonstrated in pre-invasive stages of LUSC. Four potential mechanisms are depicted. (A) Impaired antigen processing and presentation of epithelial cells. (B) Reduced IFNγ signalling. (C) The expression of immune checkpoint mechanisms. (D) Pre-invasive disease release of cytokines resulting in less T-cell activation and promoting myeloid derived suppressor cells (MDSCs). Figure taken from (Krysan, Tran and Dubinett 2020).
1.3.3.2. Immune profile of invasive LUSC

Characterisation of the tumour immune microenvironment of LUSC by using imaging mass cytometry has revealed infiltration of activated T-cells, including CD8+ T-cells and FOXP3+ cells, and CD33+ myeloid-derived cells. LUSC tumours displayed limited infiltration of B-cells, NK and NKT cells (Li et al. 2021).

Compared to lung adenocarcinoma, LUSC tumours display higher infiltration of neutrophils, CD4+ T-cell subsets including FOXP3+ regulatory T-cells and PD1 expressing CD8+ T-cells but less macrophages and less Th1 and Th17 CD4+ T-cell subtypes. Many of these immune cell populations have been shown to favour an immunosuppressive tumour environment (Kargl et al. 2019).

Both Th1 and Th17 CD4+ T-cell subsets have been shown to secrete IFNγ which can activate macrophages and dendritic cells and promote CD8+ T-cell adaptive immunity (Szabo et al. 2003, Galon et al. 2006). Therefore, fewer Th1 and Th17 CD4+ T-cells in LUSC tumours could favour a more immunosuppressive microenvironment with less adaptive and innate immune activation. Furthermore, higher CD4+ T-follicular helper cell infiltration overall has been associated with increased overall survival (Wang and Guo 2020).

Neutrophils have been found to be the most abundant immune cell subtype present in LUSC tumours (Kargl et al. 2017) and neutrophils have been shown to possess lymphocyte suppressive capabilities (Gabrilovich and Nagaraj 2009). Neutrophil tumour content negatively correlated with both CD8+ and CD4+ T-cell contents (particularly Th1 and Th17 subtypes), suggesting a negative impact of neutrophils on these T-cell populations (Kargl et al. 2017). The presence of high numbers of neutrophils in LUSC tumours have been linked to tumour SOX2 overexpression-mediated expression of neutrophil attracting factor CXCL6, as assessed using in vitro studies (Mollaoglu et al. 2018).
The high degree of FOXP3+ regulatory T-cell infiltration seen in LUSC tumours favours an immunosuppressive tumour environment and tumour tolerance (Adeegbe and Nishikawa 2013). In NSCLC, patients increased tumour infiltration of FOXP3+ T-regulatory cells has been associated with a decrease in recurrence-free survival (Shimizu et al. 2010). The ratio of CD8+ to FOXP3+ cells in a genetically engineered murine model of LUSC inversely correlated with tumour burden (Xu et al. 2014), further demonstrating that T-cell mediated immunosuppression favours LUSC tumour development.

Tumours have been shown to express PD-L1. Upon recognition by T-cells via their PD-1 expression, T-cell activation is inhibited and T-cell exhaustion favoured. Thus preventing immune mediated clearance of tumour cells (Vinay et al. 2015). The expression of PD1 on CD8+ T-cells presents immune checkpoint inhibition as an additional tumour influencing immunosuppressive mechanism. PD-L1 expression has been demonstrated in multiple transgenic pre-clinical LUSC models (Xu et al. 2014, Mollaoglu et al. 2018). The interaction of PD1 with PD-L1 has been shown to inhibit CD8+ T-cell survival (Barber et al. 2006), proliferation and function, whilst also promoting CD4+ regulatory T-cell differentiation (Francisco et al. 2009), further perpetuating an immunosuppressive environment.

A subset of LUSC tumours have shown to have high levels of infiltrating inflammatory monocytes, which was associated with poor survival. Using a murine pre-clinical transplantation model, these inflammatory monocytes were shown to be recruited via cancer cell-derived CCL2. The recruited inflammatory monocytes express Factor XllIA, which creates a fibrin cross-linked based scaffold that favours invasion and metastasis. The presence of high levels of intratumoural cross-linking in human patients has been associated with poor survival (Porrello et al. 2018).
Diverse immune cell subtypes have been shown to influence LUSC development and maintenance. This presence of immune-cancer cell interactions in all stages of the disease highlights the importance of the immune compartment in directing LUSC development. This also presents the opportunity to target the immune compartment to both interject in LUSC development and to treat advanced LUSC.
1.4. Treatments for NSCLC

The primary treatment for NSCLC, particularly early stage is surgery. This could be in the form of a partial or complete lobectomy. However, this is only applicable to resectable tumours in patients who are deemed fit for surgery and is not always a curative option, especially in the case of advanced or metastatic disease. The use of video-assisted thorascopic surgery has advanced lung cancer surgery by being minimally invasive, reducing patient surgery associated complications and recovery time (Howington et al. 2013, Scott et al. 2010). To reduce the risk of lung cancer relapse, patients are also often treated with adjuvant therapy.

Chemotherapy and targeted chest radiotherapy are most often applied in combination for the treatment of advanced NSCLC although both have been used to treat and manage patients with earlier resected tumours (Ohe 2004). Chemotherapy exploits the highly proliferative nature of cancer cells. For NSCLC, chemotherapy is most often applied as a platinum-based doublet therapy. This includes a platinum-based drug that binds to DNA causing DNA damage and halting replication, and a cytotoxic therapy that could for instance, interfere with cellular division. Comparisons of different combinations of chemotherapeutic drugs in the treatment of NSCLC have shown little clinical difference in response (Schiller et al. 2002).

In contrast to systemically administered chemotherapy, radiotherapy utilises high-energy beams directed to the tumour site. These high-energy beams damage DNA within cancer cells inducing cell death. Side effects of both chemotherapy and radiotherapy have limited the applied dosage in the treatment of NSCLC. The response rate of chemotherapy in the treatment of NSCLC has been reported as low as 19% (Schiller et al. 2002).
1.4.1. Targeted therapies

Efforts to develop more efficacious NSCLC treatments have utilised advances in the understanding of the genomic landscape of lung cancer, directing the development of gene targeted therapies for lung cancer. These include therapies targeting Kirsten rat sarcoma (KRAS), epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK) and ROS proto-oncogene 1 (ROS1). However, due to the great intra- and inter-tumoral heterogeneity and evolvability of lung cancer the efficacy of these targeted therapies has been limited.

1.4.1.1. KRAS inhibitors

Single amino acid substitution mutations in KRAS are common in NSCLC, predominately adenocarcinomas. In fact, 10-25% of adenocarcinomas have KRAS mutations (Zappa and Mousa 2016) whereas, KRAS mutations occur in 1-7% of LUSC (Acker et al. 2021). Recent updates in NICE guidelines recommends the treatment of patients with KRAS mutant advanced NSCLC with sotorasib (NICE. 2022). Sotorasib is a small molecule inhibitor that targets R28C mutant KRAS. A single group phase II clinical trial demonstrated that sotorasib treatment displayed durable clinical benefit (Skoulidis et al. 2021).

1.4.1.2. EGFR kinase inhibitors

Oncogenic EGFR signalling has been shown to induce uncontrolled cell division in cancers. EGFR genomic changes can occur in 27% adenocarcinomas and <9% of LUSC (Herbst et al. 2018). Activating genomic changes often occur in the kinase domain and include deletion of exon 19 and the L858R exon 21 mutation leading to oncogenic activation of EGFR (Zappa and Mousa 2016). This EGFR activation stimulates downstream signalling, favouring increased proliferation, metastasis and angiogenesis (Wheeler, Dunn and Harari 2010). When treated with Erlotinib a tyrosine kinase inhibitor, as part of a phase III trial, patients with EGFR genomic changes, display significantly longer progression-free survival compared to chemotherapy but no difference in overall survival (Zhou et al. 2015, Wen et al. 2018).
1.4.1.3. ALK inhibitors

ALK rearrangements, most commonly with echinoderm microtubule-associated protein-like 4 (EML4) occur in ~7% of NSCLC patients (Soda et al. 2007). Majority of these are classified as adenocarcinomas (Shaw et al. 2009), with only very rare occurrences in LUSC (Meng et al. 2021). Genetic fusion of the 5’ end of EML4 with the 3’ end of ALK on chromosome 2p23 results in EML4-ALK fusion proteins which favour constitutive ALK activity. Lung cancer patients with EML4-ALK rearrangements have been treated with crizotinib treatment. Compared with chemotherapy alone, previously untreated non-squamous NSCLC patients, had a 29% increase in overall response rate when treated with crizotinib (Solomon et al. 2014). In LUSC patients with rare EML4-ALK rearrangements the overall response rate to crizotinib has been reported as high as 55% (Meng et al. 2021).

1.4.1.4. Targeting ROS1

ROS1 genomic rearrangements can result in fusion with gene partners that result in constitutive kinase activity which can drive tumour progression. ROS1 genomic rearrangements occur in ~2% of lung adenocarcinomas and are extremely rare in LUSC (Herbst et al. 2018, Yang et al. 2021). Since the ROS1 kinase domain shares a high degree of homology with ALK, ALK targeted therapies have also been used for lung cancers with ROS1 genetic changes (Herbst et al. 2018). Crizotinib has shown efficacy in NSCLCs that have activated oncogenic ROS-1 rearrangements (Shaw and Solomon 2015).

The application of these kinase inhibitors shows therapeutic benefit only in a subset of patients with the respective genomic changes. When applied to the correct NSCLC target patient populations these kinase inhibitors prolong progression-free survival but often have little to no effect on overall survival. The therapeutic potential of these targeted kinase inhibitors is often limited by acquired resistance mechanisms. Resistance can be acquired through the
acquisition of additional kinase mutations or altered pathway reliance (Cooper, Sequist and Lin 2022, Pan et al. 2021).

In addition, many of these targeted therapies and associated therapeutic benefit are biased to the treatment of adenocarcinoma. As mentioned above, genomic changes that induce oncogenic activation of KRAS, EGFR, ALK and ROS1 are consistently more frequent in adenocarcinoma than LUSC (Herbst et al. 2018). Making these targeted therapies much less applicable to patients suffering with LUSC. To identify new targeted therapies for LUSC targetable mutations that occur in LUSC need to be identified. One of the limitations of identification of targetable mutations in LUSC has been the lack of effective pre-clinical models that accurately depict the molecular heterogeneity of LUSC. Therefore, better LUSC pre-clinical models are required to explore new therapeutic avenues.

1.4.2. Immune checkpoint inhibition

Immune checkpoints are one such mechanism that protects tissues by maintaining tolerance to self and regulating immune reaction. However, expression of immune checkpoints have been shown to be exploited by tumour cells as a mechanism to evade immune detection in a wide variety of cancers, including lung cancer (Vinay et al. 2015).

The use of PD-L1 targeted immune checkpoint therapies has dominated in the treatment of NSCLCs. Upon diagnosis, NSCLC biopsies are histologically assessed for PD-L1 expression. If sufficient PD-L1 expression is noted, patients are recommended for PD-1/PD-L1 targeted monoclonal antibodies. Nivolumab, pembrolizumab, atezolizumab, and durvalumab are but a few PD-1/PD-L1 targeted antibodies successfully applied to the treatment of NSCLC (Onoi et al. 2020). Treatment with PD-1/PD-L1 monoclonal antibodies disrupts immune checkpoint inhibition, allowing immune cell activation and tumour cell clearance. Pembrolizumab is recommended as a first line therapy to patients with non-squamous NSCLC with no targetable mutations and has displayed
an increased survival of 10 months in a phase III clinical trial (Gandhi et al. 2018).

However, the response rate is only ~15–25% of patients and treatment is associated with high incidence of immune-mediated side-effects (Johnson, Rioth and Horn 2014). Therefore, patient stratification is important to predict therapeutic benefit of immune checkpoint inhibitors in NSCLC. Recent studies have suggested that tumour immune infiltration can predict immune checkpoint response with poor response indicated by high levels of neutrophil infiltration in a subset of myeloid-rich NSCLC tumours. Tumour neutrophil count as a ratio of CD8+ T-cell infiltration distinguished immune checkpoint responsive patients (Kargl et al. 2019). Efficient patient stratification is still ongoing.

Unfortunately, patients that do respond to immune checkpoint inhibition often acquire resistance through other mechanisms of tumour immunosuppression and altered checkpoint inhibitor expression (Boyero et al. 2020).
1.4.3. The use of interventional treatments to interrupt LUSC development

Due to the dynamic nature of pre-invasive squamous lung lesions, currently there is no consensus as to at what stage pre-invasive disease should be treated. However, if they were to be treated, there have been several suggested strategies and molecular processes that could be targeted to induce regression of dysplastic lesions. These include the removal of mutagens, targeting of driver mutations to remove mutated clones, promoting immune activation and rejuvenating unmutated progenitor cells (Keith et al. 2022).

One of the most obvious is the removal of mutagens. Indeed, the cessation of smoking has been shown to not only lower risk of lung cancer (Tindle et al. 2018), but the epithelial population with near-normal mutational burden has been shown to regenerate (Yoshida et al. 2020).

Efforts to target driver mutations in pre-invasive disease lesion have been limited. A phase I clinical trial investigated the chemopreventative potential of myo-initosol, an indirect inhibitor of PI3K signalling, in patients with a smoking history and one or more dysplastic bronchial lesions. Myo-initosol treatment increased the regression of dysplastic lesions from 48% to 91% (Lam et al. 2006). However, a later phase IIb clinical trial demonstrated that myo-initosol treatment had no statistically significant effect on bronchial dysplasia despite reduced PI3K gene expression in bronchial dysplastic lesions (Lam et al. 2016).

Additional LUSC associated changes have been targeted. Low levels of prostacyclin have been associated with lung cancer. Oral iloprost administration, which acts to increase prostacyclin levels, has been shown to improve bronchial histology over 6 months of treatment, in former smokers only (Keith et al. 2011). This potentially highlights iloprost treatment as a potential avenue of therapeutic value for the prevention of LUSC.
The recent advancements in the molecular, genomic and transcriptomic landscape of pre-invasive disease fate can now better direct the development of driver-targeted therapies to modulate the progression of pre-invasive disease to LUSC.

The potential of dietary supplements including vitamins and minerals to prevent the progression of pre-invasive lesions to invasive LUSC have been extensively studied in clinical trials (Keith et al. 2022). A systematic review of these trials has concluded that they have no therapeutic benefit in this context (Cortés-Jofré et al. 2020). Two current clinical trials are investigating the potential of dietary supplements to influence the fate of bronchial dysplastic lesions in high-risk patients, these include the use of Sulforaphane (NCT03232138) and Lovaza with Curcumin C3 Complex (NCT03598309).

The potential of immunomodulatory interventional therapies, to regulate the progression of pre-invasive LUSC lesions has just started to be thoroughly investigated. There has been one such completed, phase II trial that aimed to target inflammation associated with dysplastic bronchial lesions by long-term administration of inhaled corticosteroid budesonide. However, there was no difference in lesion fate observed (Lam et al. 2004) or in nodule size (Veronesi et al. 2011). Currently recruiting clinical trials investigate the efficacy of immunotherapies in modulating the fate of pre-invasive lesions. There is currently a phase II clinical trial investigating the efficacy of PD-1 inhibitor Nivolumab in influencing dysplastic bronchial lesions in patients with high risk of developing lung cancer (NCT03347838). Another phase II clinical trial investigates the potential of pembrolizumab in the fate of pre-neoplastic bronchial lesions in patients that are of high-risk of developing lung cancer (NCT03634241).

So far, interventional therapies have shown no lasting clinical benefit in trials meaning there is still no therapeutic option for high-risk patients with pre-
invasive LUSC. However, this interception strategy remains an extremely important possible route to reduce LUSC prevalence and therefore patient suffering and mortality. Improved clinical benefit could be more likely in the PD-1/PD-L1 clinical trials mentioned, as PD-L1 expression has been documented in pre-invasive LUSC and immune evasion is critical in pre-invasive lesion fate (as previously discussed). Additional studies applying targeted therapies to the genomic and molecular alterations present in pre-invasive LUSC could also much more efficacious.

One important requirement of these studies will be comprehensive patient cohorts. This will rely on reliable identification of high-risk patients as pre-invasive disease have little to no associated symptoms. Patient identification would likely rely on smoking status/history records and extensive lung screening programmes.
### 1.5. Phosphoinositide 3-kinase signalling

#### 1.5.1. PI3K- an overview

PI3Ks are lipid kinases that regulate signalling and vesicle traffic by phosphorylating the 3’ position of intracellular inositol lipids. Eight distinct isoforms of PI3K are found in mammals, reflecting the diversity of signalling processes PI3Ks are involved in. These 8 isoforms can be separated into 3 classes, Class I, II and III, depending on structure- and substrate-specificity (Bilanges, Posor and Vanhaesebroeck 2019).

Class I PI3Ks are a major regulator of signal transduction from the plasma membrane and have been extensively studied due to their frequent genetic activation in cancer, benign overgrowth disorders and their role in inflammation and autoimmunity. While the roles of both class II and III PI3Ks are still being fully elucidated, they have been shown to be regulators of membrane trafficking with an indirect effect on cell signalling (Bilanges et al. 2019).

In addition to PI3K signalling by catalytic kinase activity, PI3Ks have also been shown to have scaffolding roles in stabilising protein partners (Bilanges et al. 2019).

#### 1.5.1.1. Class I PI3K

**Structure and function**

Class I PI3Ks are activated by plasma membrane bound tyrosine kinases, G protein-coupled receptors (GPCRs) and monomeric small GTPases. When catalytically inactive, class I PI3Ks exist as heterodimers of a catalytic p110 subunit and a regulatory subunit. Class I PI3Ks consist of 4 catalytic isoforms in mammals, which include p110α, p110β, p110δ and p110γ. Each of which
are encoded by distinct genes; *PIK3CA, PIK3CB, PIK3CD and PIK3CG*, respectively (Bilanges et al. 2019).

*PIK3CA* and *PIK3CB* display a broad tissue expression. The broad tissue expression of *PIK3CA* likely reflects the role of PI3Kα in metabolism and insulin signalling (Foukas et al. 2006, Knight et al. 2006). On the other hand, the expression of both *PIK3CD* and *PIK3CG* are predominately limited to leukocytes, reflecting their role in immunity (So and Fruman 2012).

Class I PI3K signalling can further be subdivided into class IA and IB signalling, varying by the size of the paired regulatory subunit. Class IA subunits include p110α, p110β and p110δ and form catalytically inactive heterodimers with p85 regulatory subunits. There are two p85 regulatory subunits p85α and p85β, encoded by *PIK3R1* and *PIK3R2*, respectively. Whereas class IB subunits exclusively include p110γ, which associates with a p101 or p84 regulatory subunit, encoded by *PIK3R5* and *PIK3R6*, respectively (Bilanges et al. 2019).

Differential promoter usage of *PIK3R1* also results in p50α and p55α regulatory subunits and *PIK3R3* encodes p55γ. Regardless of size, all regulatory subunits include three SH2 domains, an inter-SH2 (iSH2) domain flanked by an nSH2, and cSH2 domain. The p85 regulatory subunits have additional proline rich regions, a breakpoint cluster region-homology domain (BCR) homology domain and a SH3 domain. These regions bind to GTPases and PTEN respectively (Bilanges et al. 2019).

Class IA PI3Ks are comprised of three main structural domains, whereas class IB PI3Ks contain two. Class IA p110 catalytic subunits include a N-terminal adapter binding domain (ABD), that binds the regulatory subunit, but is not present in class IB PI3Ks. All class I PI3Ks have a GTPase binding domain ras binding domain (RBD). This domain principally binds Ras in the case of p110α, p110δ and p110γ isoforms, but RAC1 and CDC42 in p110β. The PI3K
core, which encompasses a C2, helical and catalytic domain is present in all PI3Ks (Bilanges et al. 2019).

<table>
<thead>
<tr>
<th>Lipid substrate in vivo</th>
<th>Catalytic subunit</th>
<th>Regulatory subunit</th>
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<tr>
<td>Class I PI3Ks</td>
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<tr>
<td>Phosphoinositide (PI3P)</td>
<td>p110α, p110β and p110δ</td>
<td>p110α and p110β</td>
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<td>Phosphatidylinositol (PI4P)</td>
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<td>Phosphatidylinositol (PI4,5P2)</td>
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**Figure 1.4 - Domain organisation and lipid specificity of PI3K isoforms.** Class substrate specificity is indicated with domain organisations of both catalytic and regulatory subunits depicted for each isoform. The p101/p87 regulatory subunit displays no specific domain structures. C2- protein kinase C conserved region, p-proline rich region, B-BCR homology domain, PX-phox homology, SH-src homology domain 2/3. Figure adapted from (Vanhaesebroeck et al. 2010).
Signalling and regulation

As a heterodimer, the p110-p85 complex is inactive and remains in the cytosol. Upon receptor stimulation, the p85 regulatory subunit binds to phosphorylated tyrosine proteins via its SH2 domains, ultimately releasing the p110 catalytic subunit. The p110 catalytic subunit is then able to phosphorylate phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$) to phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$). PIP$_3$ is further metabolised by 5’ phosphatases Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1 (SHIP1) and SHIP2 to phosphatidylinositol 3,4-bisphosphate (PI(3,4)P$_2$). Both PIP$_3$ and PI(3,4)P$_2$ act as important lipid secondary messengers by interacting with pleckstrin homology (PH) domains of downstream protein effectors (Bilanges et al. 2019).

Class I PI3Ks generate pools of phosphorylated inositol lipids, PIP3 exclusively at the plasma membrane and P(3,4)P$_2$ at the plasma membrane and early endosomes. The cellular levels of phosphoinositides are regulated by PTEN. PTEN is a lipid 3-phosphatase that converts PIP3 and PI(3,4)P$_2$ to PI(4,5)P$_2$ and PI(4)P respectively. PTEN is kept in an inactive phosphorylated conformation but upon growth factor stimulation, dephosphorylation of PTEN occurs, allowing interaction with lipid species (Bilanges et al. 2019). When in excess, p85α can homodimerise and bind PTEN stabilising the PTEN complex favouring its activity (Chagpar et al. 2010).

Downstream protein effectors include protein kinases, scaffolding proteins, GTPase activating proteins and guanine nucleotide exchange factors, allow regulation of a broad range of cellular processes (Vanhaesebroeck et al. 2010).

One key downstream effector is the protein kinase AKT (also known as PKB). AKT is recruited to the plasma membrane by binding of PIP$_3$/PI(3,4)P$_2$ to its PH domain. Upon recruitment, AKT is then activated by serial phosphorylation at positions Thr308 by phosphoinositide-dependent kinase 1 (PDK1) and at
Ser473 by mTOR Complex 2 (mTORC2). Both PDK1 and mTORC2 are also recruited to the plasma membrane by their PH domains. Dual phosphorylation of AKT mediates full activation and triggers AKT to phosphorylate its substrates, which most often renders them functionally inactive. AKT substrates include p21/p27, which inhibit cell cycle progression, apoptosis inducer BCL2 associated agonist of cell death (BAD), the protein kinase Glycogen synthase kinase-3 beta (GSK3β) and select forkhead box transcription factors (FOXO) transcription factors. In addition, AKT inhibits the GTPase activating protein Tuberous Sclerosis Complex 2 (TSC2) which mediates the activation of mTORC1 via activation of the small GTPase Ras homolog enriched in brain (RHEB). mTORC1 regulates cellular growth and metabolism in response to environmental and nutrient cues (Bilanges et al. 2019). In summary, active PI3K signalling mediates a cascade of downstream effects, regulating cell growth, survival and proliferation.

PI3K/AKT signalling is inactivated by internalisation of the activated tyrosine kinase receptors by clathrin-mediated endocytosis. The GTPase Rab5 mediates this endocytosis. The deactivated tyrosine kinase receptors are then recycled back to the plasma membrane or processed for degradation via the lysosome (Mellor et al. 2012, Marshall et al. 2019).

Class IB PI3K p110γ associates to the membrane and is activated by Gβγ subunits produced by activated GPCRs. p110β is a unique member of class I PI3Ks in its ability to be activated by Gβγ subunits of GPCRs, further complicating its signalling regulation (Bilanges et al. 2019).
Class II PI3K

Mammals have three isoforms of Class II PI3Ks, which include PI3KC2α, PI3KC2β and PI3KC2γ. Class II PI3Ks act to indirectly influence cell signalling by regulating intracellular membrane dynamics and membrane traffic. Both PI3KC2α and PI3KC2β are expressed ubiquitously, whereas PI3KC2γ is exclusively expressed in the liver (Bilanges et al. 2019).

Catalytic subunits of class II PI3Ks are composed of the PI3K core, as described for class I PI3K’s, with additional C-terminal phox and C2 domains. Additional N-terminal structures are largely disordered and include a RBD, as seen in class I PI3Ks (Bilanges et al. 2019).

Figure 1.5 - Overview of signalling by class I PI3K isoforms. The p110 catalytic subunit is bound by the p85 regulatory subunit. p85 regulatory subunit binds phosphorylated residues in membrane associated proteins illustrated. p101 in the case of p110γ, engages with Gβγ subunits. These events along with engagement with small GTPases mediate catalytic activity. Catalytically active p110 then phosphorylate phosphatidylinositol lipids to triggers downstream signalling via effectors indicated. Figure copied from (Varhaesebroeck et al. 2021).
There are no known regulatory subunits of class II PI3Ks and instead it is thought that autoinhibition is important with a series of conformational changes occurring upon activation. Class II PI3Ks are involved in endosomal sorting and exocytosis and generate pools of phosphorylated inositol lipids, PI(3,4)P2 and PI3P both at the plasma membrane, early and late endosomes (Bilanges et al. 2019).

The organismal role of PI3KC2α has not yet been fully elucidated and research has been hampered by the lack of PI3KC2α specific inhibitors. Yet, studies in mice have demonstrated that PI3KC2α is critical for embryonic development with suggested roles in cilia dynamics (Franco et al. 2014).

1.5.1.2. Class III PI3K

Class III is represented by a single isoform of vacuolar protein sorting 34 (VPS34). VPS34 is highly evolutionarily conserved and plays a role in membrane trafficking. VPS34 occurs as two multiprotein complexes, complex I or II. Complex I is made up of VPS34, VPS15 (regulatory subunit), beclin 1 and autophagy related-14 (ATG14). Whereas Complex II consists of the same multiprotein complex but with UV radiation resistance associated (UVRAG) instead of ATG14 accessory protein. Each complex has distinct roles in stages of vesicle trafficking with catalytic activity of VPS34 regulated by conformation changes in the helical and kinase domains (Bilanges et al. 2019).

Class III PI3Ks generate pools of phosphorylated inositol lipids, PI(3)P at the early, late endosomes and autophagosomes and regulates autophagy, endosomal sorting and phagocytosis. VPS34s role in vesicle trafficking can indirectly regulate signalling through downstream effectors such as serum- and glucocorticoid-regulated kinase 3 (SGK3) (Bilanges et al. 2019).
1.5.2. PI3K dysregulation in cancer

Dysregulation of class I PI3K signalling, leading to aberrant activation has been linked to the development of a variety of cancers (Fruman et al. 2017). Oncogenic activation of PI3Kα has been shown to not only increase cell growth and proliferation, but also to favour stem-like cell behaviour, increase epithelial to mesenchymal transition (EMT) (favouring invasion and metastasis) and increase tolerance to chromosomal instability; all of which play a role in cancer development. In addition, emerging evidence suggests that oncogenic PI3Kα, in particular contexts, can alter both paracrine signalling and the immune microenvironment to favour tumour development (Vanhaesebroeck et al. 2021, Madsen et al. 2019, Vanhaesebroeck et al. 2019).

Oncogenic PI3K signalling can occur through multiple mechanisms with alterations occurring at diverse points in the PI3K/AKT pathway.

1.5.2.1. Genetic changes in PIK3CA

Activating mutations in the catalytic subunit p110 are most commonly found in PIK3CA. Exactly why mutations almost exclusively occur in p110α is unknown, but it has been suggested that this preference could be due to its broad tissue distribution and its role in metabolic signalling downstream of growth factors (Vanhaesebroeck et al. 2010).

A subset of hotspot mutations in PIK3CA have been implicated in cancers. These include E542K and E545K that occur in the helical domain and H1047R in the kinase domain. Both E542K and E545K result in a replacement of a negative amino acid with a positive lysine. This disrupts interactions between the nSH2 domain of p85 with the ABD domain of p110α, resulting in regulation loss and subsequent constitutive catalytic activity (Leontiadou et al. 2018). In addition to impaired association of p110α with p85, the substitution of a histidine at position 1047 to an arginine results in independent membrane
binding of p110α and inappropriate activation (Sharma, Bhardwaj and Purohit 2019).

Although these hotspot mutations both result in activated PI3K there are mutation specific phenotypic effects. For instance, E545K but not H1047R mutations in PIK3CA have been linked to an increase in metastatic potential of breast cancer cells (Pang et al. 2009).

PIK3CA amplifications have been well documented in LUSC (Mendez and Ramirez 2013), gastric (Shi et al. 2012), endometrial and oesophageal adenocarcinoma (Zhang et al. 2017). As with oncogenic activation, PIK3CA amplifications are not always necessarily associated with an increase in PI3K signalling activity and hence are potentially very context-dependent. In gastric cancer, amplifications of PIK3CA, were associated with increased phosphorylation of pAKT at Ser473 and shorter patient survival time (Shi et al. 2012). Whereas PIK3CA amplifications in uterine corpus endometrial carcinoma have been linked to an aggressive phenotype, but no increased levels of pAKT1, 2, 3 or mTOR were evident in affected tumours (Holst et al. 2019).

Timing of genetic changes in PI3K are also very context-dependent and therefore influence their role in tumour development and their therapeutic potential. For instance, in both breast (Berenjeno et al. 2017) and colon cancers (Gerstung et al. 2020) PIK3CA mutations are early clonal events, but in melanoma (Harbst et al. 2016) and oesophageal squamous cell carcinoma PIK3CA mutations are present sub-clonally, as they arise later in development (Hao et al. 2016). In LUSC it has been shown that PIK3CA amplification as part of distal 3q is an early clonal event but additional PIK3CA driver mutations occur later in tumour development, and are sub-clonal. This could indicate that PIK3CA driver mutations have roles in tumour maintenance as opposed to a tumour initiation role of PIK3CA amplifications (Jamal-Hanjani et al. 2017).
1.5.2.2. Dysregulation of PI3K signalling

In addition to activating mutations, loss-of-functional PTEN is often implicated in cancer, with PTEN being the second most common tumour suppressor altered in cancer (Fusco et al. 2020). This can occur through acquisition of loss-of-function mutations or genomic deletion of PTEN. Both of which result in inappropriate sustained activation of PI3K signalling in cancer cells due to sustained PIP3 and PI(3,4)P2 phosphoinositide levels.

Dysregulation of PI3K signalling has been shown in some cancers with mutations occurring in PIK3R1, which encodes the p85α regulatory subunit. Mutations most often occur in the nSH2 domain, which disrupts the association of p85α to the p110α catalytic subunit thereby alleviating regulation and resulting in constitutive PI3K activity. In addition, it has been suggested that p85α may have p110α-independent roles in cancer, either as an independent protein or from acquisition of gain-of-function mutations (Marshall et al. 2019). Particular p85α mutations have been shown to inactivate its Rab5 regulatory activity, resulting in increased PI3K/AKT activity through less internalisation of activated tyrosine kinase receptors (Mellor et al. 2018). In addition to effects on receptor internalisation, loss of p85a regulation of Rab5 has been linked to increased cell migration, favouring metastasis (Frittoli et al. 2014).

Additional mutations in both the SH3 and BCR homology (BH) domains of p85α have also shown to disrupt homodimerisation and binding to PTEN. This results in lower total PTEN levels by increased ubiquitination, favouring PI3K/AKT activity (Mellor et al. 2018). Downregulated PIK3R1 expression has been linked to a poor prognosis in breast cancer (Cizkova et al. 2013).

Growing evidence demonstrates that single changes in the PI3K pathway are not sufficient to induce activated oncogenic PI3K signalling. Indeed, just the presence of hotspot mutations in cells do not necessarily always lead to
dysregulated PI3K signalling (Madsen et al. 2019, Madsen and Vanhaesebroeck 2020).

1.5.2.3. Alterations in other isoforms in cancer

Alterations in PI3Kδ have also been linked to cancer. Inappropriate high expression of PI3Kδ has been demonstrated in some solid tumours including breast, melanoma and liver cancers (Sawyer et al. 2003, Wang et al. 2014, Ko et al. 2018). In addition, E1021K mutant PIK3CD, which triggers activation of PI3Kδ, is found at very low frequency in large B cell lymphoma (Zhang et al. 2013) and acute lymphoblastic leukaemia (ALL) (Liu et al. 2017). In contrast, amplifications of PIK3CB, PIK3CD and PIK3CG are rarely documented in cancer.

However, dysregulation of class I PI3K signalling is not always associated with an increased risk of cancer. For instance, PIK3CA-related overgrowth spectrum (PROS) results in overgrowth of tissues that have PIK3CA mutations that have been acquired postzygotically and therefore result in genetic mosaic tissues. These PROS related overgrowths are benign and have no related predisposition to cancer (Madsen, Vanhaesebroeck and Semple 2018). Whereas PTEN hamartoma syndrome (PHTS), triggered by heterozygous PTEN dysfunction in all cells heavily predisposes the individual to a well characterised spectrum of cancers (Hollander, Blumenthal and Dennis 2011). This demonstrates that the potential of PI3K dysregulation to trigger cancer is very context- and timing-dependent (Bilanges et al. 2019). (Tindle et al. 2018)
1.6. Hypothesis

I hypothesise that PI3K signalling is involved in the development and pathogenesis of LUSC and that PI3K inhibitors can be applied to intervene in disease development by targeting both cancer-cell intrinsic signalling and the tumour immune-microenvironment.

1.7. Aims

- To develop an \textit{in vitro} strategy to study cell phenotypic changes associated with PI3K genomic alterations

- To characterise the involvement of both PI3K signalling and the immune microenvironment in a pre-clinical model of LUSC

- Assess the potential of two PI3K-targeted therapeutics as interventional treatments of LUSC development in this pre-clinical model

- To begin to characterise the mechanism by which successful interventional therapies have modulated disease progression
2. **Methodology**

All chemicals were purchased from Sigma unless stated otherwise. Millipore Q Plus water purification system deionized and distilled water was used for buffer preparation unless otherwise stated.

2.1. **Cell Culture**

I conducted all cell culture in a Class 2 facility in laminar flow hoods using aseptic techniques. All cells were grown in a humidified incubator at 37°C, 5% CO₂ and routinely tested to ensure cultures were mycoplasma free.

2.1.1. **Tissue culture plastic and coating**

All flasks, dishes and plates were tissue culture grade and purchased from Nunc.

For collagen coating, plates were incubated with a 0.05µg/mL collagen glacial acetic acid mix for 1h at room temperature, before aspiration and serial PBS washes prior to cell seeding.

For Poly-D-lysine (PDL) coating, plates were incubated with 0.1% PDL diluted in PBS for 15 minutes at room temperature, before aspiration and serial PBS washes, prior to cell seeding.

2.1.2. **Media and Reagents**

All media, trypsin/EDTA, FBS, and tissue culture supplements were ordered from Thermo Fisher/Invitrogen unless stated otherwise.
2.1.2.1. Culture media

3T3-J2-

Complete 3T3-J2 media: 500mL DMEM with 4.5g/L D-glucose, L-glutamine, and pyruvate.

Primary HBECs FMED-

Complete epithelial media: 373mL serum-containing DMEM (10% FBS, 1% Penicillin/Streptomycin), 125mL Ham’s F-12, 0.5mL gentamicin, 0.5mL amphotericin B, 0.5mL hydrocortisone/EGF, 0.5mL insulin, 0.5mL Y-27632, 4.3µL Cholera toxin.

ChaGoK1s-

RPMI media supplemented with 10% FBS and penicillin/ streptomycin

HBEC-3KT-

KSFM supplemented with 50µg/mL bovine pituitary extract supplied with the kit, supplemented with 5ng/mL (epidermal growth factor) EGF freshly.

HEK-293T-

RPMI media supplemented with 10% FBS and penicillin/ streptomycin

2.1.3. Cell lines

ChaGo-K-1-

ChaGo-K-1 cells were purchased from ATCC. Human bronchogenic carcinoma epithelial cell line from 45 year old male smoker (Rabson et al. 1973).
HBEC-3KT-

HBEC-3KT cells were derived from primary human bronchial epithelial cells isolated from a 65-year-old female immortalised by CDK4 and hTERT retroviral expression (Ramirez et al. 2004). Were obtained from the Francis Crick Institute via Dr Rob Hynds and Jess Orr.

HEK-293T-

HEK-293T cells were derived from human embryonic kidney 293 cells that express the SV40 T-antigen (DuBridge et al. 1987).

2.1.3.1. Cell line maintenance

Cell lines were routinely passaged 1:5 to 1:10 once to twice a week to maintain confluency below 80%. For passaging: culture media was aspirated, cells washed twice with PBS then enough 0.5% trypsin-EDTA was added to cover cells and incubated at 37°C for 2-10 minutes. Cells were detached from the flask by gently tapping. 5 volumes of culture media were added, mixed and transferred to a 15mL centrifuge tube. Cell mixtures were centrifuged at 300g for 5 minutes. Cell pellets were resuspended in culture media and an appropriate cell mixture volume was transferred to a new flask.

2.1.4. Establishing human basal epithelial cell primary cultures

Bronchial samples were collected using autofluorescence bronchoscopy at UCLH with ethical approval from the National Research Ethical committee (REC reference 06/Q0505/12) and patient consent. Human basal epithelial cells, HBECs, were expanded from biopsies and grown in 2D culture following the methods as described in Hynds et al. 2016 Meth. In Mol. Bio with the following modifications:

- Instead of digesting biopsies, biopsies were cut into 5/6 pieces using a scalpel and placed onto a mitomycin treated 3T3-J2 feeder layer.
- To create feeder layers 3T3-J2 cells were treated with mitomycin C (4µg/mL) for 3h rather than 2h.
- The 3T3-J2 feeder layer was seeded at 30,000cells/cm² as opposed to 25,000cells/cm². This provided improved growth support to HBECs.

3T3-J2 cells were passaged 1:8-1:10 every week, ensuring confluency does not surpass 80%. Cultures were used up to and including passage 12 then discarded. To prepare 3T3-J2 feeder layers for primary HBEC cultures, 70-80% 3T3-J2 cultures were incubated with 4µg/mL mitomycin C in culture media for 3h. Following the 3h incubation, the now mitotically inactive 3T3-J2 cells were detached by a 2-minute incubation with 0.5% trypsin-EDTA at 37°C. At least 5 volumes of J2 media were added to the cell mixture, mixed and counted. Cells were replated at 30,000 cells/cm² in J2 media. Feeder layers were used within 48h after seeding.

Bronchoscopic bronchial biopsies were cut with sterile scalpels into 5-6 pieces and placed onto a mitomycin treated 3T3-J2 feeder layer in a T25. biopsies cultures were left to outgrow in FMED media for up to 2-weeks. Co-cultures were subsequentially always maintained in FMED media.

Once cell outgrowths covered more than 50% of culture area, cells were detached using differential trypsinisation. Remaining mitotically inactive 3T3-J2 feeder cells were detached using a 2-minute incubation with 0.5% Trypsin-EDTA at 37°C. Following two washes with PBS, the remaining HBECs were detached with a further 5-minute incubation at 37°C with 0.5% Trypsin-EDTA. Primary HBECs were maintained in culture on a mitotically-inactivated 3T3-J2 feeder layer, in FMED media, with 1:8 weekly passaging.
2.1.5. Cryopreservation of cells

All cells were frozen in cryovials at -80°C for a minimum of 2 days then transferred to -150°C for long-term storage.

Primary HBECs-

1 million epithelial cells were resuspended in 400µL of freezing media (50% Profreeze media (BP12-769E), 42.5% FMED and 7.5% DMSO).

3T3-J2-

1 million cells were resuspended in 3T3-J2 culture media containing 10% DMSO.

ChaGOK1-

1 million cells were resuspended in culture media containing 10% DMSO.

HEK-293T-

1 million cells were resuspended in culture media containing 10% DMSO.

HBEC-3KT-

Resuspended in 1ml of freezing media (50% Profreeze media, 42.5% KSFM and 7.5% DMSO)
2.2. Modulation of gene expression *in vitro*

2.2.1. Transfection

To optimise transfection of primary and immortalised HBECs I tested both nucleofection and Polyplus transfection reagents jetPRIME (101000015, Polyplus Transfection) and jetOPTIMUS (101000051, Polyplus Transfection).

2.2.1.1. Nucleofection

Nucleofection of primary HBECs was performed using the P3 Primary Cell 4D-Nucleofector™ X kit L (V4XP-3024, Lonza). Per transfection 200,000 cells were resuspended in 100µL Nucleofector 4D-primary solution at room temperature. 2.5µg of total plasmid DNA was added and transferred to Nucleocuvette vessels. The following programmes were run using the 4D Nucleofector X unit: EL-110, CM-113 and DS-109. Cell mixtures were then seeded into 6-well plates (collagen coated) with mitotically-inactivated J2-3T3 feeder layers.

2.2.1.2. jetPRIME transfection

400,000 cells/well primary HBECs or immortalised HBEC-3KT cells were plated in a 6-well plate (collagen coated) one-day prior to transfection. A total of 2.5µg/well of plasmid DNA was mixed with 200µL jetPRIME buffer. 10µL of jetPRIME reagent was then added, vortexed and incubated at RT for 10 minutes. This mixture was added dropwise to the plated cells and incubated at 37°C. Media was replaced after 5h.

2.2.1.3. jetOPTIMUS transfection

250,000- 400,000 cells/well were plated in a 6-well plate (collagen coated) two-days prior to transfection. A total of 2.5µg/well of plasmid DNA was mixed with 200µL/well jetPRIME buffer. jetOPTIMUS reagent was then added 1:1, vortexed and incubated at room temperature for 10 minutes. This mixture was
added dropwise to the plated cells and incubated at 37°C. Media was replaced after 4h.

2.2.1.4. Selection of modified cells

24-48h post transfection, cells were selected with antibiotics encoded by the targeting vector and included Geneticin (10131035, Invitrogen), Puromycin (A1113803, Gibco) or Blasticidin (A1113903, Gibco). All antibiotic selection concentrations were selected by the dose that achieved complete culture death in 4-5 days. This was specific to each cell type.

Table 2.1 Antibiotic selection

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Cell culture</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geneticin</td>
<td>Primary HBEC</td>
<td>40µg/mL</td>
</tr>
<tr>
<td></td>
<td>ChaGOK1</td>
<td>200µg/mL</td>
</tr>
<tr>
<td>Puromycin</td>
<td>Primary HBEC</td>
<td>1µg/mL</td>
</tr>
<tr>
<td>Blasticidin</td>
<td>HBEC-3KT</td>
<td>4µg/mL</td>
</tr>
</tbody>
</table>

2.2.2. shRNA knockdown of TP53 in HBEC-3KT cells

2.2.2.1. Generation of lentiviral vector to silence TP53

A shRNA knockdown plasmid for TP53 (shp53 pLK01, Addgene #19119) was utilised to silence TP53. Due to their derivation (Ramirez et al. 2004), HBEC-3KT cells are inherently resistant to puromycin, the existing shRNA TP53 plasmid and empty vector control (PLK01) were modified to confer resistance to hygromycin.
2.2.2.2. Lentiviral production

HEK-293T cells were left to grow to 80-90% confluency in RPMI+10% FBS. Envelope plasmid pMD2.G and packaging plasmids pMDlg/pRRE and pRSV-Rev where transfected using JetPEI (101000053, Polyplus Transfection) into HEK-293T cells. Virus containing media was harvested at 48 and 72h post transduction and filtered through a 0.45µm filter system. PEG-it virus precipitation solution (LV810A-1, System Biosciences) was used to concentrate virus from filtered HEK-293T supernatants. Concentrated virus was aliquoted and stored at -80°C.

2.2.2.3. Viral transduction

Passage 4 immortalised HBEC-3KT cells were thawed and 500,000 cells plated into a T25. The following day the media was replaced with 2mL of fresh K-SFM with 4µg/mL polybrene and 30µL of virus (either TP53 knockdown or PLK01 empty vector control). After 7h at 37°C the media was replaced with fresh media.

2.2.2.4. Selection of transduced cells

48h after viral transduction cells were selected with 40µg/mL of hygromycin (10687010, Invitrogen). Cells were left under selection until the mock transduction (no virus added) was completely clear. Cultures were grown in the absence of hygromycin but 30µg/mL hygromycin was used to re-select 24h after thawing cryopreserved cells. Knockdown was verified at both the protein and RNA level by western blot, immunocytochemistry and qPCR.
2.3. Molecular Biology

2.3.1. Cloning

Targeting of the AAVS1 locus was achieved using the strategy described in (Bressan et al. 2017) and displayed in Figure 1. To achieve this, I generated new targeting vectors encoding open reading frames (ORFs) of interest including, wildtype PIK3CA, PIK3CA\textsuperscript{E545K}, PIK3CA\textsuperscript{H1047R} and AKT2.

![Figure 2.1 - AAVS1 locus targeting](image)

Upon homologous recombination an antibiotic selection cassette (AR), fluorescent protein (FP) with expression linked by a P2A sequence to the ORF of interest (either PIK3CA or AKT2) will be inserted into the locus at sites indicated by green triangles.

2.3.1.1. DNA Vectors

pCas9D10AGFP (Addgene #44720), U6 AAVS1 sg1 and sg2 sgRNAs (Bressan et al. 2017) were used in conjunction with targeting vectors generated using gateway cloning, as described below.

To generate targeting vectors containing the ORFs of interest for this study the following vectors were purchased from Addgene:

- pCIG PIK3CA wildtype- #73056,
- pDONR223.PIK3CA_p.H1047R- #82824,
- pDONR223.PIK3CA_p.E545K- #82881,
- R777-E003 Hs.AKT2- #70287.

Additionally, a tGFP pDONR plasmid was utilised that Dr Sandra Gómez-López had previously cloned.
For plasmid amplification the QIAGEN miniprep (27104) and Zymo Maxiprep (D4202) kits were used.

2.3.1.2. Targeting vector generation

Targeting vectors were generated using gateway cloning technology (Invitrogen). ORFs of interest were amplified using PCR with added gateway att sequences (section 2.3.2). Single PCR products were then recombined into pDONR vectors using BP clonase to generate pDONR vectors encoding fluorescent proteins or ORFs. Fluorescent protein and ORF encoding pDONR vectors were combined by restriction enzyme (RE) digest and ligation reactions to generate pDONR vectors that contained a CAG promoter controlled fluorescent protein, linker, P2A sequence and ORF of interest.

pDONR vectors were LR recombined into destination vectors resulting in insertion of the fluorescent protein, P2A and ORF of interest between homologous recombination arms, specific to the AAVS1 locus. Destination vectors encoded antibiotic resistance.

2.3.2. PCR

ORFs have been amplified using KOD (71842, Novagen) and LongAmp Hotstart Taq (M0287S, NEB) following recommended guidelines.

KOD PCR conditions:

- Polymerase activation: 95°C for 2 min
- Denature: 95°C for 20s
- Annealing: See Table 2.2
- Extension: See Table 2.2
- Cycles: 30 cycles
LongAMP Taq PCR conditions (2-step PCR):

- **Initial denaturation**: 94°C for 30s
- **Denature**: 94°C for 30s
- **Annealing/Extension**: See Table 2.2
- **Cycles**: 30 cycles
- **Final Extension**: 65°C for 10 min

For cloning ORFs with gateway technology (Invitrogen), the following attB, P2A and linker sequences were added to the PCR primers (Table 2.2):

- attB1 (aB1)- GGGGACAAGTTTGTACAAAAAAGCAGGCTT
- attB2 (aB2)- GGGGACCACCTTTGTACAAAGAAAGCTGGGT
- Linker (L)- GGTTCCGGA
- P2A-GCAACAAACTTCTCACTACTCAAACAAAGCAGGTGACGTGGAGGAGAATCCCGGCCCT
Table 2.2 Cloning primers

<table>
<thead>
<tr>
<th>Gene/ ORF</th>
<th>Primers (5’ to 3’)</th>
<th>Template</th>
<th>Extension (°C/s)</th>
<th>Annealing (°C/s)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LongAMP-Taq</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mCherry_Li nk</td>
<td>F</td>
<td>aB1-</td>
<td>mCherry</td>
<td>65, 36s</td>
<td>720</td>
</tr>
<tr>
<td>R aB1-L-TTGTAAGCTCGTCCATG</td>
<td></td>
<td>CACCATTGTGAGCAAGGCGAGGAG</td>
<td>pDONR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Link_P2A_PIK3CA</td>
<td>F</td>
<td>aB1-L-P2A-</td>
<td>Addgene</td>
<td>65, 164s</td>
<td>3,276</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>R</td>
<td>aB2-</td>
<td>#73056</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Link_P2A_AKT2</td>
<td>F</td>
<td>aB1-L-P2A-</td>
<td>Addgene</td>
<td>65, 75s</td>
<td>1,515</td>
</tr>
<tr>
<td>AKT2</td>
<td>R</td>
<td>aB2-</td>
<td>#70287</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R CACCATTGTGAGCAAGGCGAGGAG</td>
<td></td>
<td>PCR_iCherry</td>
<td>70,15s</td>
<td>85, 10s</td>
<td>708</td>
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<tr>
<td>R aB2-CTTACTTCGGAGATGCTGC</td>
<td></td>
<td>PCR_EF1a</td>
<td>70, 12s</td>
<td>77, 10s</td>
<td>1182</td>
</tr>
<tr>
<td>R ACCTGGCACTCTCCGGGTCCCGATTGCGCGTGCA</td>
<td></td>
<td>GATCTGGCAGGTACGCACCTGAAAT</td>
<td>PCR_EF1a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R GGAAG</td>
<td></td>
<td>GGAAG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In some instances, despite using the proof-reading LongAMP Taq polymerase, sequencing the PCR amplifications showed that amplicons contained multiple point mutations. In these cases, restriction enzyme digests were used to piece together complete mutation-free vectors.

Amplicons were verified by Sanger sequencing (Source Bioscience/Genewiz).
2.3.2.1. Genotyping modified cultures

Genomic DNA was isolated from antibiotic selected cultures using PureLink genomic mini kit (K182000, Invitrogen). Selected transfected cells were genotyped via PCR, either using 2-step PCR as described above or touchdown PCR below (Table 2.3).

LongAMP Taq PCR conditions (Touchdown-PCR):

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C for 3 mins</td>
</tr>
<tr>
<td>Denature</td>
<td>94°C for 15s</td>
</tr>
<tr>
<td>Annealing</td>
<td>55-65°C for 30s</td>
</tr>
<tr>
<td>Extension</td>
<td>65°C for 2 mins</td>
</tr>
<tr>
<td>Cycles</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Final Extension</td>
<td>65°C for 10 min</td>
</tr>
</tbody>
</table>
Table 2.3. Genotyping primers

<table>
<thead>
<tr>
<th>Genotyping primers</th>
<th>Primers (5’ to 3’)</th>
<th>Polymerase</th>
<th>Annealing (°C)</th>
<th>Amplicon (bp)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targeting WT AAVS1 locus</td>
<td>803 TCGACTTCCCCTCTTC CGATG</td>
<td>LongAmp</td>
<td>60, 70s</td>
<td>1400</td>
<td>Bressan et al. 2017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Taq</td>
<td>(2-step)</td>
<td></td>
<td>Oceguera-Yanez et al 2017</td>
</tr>
<tr>
<td></td>
<td>183 CTCAGGTTTCGGGAGA GGGTAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Targeting homologous recombinant AAVS1 locus</td>
<td>803 TCGACTTCCCCTCTTC CGATG</td>
<td>LongAmp</td>
<td>55-65, 30s</td>
<td>2155</td>
<td>Bressan et al. 2017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Taq</td>
<td>(Touchdown)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAG_geno GGCGGTACTTGGCATA TGAT</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

2.3.3. Western Blotting

Cultured cells were harvested by incubation with trypsin at 37°C for 2-5mins. Trypsin was neutralised with a minimum of 3x full culture media, this mixture was counted using a cell cytometer and centrifuged at 300g for 5 minutes. The cell pellet was lysed in RIPA buffer (R0278, Sigma) with protease and phosphatase inhibitors (78440, Invitrogen). Samples were centrifuged at 16,200g for 15 minutes at 4°C in a microcentrifuge. The supernatant was then collected, and protein concentration was determined using a BCA assay (23225, Invitrogen).

25µg (25µL) of protein was loaded per well containing 10x reducing agent (RA) (NP0004, Invitrogen) and 4x loading buffer (LB) (NP0007, Invitrogen) after boiling for 10mins at 70°C. Sample mixture was loaded onto a 4-12% SDS
page gels (NP0322, Invitrogen) and run at 130V for 10 mins and then 160V until adequate ladder separation. Protein transfer was performed using the iBlot 2 dry gel transfer system (Invitrogen). Membranes were blocked for 2h in 5% milk/BSA in tris-buffered saline 0.1% tween (TBS-T) at room temperature, washed in TBS-T and incubated with primary antibody overnight at 4°C (Table 2.3).

The following day the membrane was washed twice for 15 minutes in TBS-T on a plate shaker then incubated for at least 1.5h at room temperature with secondary antibody diluted in 5% milk/BSA in TBS-T. Following 2x15 minutes TBS-T washes membranes were incubated in ECL solution for 3 minutes. Membranes were developed using ImageQuant (GE Healthcare) and band intensity measured using FIJI ImageJ2 software (version 2.3.0/1.53q).

Table 2.4. Antibodies used for western blotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Dilution</th>
<th>Source</th>
<th>Cat. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Cell Signalling</td>
<td>2527</td>
</tr>
<tr>
<td>Alpha-tubulin</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Cell Signalling</td>
<td>9099</td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit-HRP</td>
<td>Goat</td>
<td>1:2000</td>
<td>Cell Signalling</td>
<td>7074P2</td>
</tr>
</tbody>
</table>

2.3.4. Quantitative PCR

Total RNA was extracted from cells using RNA extraction kit (R1054, Zymo). Cells were scraped from the culture flask in lysis buffer after washing with PBS. cDNA was generated using High-capacity reverse transcription kit (4368814, Applied Biosystems). cDNA was diluted 5-fold in nuclease-free H₂O before PCR. qPCR was performed using TaqMan gene expression mastermix (4369016, Applied Biosystems) and analysed on an Eppendorf real-time PCR
machine, following manufacturers recommended conditions. Expression of target genes were standardised to housekeeping gene GAPDH.

Table 2.5. Primer pairs used for qPCR

<table>
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<th>Gene</th>
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<th>TaqMan assay ID</th>
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<tr>
<td>GAPDH</td>
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<td>HS99999905_m1</td>
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<tr>
<td>TP53</td>
<td>NM_001126112.3</td>
<td>HS01034249_m1</td>
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</table>
2.4. *in vivo* experiments

All animal work has been approved by the University College London Biological Services Review Committee and conducted following the UK Home Office procedural and ethical guidelines Animal [Scientific Procedures] Act 1986, in a specific pathogen free facility.

All mice were housed in Individually ventilated cages (IVCs) on a 12h light/dark cycle at 25°C, 45-65% relative humidity with food and water available ad libitum. All female FVB/n mice were 5-weeks old at transfer from Charles River.

2.4.1. Animal study size calculations

Study size calculations were calculated by Adam Pennycuick using the pwr.t.test and pwr.anova.test functions from the Bioconductor pwr package using a Cohens f statistic of 0.75 and a Cohens d statistic of 1.5 assuming a large difference between NTCU and control cohorts (Hudson et al. 2012). It was concluded that a group size of 11 would be required. I included 12 for each group for even cage numbers and to have one extra for any NTCU-related adverse reactions that would require removal from the study (36 mice total).

2.4.2. NTCU treatment

At 6 weeks old, the backs of female FVB/n mice were shaved a day prior to topical application of 75µL of 13mM NTCU (SC-2112265, Insight Biotechnology) dissolved in acetone. Topical application of NTCU, via micropipette, was repeated twice weekly for 12 weeks. Mice were maintained through an observational period of up to 12 weeks for analysis of pre-invasive and invasive disease.
Weights of NTCU treated mice were recorded weekly ensuring weight loss did not drop below 10% of starting body weight or below 15-20% of an appropriate age-matched control. Any side-effects or adverse reactions were monitored.

2.4.3. Inhibitor administration

At 15-weeks post first NTCU application (3-weeks into observational period) conventional rodent diet was replaced with either control 2018 rodent diet, 2018 rodent diet containing 10mg/kg BYL-719 or 75mg/kg PI-3065, formulated commercially by Envigo. This food was available ad libitum for the duration of the NTCU experiment.

Mice were housed in IVCs 3 per cage and the average cage weight was constant across the experimental groups at time of diet change (22.16-22.3g). The study was run blinded to avoid experimental bias.

The same batch of inhibitor containing diet was given to 21-week aged FVB/n mice, equivalent to the 15-week NTCU timepoint, as age-matched inhibitor only controls (diet-only). These were housed 5 mice per cage (25 mice total).

2.4.4. Tissue collection

For tissue collection, mice were terminally anaesthetised by intraperitoneal injection of an overdose of 200mg/mL pentobarbital (Dolethal, Vetoquinol UK), and transcardially perfused with PBS, unless otherwise specified.

2.4.4.1. Blood

For studies of diet-only treated mice, 25µL of blood was taken from the tail vein periodically, using capillaries. Whole blood was frozen and stored at -80°C.
Prior to perfusion, a cardiac puncture was performed and ~400µL of blood drawn for downstream analysis. 250µL of blood was spun down at >2000g for 20 minutes 4°C to isolate plasma. Whole blood and plasma was stored at -80°C for downstream analysis.

2.4.4.2. Spleen

Prior to PBS perfusion the spleen was taken for immune cell isolation in RPMI + 10% FBS. Non-spleen tissue was removed macroscopically and the organ weighed. The spleen was mashed into a strainer with 2mL of 1xRBC lysis buffer (00-4300-54, Invitrogen). RBC lysis buffer was quenched with RPMI + 10% FBS. Isolated cells were centrifuged at 4°C at 500g for 5 minutes and washed with 4°C PBS. Cell pellet was resuspended in 4mL of FBS + 10% DMSO freezing media (4x1mL Cryovials). Cells were stored at -150°C long-term for downstream analysis.

2.4.4.3. Lung

For histological analysis, after perfusion with PBS, the lungs were insufflated with either PAXgene fixative (765312, QIAGEN) or 4% paraformaldehyde (PFA). The lungs were then placed in 15mL of the respective fixative for downstream tissue processing.

For single cell digestion, lungs were harvested directly after perfusion into ice-cold PBS. After microscopic dissection to separate lobes and remove any non-lung tissue, whole lungs were shredded into small tissue fragments and incubated in collagenase type IV (500U/ml) DNase I (0.02mg/ml) HBSS at 37°C for 30 minutes. The tissue mixture was mixed vigorously every 10 minutes. The tissue mixture was then passed through a 40µm cell strainer, with any remaining tissue pieces mashed onto the strainer using a syringe plunger. After straining the mixture was centrifuged at 400g for 5 minutes at 4°C. Cell pellets were resuspended in PBS 10% FBS and counted with viability
assessment using trypan blue. Lung cell isolates were frozen in Recovery™ Cell Culture Freezing Medium (12648-010, Gibco) until ready to process.
2.5. Cell Analysis

2.5.1. Flow Cytometry

2.5.1.1. Cell preparation
Frozen spleen cells were thawed with warm RPMI+10% FBS+ DNase (1:100). Dead cell removal (130-090-101, Miltenyi Biotec,) using LS positive selection columns (130-042-401, Miltenyi Biotec) was performed on spleen cells frozen for over 1 month.

2.5.1.2. Washing, buffers and blocking solutions
Staining was performed in 96-well plates and all centrifugation steps were at 400g for 3 minutes. Washing refers to resuspension of cell pellets in 200μL of 4°C PBS.

FACS buffer: 2% fetal calf serum (FCS), 2mM EDTA, PBS.
Superblock: 2% FCS, 5% Normal Rat Serum, 5% Mouse serum, 5% Rabbit serum, 25μg/ml 2.4G2 anti-Fcr mAb, 0.1% (wt/vol) sodium azide (NaN3), PBS

2.5.1.3. Viability staining
APCeFluor780 fixable stain was used to distinguish live from dead cells. Prior to extracellular staining, 100μL of 1:1000 diluted fixable dye was added to each sample and incubated in the dark at 4°C for 30 minutes.

2.5.1.4. Extracellular staining
After washing, cells were blocked for 30 minutes in Superblock, BV staining buffer (563794, BD Biosciences) and 25μg/mL FcBlock (14-9161-73, Invitrogen). All primary extracellular antibodies were incubated at 1:100 in
block solution for at least 45 minutes, protected from light (Table 2.5). After incubation, cells were washed twice and centrifuged for intracellular staining.

2.5.1.5. Intracellular staining

Cell pellets were resuspended in fix/permeabilization solution (00-5123, Invitrogen) and incubated for 40 minutes at 4°C. Intracellular antibodies were incubated in permeabilization buffer (00-8333, Invitrogen), at 1:100 with 10% Superblock for 30 minutes at 4°C. After washing with perm buffer cell pellets were resuspended in FACS buffer, 1% PFA.

2.5.1.6. Acquisition and analysis

UltraComp eBeads (01-2222-41, Invitrogen) were used for compensation of antibody conjugated fluorophores. Viability compensation was performed using a mixture of live and dead cells. Cells were heated at 90°C for 10 minutes to kill cells for dead cell mixture. Samples were acquired on the BD FACSymphony recording 200,000/500,000 events for each sample. Data was analysed using FlowJo software v10.8.1.

2.5.2. Fluorescence-activated cell sorting

Frozen whole lung cell isolates were thawed at 37°C with RPMI+10% FBS added dropwise. Cells were spun down at 300g for 5 minutes then resuspended in 200μL FACS buffer (PBS containing 1% FBS, 25mM HEPES and 1mM EDTA) and plated into a 96-well plate for staining. Cells were incubated in primary antibody in FACS buffer for 20 minutes at 4°C, protected from light. Cells were washed resuspended in FACS buffer with 1:10000 DAPI/PBS (10mg/mL) and strained using Flowmi 40μm strainers.

Gating parameters and compensations were performed using lung cell isolates stained with all but one fluorophore and DAPI was used as a viability marker. Sorting was performed at the Cancer Institute Flow core facility.
(machine details) using a 100µm nozzle by George Morrow. DAPI negative EPCAM+ or CD45+ cells were sorted into DNA LoBind 2.0mL tubes (Eppendorf) containing 0.04% UltraPure BSA PBS (AM2616, Invitrogen). Sorted cells were kept on ice and immediately proceeded to downstream processing.
### 2.5.2.1. Flow cytometry antibodies

Table 2.6. Antibodies used for flow cytometry

<table>
<thead>
<tr>
<th>Specificity</th>
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<td>Invitrogen eBioscience</td>
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<td>Viability</td>
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2.5.2.2. Flow cytometry gating strategy

Figure 2.2 - Gating strategy for flow cytometry of murine spleens. Lymphocytes were selected and then doublets and dead cells were excluded. B cells (CD19+ B220+) were gated and divided by CD21/CD23 expression into marginal zone B-cells (MZB)(CD21+CD23-), follicular B-cells (CD21+CD23+) and B1 B-cells (CD21- CD23). T-cells (TCRb+) were gated and divided into cytotoxic T-cells (CD8+), T-helper cells (CD4+) and T-regulatory cells (CD4+CD25+FOXP3+).
2.5.3. In situ cell analysis

2.5.3.1. Tissue processing

Isolated tissue samples were either fixed with 4% PFA overnight at 4°C or PAXgene tissue fixative (765312, QIAGEN) for 24h at room temperature.

PFA-fixed tissues were then washed in PBS and transferred to 70% EtOH overnight before tissue processing into paraffin wax using an automated system (Leica TP 1050). Tissues were then orientated and embedded in paraffin wax.

PAXgene fixed tissues were transferred to PAXgene tissue stabilizer solution (765512, QIAGEN) for a minimum of 24h followed by a transferal to a 30% sucrose solution for 24h at 4°C. The following day the tissues were transferred to a 1:1 solution, 30% sucrose:OCT solution for 24h at 4°C, prior to OCT embedding on dry-ice and storage at -80°C.

2.5.3.2. Sectioning and haematoxylin and eosin staining

Paraffin embedded tissues were sectioned using a microtome at 4μm whereas OCT embedded tissues were sectioned at 8-10μm using a cryostat. Both were mounted onto SuperFrost Plus charged slides. Haematoxylin and eosin (H&E) staining was performed using an autostainer (TissueTek). Prior to H&E staining, frozen sections were allowed to dry at room temperature for 2h then fixed with 4% PFA for 20 minutes.

2.5.3.3. Immunostaining

For all immunostaining, PFA-fixed paraffin embedded (FFPE) slides were dewaxed using an autostainer (TissueTek), washed in PBS, then antigen retrieval was performed by submerging slides in 10 mM sodium citrate buffer (pH 6.0) or EDTA antigen retrieval solution (pH 9.0) (00-4956-58, Invitrogen)
and heating in the microwave for a total of 20 minutes. Frozen sections were allowed to dry at room temperature for 2h.

Slides were blocked with 5% donkey serum/3%BSA/0.1-0.25% Triton/PBS blocking solution then incubated with primary antibodies diluted in blocking solution overnight at 4°C (Table 2.6). Sections were rinsed with PBS and then followed by two 0.1% Triton/PBS washes, each for 15-minutes. Secondary antibodies were conjugated to Alexa Fluor dyes (Life Technologies) and incubated at 3-4h at room temperature or overnight at 4°C. Following a PBS rinse and two 15 minute 0.1% Triton/PBS washes, nuclei were counterstained with 1:10000 DAPI/PBS (10mg/mL).

2.5.3.4. Immunohistochemistry

Post antigen retrieval, sections were incubated for 10 minutes at room temperature in 3% H₂O₂ to quench endogenous peroxidase. Sections were blocked in 2% goat serum (MP-7451, Vector labs) for at least 30 minutes then incubated with primary antibody in blocking solution overnight at 4°C. Slides were washed twice with PBST then incubated in Immpress polymer reagent (MP-7451, Vector Labs) for 30 minutes following recommended procedures. After twice washing with PBST slides were incubated with ImmPact NovaRed substrate kits (SK-4805, Vector Labs) following recommended procedures and then counterstained with haematoxylin via an autostainer (TissueTek).

Some primary antibodies required amplification to achieve perceptible immunofluorescent signal and are denoted with an (A) in section 2.5.3.6. For this a TSA signal amplification kit (NEL70000KT, PerkinElmer) was used. The following modifications were made to the immunostaining protocol as described in section 2.5.3.3. Post antigen retrieval sections were incubated for 10 minutes at room temperature in 3% H₂O₂ to quench endogenous peroxidase. After secondary antibodies for co-stains had been incubated overnight and washed, a HRP conjugated antibody (A10549, Invitrogen, 170-
specific for the target primary antibody host species was applied for 45-60 minutes at room temperature in blocking solution. Slides were washed twice in 0.1% PBST and biotin solution was incubated for 8-10 minutes in the supplied amplification diluent (NEL70000KT, PerkinElmer). After washing, slides were then incubated with a streptavidin specific 555 secondary antibody (S32355, Invitrogen). The blocking solution supplied with the kit was not used but substituted by 5% donkey serum/3%BSA/0.1-0.25% Triton/PBS blocking solution throughout.

2.5.3.5. Immunocytochemistry

Cell cultured monolayers were fixed in 4% PFA for 10 minutes at room temperature. After twice washing in PBS immunofluorescence staining was performed as described in section 2.5.3.3, without antigen-retrieval.
## 2.5.3.6. Primary antibodies

### Table 2.7 Primary antibodies used for immunostaining

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<th>Host</th>
<th>Dilution</th>
<th>Source</th>
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<td>BioLegend</td>
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<td>Citrate Buffer</td>
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<td>BioLegend</td>
<td>905901</td>
<td>Citrate buffer</td>
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2.5.3.7. Imaging

Fluorescent images were taken using a Leica DMi8 widefield fluorescent microscope or a Carl Zeiss Airyscan 880 confocal. Images were analysed using FIJI ImageJ2 software (version 2.3.0/1.53q).

Chromogenic immunostains and H&E’s were imaged using a Nanozoomer slide scanner (Hamamatsu UK) and analysed using NDP.view2 software (version 2.9.29).
2.6. Assessment of inhibitor administration effect

2.6.1. Assessment of the systemic effects of inhibitors

Blood glucose measurements of diet-only treated mice were taken using a glucometer (Contour Next) at 9am, for five consecutive days, starting on the day of diet change, to monitor insulin feedback in response to BYL719 administration.

An insulin tolerance test (ITT) was conducted on diet-only treated mice on day 8 after diet change, to investigate the impact of BYL719 administration on insulin responsiveness. For this a basal blood glucose measurement was taken at time zero. Insulin (Hypurin, fast glucose release) was injected intraperitoneally at 0.75U/kg and blood glucose measured at 15, 30, 45, 60, 90 and 120 minutes post insulin injection.

The insulin levels present in ex vivo blood samples at time of harvest were measured using the ultra-sensitive mouse insulin ELISA kit (90080, Crystal Chem), low detection range. ELISA absorbances were quantified using a SpectraMax M5 light imaging plate reader (Molecular Devices).

Pharmacokinetic (PK) analysis was performed by Sygnature Discovery on 20μL of blood plasma for either PI-3065 or BYL-719, using vehicle treated blood plasma as blank matrix.

2.6.2. Assessment of NTCU-induced disease

With the help of Pascal F. Durrenberger, lung blocks were sectioned to expose the bronchial tree. Consecutive sections were H&E stained and immunostained for KRT5 (IHC for PFA, IF for PAXgene). At a further depth, 200μm from the first H&E slide, two more consecutive sections were again H&E stained and immunostained for KRT5. Both tissue depths were used to
quantify invasive disease. Representative tissue depths are displayed in Figure 2.2A.

2.6.2.1. Pre-invasive disease quantification

Using NDP.view2 (Hamamatsu UK, version 2.9.29) or FIJI ImageJ2 software (version 2.3.0/1.53q), intrapulmonary KRT5 expressing epithelium and total exposed airway was quantified. The proportion of total airway expressing KRT5 was calculated (Figure 2.2C).

Comparison to the corresponding H&E allowed intrapulmonary KRT5 expressing epithelium to be separated into pre-invasive lesion grades: flat atypia, low grade and high grade. Flat atypia was defined as a single cell layer with flattened enlarged nuclei and increased nuclear-cytoplasmic ratio. Low grade lesions were comprised of a well-ordered multi-layered epithelium. High grade lesions were defined as a multi-layered disorganized epithelium with enlarged nuclei. Characteristic pre-invasive lesions are displayed in Figure 2.2B. This follows the grading criteria performed in L. Succony et al. ERJ 2022 (Succony et al. 2022). The percentage of total airway coverage corresponding to each grade was then calculated.

2.6.2.2. Invasive disease quantification

Tumours were defined as KRT5 positive cells that had broken through the basement membrane, within the alveolar space. Total and individual tumour area was measured using NDP Viewer Software or FIJI ImageJ2 software (version 2.3.0/1.53q). Independent tumours were defined when separated by 200µm of normal histology, non-KRT5 expressing tissue. Individual and average tumour size and number were calculated across both tissue depths (200µm apart).
Figure 2.3 - NTCU-induced disease quantification. (A) Representative H&E stained sections of the two tissue depths quantified for invasive disease. (B) Representative examples of NTCU-induced pre-invasive lesion grades. (C) An example of a KRT5 stained tissue section with total exposed airway annotated into either Non-KRT5, Flat Atypia, Low or High grade epithelium.
2.7. Single cell RNA sequencing

Following fluorescence-activated cell sorting EPCAM+ epithelial and CD45+ immune cells were mixed at a 1:1 ratio. Cells were washed twice with ice-cold 0.04% Ultrapure BSA/PBS, cells were centrifuged at 400g for 3 minutes each time. Cell suspensions were counted, using trypan blue to estimate viability. Cell mixtures were resuspended at 1000cells/µL and submitted to the Cancer Research UK, City of London single cell genomics facility for immediate loading on to a 10x Chromium controller and subsequent TCR amplification and library preparation, by Imran Uddin, facility manager.

Gel Beads in Emulsion (GEMs) were generated from cell suspensions, facilitating individual cell barcoding, using the Chromium Next GEM Single Cell 5' Kit v2 and Chip K Single Cell kit (10x Genomics). T-cell receptor (TCR) amplification was performed using the Chromium Single Cell Mouse TCR Amplification Kit (10x Genomics). Both TCR and gene expression cDNA libraries were generated and library quality was assessed using a bioanalyser.

Both TCR and gene expression cDNA libraries were sent to the Wellcome Sanger Institute for sequencing on a Illumina NovaSeq 6000, 150bp paired-end sequencing. When available FastQ files will be analysed by Moritz Przybilla, Wellcome Sanger Institute/ Dr Ahmed Alhendi, UCL Respiratory.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad PRISM (Graphpad software v9.2.0). Data was tested for normality and then an appropriate parametric or non-parametric test performed. The threshold for statistical analysis was defined as p≤0.05.
3. Modelling LUSC-associated PI3K dysregulation in human bronchial epithelial cells

3.1. Background

Both gain of distal chromosome 3q and TP53 mutations are the most common genetic changes associated with both pre-invasive and invasive LUSC (Mendez and Ramirez 2013, Jamal-Hanjani et al. 2017, Ooi et al. 2014, Teixeira et al. 2019). This distal 3q amplification includes, but is not limited to, PIK3CA, SRY-box 2 (SOX2) and protein kinase C 𝜏 (PRKCI). Many of which have been investigated as potential LUSC drivers as 3q amplification is associated with progression of pre-invasive lesions to invasive LUSC (Mendez and Ramirez 2013, Teixeira et al. 2019).

The limited available in vitro models of LUSC have been vital in gaining mechanistic insight into how these genomic changes influence the development of LUSC. In contrast to other cancers, LUSC have very few representative cancer cell lines. Instead, the majority of LUSC in vitro models focus on the use of either primary mouse or human airway epithelial cell cultures. Human bronchial epithelial cells (HBECs) can be isolated from bronchial brushings or biopsies and grown on mitotically-inactivated mouse 3T3-J2 feeder layers in 2D culture in the presence of Rho Kinase inhibitor (Y-27632). These culture conditions, enrich for basal cells that can differentiate into both ciliated and goblet cell lineages (Butler et al. 2016).

Primary airway epithelial cells isolated from either mouse or human can also be grown in 3D culture. When cultured in non-adherent conditions and provided with a basement membrane matrix, like Matrigel, bronchial epithelial cells give rise to 3D-sperichal structures, containing both progenitor basal cells and differentiated progeny surrounding a hollow lumen (Barkauskas et al. 2016).
Primary airway epithelial cells can also be studied in air-liquid interface (ALI) cultures. For ALI cultures airway epithelial cells are seeded in cultivation chambers and initially grown submerged in culture media. After the initial epithelial growth phase, the apical surface of the epithelial cells are exposed to air whilst the basal surface retains nutrient delivery through the permeable support layer of the cultivation chamber. Continued culture growth whilst exposed to air triggers differentiation of the epithelial cells into both ciliated and mucinous cell types, resulting in a polarised epithelial cell sheet (Whitcutt, Adler and Wu 1988). These 3D models better recapitulate the epithelium structure and can be used to assess differentiation dynamics.

Genetic modification of these primary airway epithelial cells has been used to understand how the common LUSC genetic changes contribute to LUSC development by altering cellular phenotype. Importantly, the use of these in vitro models have demonstrated how characteristic genetic changes can cooperate to favour LUSC development.

TP53 gene silencing in HBECs, leads to the formation of epithelial sheet focal outgrowths which were more diffuse with additional SOX2 overexpression (Correia et al. 2017). HBECs overexpressing SOX2, cultured in ALI conditions display a dysplastic phenotype and a shift in differentiation from ciliated to mucinous cell progeny (Kim et al. 2016). This SOX2-driven development of squamous metaplasia and dysplasia has been shown to be PI3K signalling dependent. Either pharmacological inhibition of PI3K activity via AKT (Correia et al. 2017) or knockdown of PIK3CA prevented SOX2 overexpression induced squamous differentiation (Kim et al. 2016). This demonstrates the importance of PI3K signalling in driving the development of pre-invasive LUSC and the functional relevance of the amplification of both SOX2 and PIK3CA as part of distal 3q.

Additionally, mouse tracheal epithelial cells have been engineered to overexpress SOX2 alongside loss-of-function Trp53, CDKN2 and PI3K
negative regulator, PTEN. When these modified mouse tracheal epithelial cells are cultured as organoids, organoids display a growth advantage and engraft into mice to develop LUSC-like tumours (Hai et al. 2020). This further demonstrates the co-operation of the early genetic changes associated with LUSC development.

Although these models have demonstrated an important role of PI3K signalling in LUSC development, they have not directly investigated the contribution of commonly found genomic amplifications and PIK3CA alterations to LUSC development. They have instead utilised strategies to negatively impact PI3K signalling by using pharmacological inhibition and knockdown. Therefore, the question remains as to how PIK3CA amplifications influence PI3K signalling and ultimately phenotypically influence HBECs to favour LUSC development.

In this chapter, I describe my efforts to optimise HBEC gene-editing to develop an in vitro model to study the phenotypic effects of PI3K signalling changes associated with LUSC development using HBECs. Understanding the impact of molecular changes that occur in the early stages of LUSC development, such as PIK3CA amplification is vital in evaluating their potential to be targeted as interventional therapies.
3.2. Aims

- Investigate the occurrence of genomic and transcriptomic changes in PI3K signalling throughout LUSC development

- Optimise the gene editing of HBECs using a CRISPR/Cas9n strategy.

- To set up an *in vitro* model to investigate the phenotypic effect of PI3K genetic changes in HBECs.
3.3. Results

3.3.1. PI3K pathway changes in LUSC samples

The amplification of \textit{PIK3CA} as part of distal 3q has been well characterised in both pre-invasive and invasive LUSC. Recent studies illustrate the importance of PI3K pathway perturbation at multiple levels to be able to induce cancer development. One such study documented that 25\% of PIK3CA-associated cancers have additional changes in proximal components of the PI3K pathway, \textit{PIK3CA}, \textit{PIK3R1}, \textit{PTEN}, \textit{AKT1,2,3} (Madsen et al. 2019). To assess if there are additional PI3K pathway changes in LUSC, I began by looking into both the transcriptional and genomic changes that occur in 466 LUSC patient samples included in the TCGA PanCancer Atlas. I have assessed the frequency of genetic changes \textit{PIK3CA}, \textit{PIK3R1}, \textit{PTEN} and \textit{AKT2}, which are key components of the PI3K signalling pathway (Figure 3.1A).

Changes in \textit{TP53} were present in 95\% of LUSC samples, demonstrating this as an important context for which other genetic changes cooperate with to favour LUSC development and maintenance (Figure 3.1A). \textit{PIK3CA} was altered in 66\% of LUSC samples, with most cases being gene amplifications. All samples with amplifications in \textit{PIK3CA} also had higher transcription of \textit{PIK3CA} than adjacent normal regions. In addition to amplifications, activating mutations of \textit{PIK3CA}, were also present in LUSC samples. These activating mutations include H1047R, E545K and E542K (Figure 3.1B). These activating mutations have been associated with other cancer types including breast cancer (Samuels and Waldman 2010). Activating mutations in \textit{PIK3CA} in LUSC occurred both in samples that have additional amplification of \textit{PIK3CA} and those that do not (Figure 3.1A).

In addition to changes in \textit{PIK3CA}, changes in \textit{AKT2} occurred in 51\% of LUSC samples (Figure 3.1A). The vast majority of samples with changes in \textit{AKT2}
displayed transcriptional upregulation when compared to normal matched regions. Amplification of AKT2 was present in ~6% of LUSC samples. It has not been demonstrated how these transcriptional and genomic changes of AKT2 impact PI3K signalling in LUSC however they likely favour PI3K signalling activity, further highlighting the relevance of PI3K signalling activity in LUSC.

To better understand how early these alterations occur during LUSC development, I have assessed the presence of both changes in PIK3CA and AKT2 in a cohort of pre-invasive longitudinally tracked CIS samples, studied within the Janes laboratory (Figure 3.1B) (Teixeira et al. 2019). Of 39 CIS samples, 32 bore amplifications of either PIK3CA or AKT2. 25/39 had amplifications in both PIK3CA and AKT2 and 23/39 had these amplifications in the context of loss of TP53. AKT2 amplifications occurred in ~67% of pre-invasive CIS samples and this 19q focal amplification of AKT2 has been identified as a driver of CIS progression (Teixeira et al. 2019). The presence of AKT2 amplifications in pre-invasive samples is in contrast to its low occurrence in invasive LUSC samples. If this is a consistent observation across pre-invasive samples, this could indicate that AKT2 amplifications are influential in the development of pre-invasive lesions but are then selected against, after invasion. However, this has not been studied.

Longitudinal sampling has allowed the tracking of the fate of these CIS regions (Teixeira et al. 2019). After separating the CIS lesions that later progressed to invasive disease and those that regressed, I found that the co-occurrence of changes in TP53 with amplification of PIK3CA and AKT2 was associated with progressive rather than regressive CIS lesions (p=0.0127) (Figure 3.1C). However, it is of note that the regressive CIS lesions were overall much less genetically changed then progressive CIS lesions.

In addition to amplification and upregulation of genes that favour PI3K activity, LUSC samples showed lower expression of some negative regulators of PI3K
signalling than adjacent normal tissue. PIK3R1 encodes the p85 regulatory subunit that binds the p110α catalytic subunit to maintain it in an inactive conformation and was altered in 53% of invasive LUSC samples (Figure 3.1A). The majority of these changes were transcriptional down-regulation of PIK3R1, which would favour PI3K signalling activity. PTEN regulates 3’phophoinositide lipid levels to prevent inappropriate sustained activation of p110α and was changed in 62% of LUSC samples (Figure 3.1A). Most of these changes are transcriptional down-regulation or genomic deep deletions of PTEN. Both changes would result in lower PTEN mediated negative feedback on PI3K signalling, favouring sustained p110α kinase activity.

The demonstration that multiple genetic and transcriptomic changes, that favour oncogenic PI3K signalling, co-occur in LUSC samples further indicates the importance of PI3K signalling in LUSC. In addition, the fact that multiple of these changes are present at pre-invasive disease stages demonstrate that these changes are important in the LUSC development and further supports the development of strategies to model these changes in combination.
Figure 3.1 - Genetic and transcriptomic changes of TP53 and the PI3K pathway in pre-invasive and invasive LUSC. (A) Oncoprint displaying frequency of transcriptomic and genomic alterations in TP53 and genes encoding proximal components of the PI3K pathway across 466 human LUSC donor samples in The Cancer Genome Atlas (TCGA) Pan-Cancer atlas dataset. Gene expression changes are given relative to normal adjacent tissue with z-score threshold ± 2.0. (B) Lollipop plot displaying PIK3CA mutations found in 466 human LUSC donor samples in The Cancer Genome Atlas (TCGA) Pan-Cancer atlas dataset. The height of the lollipop represents mutation frequency. The three most common recurring mutations are named (downloaded from cBioPortal https://www.cbioportal.org). (C) Venn diagrams showing the frequency of genomic changes of interest documented in all 39 CIS bronchoscopy biopsies (left panel). Comparison of the frequency of genomic changes between progressive and regressive samples (right panel) shows that all three genomic changes co-occur more often in progressive samples than regressive (Fisher exact test statistic value 0.0127 significant at p<0.05).
3.3.2. CRISPR/Cas9n gene editing strategy in HBECs

Modelling of PIK3CA and AKT2 amplifications and PIK3CA activating mutations in the context of loss of TP53 in HBECs could elucidate the role of PI3K signalling in triggering LUSC disease development.

To be able to model PIK3CA/AKT2 amplifications and PIK3CA activating mutations in the context of loss of TP53 in HBECs, I chose to employ a CRISPR/Cas9 strategy. CRISPR/Cas9 technology allows the insertion/replacement of DNA into a locus of choice. Guide RNAs specific to the target locus guide the Cas9 activity to the desired region exclusively. After Cas9 mediated cutting of the endogenous DNA, exogenous DNA with homology arms specific to the targeted locus can then integrate into the genome by homologous recombination (Bressan et al. 2017).

In this study, I have utilised a CRISPR/Cas9 strategy that had previously been trialled within the Janes laboratory. This CRISPR/Cas9 strategy targets genetic modifications to the adeno-associated virus integration site 1 (AAVS1) safe harbour locus in the human genome (Figure 3.2B). Targeting this locus minimises off-target effects (Oceguera-Yanez et al. 2016). Instead of a standard Cas9 enzyme I have used a Cas9 nickase (Cas9n), which generates a single strand DNA cut, rather than a double strand cut, to reduce the likelihood of non-homologous end joining (NHEJ) (Ran et al. 2013).

I have used a plasmid-based method for Cas9n, guide RNAs and gene delivery (Bressan et al. 2017). This requires the co-transfection of four plasmids into cells. Two of these plasmids were single guide RNA plasmids encoding sequences analogous to regions between exon 21 and 22 of the AAVS1 locus (Figure 3.2B). One plasmid encoded Cas9n, whose expression is also linked to GFP, to enable Cas9n expression tracking. The last plasmid was the targeting vector, which encoded the desired DNA sequence to be inserted flanked by homology arms to the AAVS1 locus to facilitate homologous recombination (Figure 3.2A).
Figure 3.2 - Schematic describing the strategy to model amplification of PIK3CA and AKT2 in HBECs. (A) Schematic detailing the CRISPR/Cas9n gene editing strategy used to isolate modified clones by antibiotic selection. (B) The AAVS1 locus targeted by the CRISPR/Cas9n system. Upon homologous recombination an antibiotic selection cassette (AR), fluorescent protein (FP) with constitutive expression linked by a P2A sequence to the ORF of interest (either PIK3CA (wild-type or mutant) or AKT2) is inserted into the locus.
3.3.2.1. Targeting vector generation

Since this CRISPR/Cas9n system to target the AAVS1 locus, had been previously used in the Janes laboratory, both the single guide RNA plasmids and Cas9n-GFP plasmid were available to use. However, I had to generate targeting vectors that would introduce the PI3K genomic changes that I wanted to study. These included: (a) wild-type PIK3CA to mimic PIK3CA amplification, (b) PIK3CA with activating mutations E545K or H1047R to reflect the few cases of mutant PIK3CA seen in both pre-invasive and invasive LUSC samples, and (c) AKT2, to model the instances of AKT2 amplifications. Modelling these changes independently and in combination would allow me to study the potentially critical multiple PI3K hits required for LUSC carcinogenesis.

To generate the different targeting vectors, I used gateway cloning technology and vectors obtained from Addgene encoding open reading frames (ORF) of interest: PIK3CA, mutant forms of PIK3CA (E545K and H1047R) and AKT2. To be able to identify modified cells, the targeting vectors also encoded fluorescent proteins, either turbo-green fluorescent protein (tGFP) or mCherry. The expression of these fluorescent proteins was coupled to the expression of the ORF of interest by a P2A self-cleaving peptide.

In addition to being able to identify modified cells with a fluorescent protein, the targeting vector included an antibiotic selection marker to be able to generate pure populations of modified cells.

The targeting vector backbone with open reading frames of interest are displayed in figure 3.3. In addition, to creating targeting vectors encoding the ORFs of interest, I also generated targeting vectors that encoded the mCherry fluorescent protein only, to control for any cellular changes that occurred with transfection, selection and expression of the fluorescent proteins. A targeting vector encoding tGFP was available in the lab already.
Figure 3.3 – Targeting vectors generated for HBEC gene editing. Targeting vector backbone shown. Key features of the vector are displayed including antibiotic resistance (AR) gene and both left and right homology arms (HA-R, HA-L), homologous to the AAVS1 locus. Highlighted area of the vector displays the fluorescent protein, P2A sequence and open reading frame under the control of the CAG promoter. Below targeting vector backbone, both the fluorescent protein only control plasmids and the targeting vectors with open-reading frames are displayed with total vector size included. Vector backbone created using SnapGene.
3.3.3. Optimisation of the transfection of primary HBECs

Whilst generating the targeting vectors, I began optimising the transfection of primary HBECs using the tGFP only targeting vector which conferred G418 antibiotic resistance. As previously mentioned, to track Cas9n expression in cells the Cas9n plasmid also encoded tGFP. Therefore, after successful transfection cells that express Cas9n are transiently tGFP+. However, upon successful homologous recombination, modified cells remain tGFP+ and are resistant to G418 antibiotic selection due to the integrated targeting vector DNA.

3.3.3.1. Nucleofection of primary HBECs

To optimise the transfection of primary HBECs I began by using nucleofection and the P3 Primary Cell 4D-Nucleofector™ X kit L (Lonza). Nucleofection is an electroporation-based technology, which involves applying a voltage to cell mixtures to create pores in the membranes of cells (Distler et al. 2005). This allows the plasmid DNA encoding the ORFs of interest (targeting), Cas9n, and sgRNAs into the cell.

To improve the efficiency of nucleofection on primary HBECs I tested three different nucleofection programmes. I chose three that had previously been shown to be applicable to primary cells: DS-109, CM-113 and EL-110. Despite trying three different programs, none provided efficient transfection of primary HBECs. All transfections showed that <1% of cells were tGFP+ after 48h and no cells retained tGFP expression long-term. After antibiotic selection, no cells survived.
3.3.3.2. Alternative transfection strategies in primary HBECs

Since nucleofection was inefficient for transfecting primary HBECs, to improve transfection efficiency I then tried the Polyplus transfection reagent jetPRIME. jetPRIME is a cationic polymer-based molecule that forms positively charged complexes with DNA. These positive complexes are then able to penetrate cells through endocytosis.

I hypothesised that the jetPRIME based transfection may be gentler on the primary HBECs due to the lack of voltage applied to the cells and the fact that the cells are not in suspension when transfected. For this method, jetPRIME and DNA transfection mixture is added dropwise onto primary HBECS growing on a 3T3-J2 feeder layer, whereas for nucleofection, HBECS are trypsinised, electroporated with the transfection mixture and then plated onto the 3T3-J2 feeder layer.

Using the jetPRIME transfection reagent I compared two constitutive promoters for inclusion in the ongoing targeting vector cloning, CMV early enhancer/chicken β actin (CAG) and elongation factor 1α (EF1α). The aim of this comparison was to pick the promoter that gave robust sustained expression of the incorporated DNA. In this experiment this was demonstrated by bright continual tGFP expression. Regardless of promoter, 24/48h after transfection, there were observably more tGFP+ cells using jetPRIME than with nucleofection.

Comparing transfection efficiency of both CAG and EF1α constitutive promoters showed that the CAG controlled tGFP showed higher expression and transfection efficiency (Figure 3.4). I therefore, chose to use the CAG promoter to drive expression of the ORFs in the targeting vectors I was generating.
Forty-eight hours post transfection, G418 antibiotic was added into the culture media and refreshed every other day. Despite the increased transfection efficiency, I was unable to isolate modified clones even after seeing evidence of modified clones growing (Figure 3.4). I saw no cells that proliferated post antibiotic selection, instead, cells that remained tGFP+ adopted a ‘fried-egg’-like phenotype, suggestive of quiescent/senescent HBECs (Figure 3.4).

I speculate that the remaining tGFP+ HBECs after antibiotic selection are not maintained in a proliferative state due, at least in part, to selection complications. The co-culture system of primary HBECs and 3T3-J2 feeder layer means that both cell types are exposed to antibiotic. These 3T3-J2 feeder layers were not antibiotic resistant and were much more susceptible to antibiotic than the primary HBECs. Without these feeder layers, primary HBECs do not actively proliferate. In an attempt to counteract this loss of the feeder layer, culture media was supplemented with 50% of conditioned media (media that had been incubated with actively growing primary HBECs on 3T3-J2 feeder layers for 2 days). This method had previously been used to support HBECs in the absence of 3T3-J2 feeder layers in the Janes laboratory. Despite the use of conditioned media, no modified primary tGFP+ HBEC clones could be isolated.

To counteract any antibiotic selection complications, I then attempted to isolate clones using fluorescence-activated cell sorting (FACS). Forty-eight hours post transfection cells were differentially trypsinised, and HBECs harvested, washed, passed through a 40μm filter and sorted for GFP expression at the Cancer Institute Flow Cytometry facility (George Morrow). Dead cells were eliminated using DAPI. Primary HBECs were sorted as single cells into 96-well plates or as a bulk population and subsequently plated into culture flasks containing 3T3-J2 feeder layers.

As characteristic of single-cell sorted primary HBECs the clone recovery rate was low, ~5-10% and none of these clones retained tGFP expression. I
hypothesise that these cells that were tGFP+ were only transiently expressing tGFP due to the Cas9n plasmid and had not undergone homologous recombination. A subset of the bulk sorted population did maintain GFP expression but did not survive subsequent antibiotic selection.

To verify that the lack of modified cells was not due to issues with the antibiotic selection cassette, I applied this transfection system to ChaGOK1 cells. ChaGOK1 cells are a commercially available bronchial carcinoma cell line. ChaGOK1 cells do not have the unchanged genetic background required to study the impact of PIK3CA/AKT2 and TP53 loss in isolation, however, they did provide a robust cell line to test the homologous recombination and antibiotic selection. I was able to isolate modified ChaGOK1 cells with antibiotic selection (Figure 3.4). This demonstrates the capacity of the transfection system to generate modified cells and the antibiotic selection cassette was functional.

This led me to conclude that both the complexity of the culture system and the low transfection efficiency of primary HBECs meant I was not able to generate modified primary HBECs. This, in combination with the limited proliferative capacity of primary HBECs led me to switch to the immortalised HBEC cell line HBEC-3KT.
Figure 3.4 - Optimisation of CRISPR/Cas9n gene editing using a targeting vector expressing tGFP.

(A) Schematic illustrating the A4VS1 targeted locus after homologous recombination when using the targeting vector driving expression of GFP during optimisation. AR- antibiotic selection cassette, L-HA- left homology arm, R-HA- right homology arm. (B) Merged phase-contrast and fluorescent images of HBEC transfections with targeting vectors with either the CAG or EF1α promoter controlled GFP expression. Control transfections, that did not receive Cas9n plasmid are displayed on the right. (C) Left panel shows transfection efficiency 17hrs post-transfection. Centre panel shows growing transfected HBECs 4 days post transfection. Right panel shows senescent/quiescent modified HBECs after 10 days of G418 selection. (D) G418 selected ChaGoK1 GFP positive clone.
3.3.4. Optimisation of the transfection of immortalised HBECs

The HBEC-3KT cell line was established from primary HBECs and immortalised using cyclin dependent kinase 4 (CDK4) and human telomerase reverse transcriptase (hTERT) (Ramirez et al. 2004). Despite these additional genomic changes, this cell line provided an alternative to primary HBECs that were genetically unchanged for PIK3CA/AKT2 and provided an increased proliferative potential critical for generating modified clones.

Due to their derivation method, HBEC-3KT cells are inherently resistant to both G418 and puromycin (Ramirez et al. 2004). I therefore modified the targeting vectors I had generated to include a blasticidin selection cassette instead of G418.

To be able to model the changes in PIK3CA/AKT2 in a background of a loss of functional p53, I used a lentiviral vector expressing a short hairpin RNA (shRNA) against TP53 as well as hygromycin resistance. I verified the knockdown of TP53 in hygromycin selected cells by qPCR, western blot and immunocytochemistry (Figure 3.5).

To better track the transfection of HBEC-3KT cells, I used a newly generated targeting vector encoding mCherry with a blasticidin resistance cassette. This allowed me to distinguish cells that post-transfection expressed Cas9n (GFP+) and those that also expressed the targeting vector and were mCherry positive as well.

Applying JetPRIME transfection to the HBEC-3KT cells allowed me to generate modified clones that retained mCherry expression throughout selection passaging and cryopreservation. However, this occurred at a low efficiency and I thought that transfection of the PIK3CA encoding constructs, which were more than 3000bp larger, would be more difficult.
Figure 3.5 Validation of TP53 knockdown in HBEC-3KT cells. (A) Immunocytochemistry staining of cultured HBEC-3KT cells. P53 is displayed in grey, KRT5 in red and DAPI nuclei staining in blue. Top panel displays empty vector HBEC-3KT cells and the bottom HBEC-3KT cells after knockdown of TP53. (B) Western blot for P53 in empty vector and TP53 knockdown cells. GAPDH was used as a loading control. (C) qPCR fold change of empty vector and TP53 knockdown cells. Single experiment displayed triplicate technical replicates in triplicate. Mean ± SD displayed.
Conveniently at this time, Polyplus released a new transfection reagent, called jetOPTIMUS, specifically designed for hard to transfect mammalian cell lines, including primary cells. Polyplus proposes that this reagent increases cellular uptake of DNA and endosomal release. I tested the jetOPTIMUS reagent to see if this would boost transfection efficiency in HBEC-3KT cells. jetOPTIMUS increased transfection efficiency drastically when compared to with jetPRIME (Figure 3.6A). Modified HBEC-3KT cells were easily selected using blasticidin antibiotic selection (Figure 3.6B). After selection, genomic DNA was isolated from cell cultures and genotyped with PCRs targeting both the unmodified wildtype AAVS1 locus and targeted AAVS1 locus after homologous recombination (Figure 3.6C). PCR genotyping revealed that both tGFP+ and mCherry+ selected cells had undergone homologous recombination. The retention of the wildtype AAVS1 locus in modified cells demonstrate that cultures had been heterozygously modified (Figure 3.6D).

Due to the drastic increase in transfection efficiency with jetOPTIMUS I applied the jetOPTIMUS transfection system to the transfection of primary HBECs to see if this would improve my previous efforts. Although the transfection efficiency was increased with jetOPTIMUS, no modified clones could be successfully isolated after antibiotic selection. This further supports my conclusion that the proliferative potential of primary HBECs limits their applicability to this method of genomic modification.

Using HBEC-3KT cell however, I have successfully optimised a transfection strategy for the study of genetic changes in the PI3K pathway. In the time-frame available however, I have been unable to generate cells harbouring the PI3K genetic modifications I had originally planned. Now that the transfection has been optimised, modified cell populations can be readily generated with immortalised HBEC-3KT cells.
Figure 3.6- Optimisation of CRISPR/Cas9n gene editing of HBEC-3KT cells using GFP and mCherry only targeting vectors. (A) Images of HBEC-3KT transfections taken 24h post transfection. GFP channel shows cells expressing cas9n-GFP. mCherry channel shows cells expressing mCherry targeting vector. Individual channels and merged image displayed. (B) HBEC-3KT transfections with tGFP only (top panel) and mCherry only targeting vectors (bottom panel) after 7-10 days antibiotic selection. Scale bar represents 100 μm. (C) Genotyping PCR strategy for wild-type and targeted loci. (D) Genotyping PCR. Genomic DNA isolated from parental and tGFP+ and mCherry+ cells. Gene edited cells were genotyped after antibiotic selection.
3.4. Discussion

In this study, I have optimised gene-editing of an immortalised HBEC cell line with insertions targeted to the AAVS1 locus. This is an important step in being able to study the common PIK3CA genomic alterations in LUSC. The CRISPR/Cas9n gene editing method used in this study, presents the opportunity of studying gene insertions to model amplifications with minimal off-target effects. This is in contrast to lentiviral techniques that have so far been commonly used to model overexpression in HBECs (Kim et al. 2016, Correia et al. 2017). This could therefore highlight this strategy as an important tool to modify HBECs to study the impact of LUSC molecular changes.

Although I was unable to investigate the phenotypic effects of PI3K changes in HBECs in combination with loss of p53, I have a range of hypotheses that going forward this in vitro system could be used to investigate.

As the PI3K pathway plays an important role in cell survival and proliferation, PI3K genomic changes could enhance HBEC colony forming ability. This could be tested by sorting single cells into 96-well plates using flow cytometry and comparing the number of colonies that grow.

If this is the case, I suspect that expression of PI3K genomic changes in HBECs would also result in a more competitive cell population than fluorescent only cells, favoured by the pro-survival, proliferative cellular phenotype. This could be tested with competition assays. Competition assays have been used in previous studies in the Janes laboratory to compare the growth advantage of paediatric over adult HBECs (Maughan et al. 2020). These cell culture assays involve plating two or more cell populations into the same culture well, each identified by a fluorescent protein. When cultures reached 80% confluency, cell proportions were measured using flow cytometry. This begins to model the dynamic relationship between both normal cells and changed cells in the bronchial airway.
In addition, these competition assays could be used to compare competitive advantage between PIK3CA genomic changes by comparing modified cells expressing additional \( PIK3CA \), \( PIK3CA^{E545K} \) or \( PIK3CA^{H1047R} \). This would require changing the co-expressing fluorescent protein of at least one of the \( PIK3CA \) variants. However, the use of gateway cloning and the fact that a unique restriction enzyme site has been included within the linker would make this very easy.

The TRACERx study which performed multi-region whole-exome sequencing of cancers, highlighted chromosomal instability as a key driver of LUSC. Oncogenic \( PIK3CA^{H1047R} \) mutant mouse cells have been shown to display duplicated centrosomes and a tolerance to genome doubling (Berenjeno et al. 2017). This could be a mechanism by which increased chromosomal changes are favoured throughout LUSC stepwise development. To investigate this, centrosome staining could be used to assess if these changes occur in HBECs harbouring PI3K genomic changes, particularly \( PIK3CA^{H1047R} \).

Introduction of oncogenic \( PIK3CA^{H1047R} \) in induced pluripotent stem cells (iPSCs) has demonstrated altered cell fate in a dose dependent manner. Double copies of \( PIK3CA^{H1047R} \) resulted in upregulation of stemness markers and impaired differentiation (Madsen et al. 2019). The observation that this differentiation impairment only occurs when \( PIK3CA^{H1047R} \) is homozygously expressed and not heterozygously, presents the idea that a minimum threshold of oncogenic PI3K activity is required to trigger this shift in cell fate (Madsen et al. 2019). It would be interesting to study if any genomic changes alone or in combination can reach a threshold of oncogenic PI3K activity and trigger cell differentiation changes. To study this, modified HBECs could be cultured in ALI and differentiated cell proportions quantified.

The use of the immortalised HBEC cell line, HBEC-3KT, does introduce artificial overexpression of CDK4 and hTERT that will not truly reflect HBEC
behaviour. These modifications skew cells to a more proliferative pro-survival cell state not present in primary HBEC cultures or importantly in vivo. This could mean that this model would overestimate the impact of genomic changes. However, to be able to robustly withstand genetic modification, selection and maintain proliferative ability, this was a necessary feature of this in vitro strategy.

This work describes important steps towards developing an improved in vitro model of LUSC that can recapitulate key genetic disease changes. Having an in vitro system that genetically reflects genomic changes that occur in pre-invasive LUSC could significantly enhance the development of efficacious interventional LUSC therapies. For instance, this would present the possibility of using high-throughput assays to screen vast numbers of compounds to identify new promising therapies to be tested for in vivo efficacy.
3.5. Summary

- I have generated targeting vectors for CRISPR/Cas9 mediated incorporation of *PIK3CA* and *AKT2* into the *AAVS1* locus.

- The transfection of primary HBECs was not possible in this study due to low transfection efficiencies and the complexity of the primary HBEC co-culture system.

- I have optimised a CRISPR/Cas9 gene-editing strategy of immortalised HBEC-3KT cells.

- These optimised methods for delivering multiple plasmids can be used to study the phenotypic effects of LUSC genomic changes.
4. Assessment of the involvement of the PI3K pathway and immune microenvironment in NTCU-induced lung squamous cell carcinoma.

4.1. Background

The lack of good in vivo preclinical models have limited the understanding of the molecular pathogenesis of LUSC development and ultimately the development of new targeted therapies. So far to study LUSC development a handful of in vivo models have been used, each of which have been shown to model aspects of LUSC development. These include transgenic, transplantation and chemical carcinogenesis models (Gómez-López et al. 2021).

Transplantation models have utilised LUSC patient samples and cell lines to investigate tumour engraftment requirements and pharmacologically modulate growth in vivo (Jeong et al. 2017, Hai et al. 2020, Liu et al. 2020). Although beneficial, these models are unable to model pre-invasive disease stages. Additionally, majority of these models, unless syngenic/humanized, utilise immunocompromised mouse strains to facilitate engraftment (Jeong et al. 2017, Hai et al. 2020). This limits their use in studies of the immune microenvironment and investigating immune-targeted therapies for LUSC.

Transgenic models on the other hand, have utilised genetic modifications that occur in human LUSC patients to increase mouse susceptibility to LUSC development. Importantly, these utilise immune competent mouse strains so can be used to model the disease immune microenvironment. However, few transgenic models have demonstrated exclusive induction of LUSC, with many
having demonstrated additional or precluding LUAD development (Ji et al. 2007, Han et al. 2014, Wang et al. 2019, Ruiz et al. 2019). There are some transgenic models that have targeted the PI3K pathway to exclusively induce LUSC development. Co-deletion of Cdkn2ab and Pten, in the context of Sox2 overexpression results in development of both pre-invasive disease and LUSC (Ferone et al. 2016). Additionally, in this genetic background Pik3ca^{H1047R} expression rather than Pten loss has also been shown to be able to trigger LUSC development (Yuan et al. 2021). Although able to model immune interaction, transgenic models can inherently genetically bias disease development and resultingly likely represent just the subset of human LUSC patients with analogous genetic changes.

Chemical carcinogenesis models have exposed mice to cigarette smoke and its toxic components to induce lung cancer development in vivo. The use of chemical carcinogenesis models can limit genetic bias, utilise immune competent mice and model the disease microenvironment within the lung. One such chemical carcinogenesis model uses N-nitroso-tris- chloroethylurea (NTCU), a nitrosamide derivative. Nitrosamides are toxic components found in cigarette smoke and have been used in several models of cancer (Lijinsky and Reuber 1988, MAGEE and FARBER 1962, Rehm et al. 1991) They are thought to be a genetically unbiased alkylating agents (MAGEE and FARBER 1962). When topically applied to the shaved dorsal region of mice, NTCU dissolved in acetone, can selectively cause the development of LUSC through stepwise progression of pre-invasive disease, displaying similar histology seen in human LUSC (Rehm et al. 1991, Tago et al. 2013, Ghosh et al. 2015). This selective induction could be due to specific lung metabolism, but this has not been studied in depth.

The susceptibility to NTCU is highly sex and strain dependent, with female mice being more susceptible than males and strains including BALB/cJ, SWR/J and FVB/NJ more sensitive than 129/svJ and C57BL/6J (Riolobos et al. 2019, Tago et al. 2013, Hudish et al. 2012). This strain susceptibility has
been linked to specific genetic loci, including D3Mit178 and D18Mit91 which are syntenic to the human chromosome loci 3q26.2-26.31 and 5q23-31 (Wang et al. 2004). The fact that both of these loci are often changed in human LUSC (Fields, Justilien and Murray 2016, Jamal-Hanjani et al. 2017) suggests that NTCU-induced carcinogenesis shares genetic similarities with human LUSC.

In addition, several studies have used RNAseq to characterise the transcriptome of NTCU-induced LUSC (Pan et al. 2018, Beane et al. 2017, Beane et al. 2019). Many highlight the similarities to the transcriptomic changes that occur in human LUSC development. One such study concluded that the top 150 dysregulated genes in human LUSC are also altered in NTCU-induced LUSC (Riolobos et al. 2019). Another study, performed RNAseq on epithelial cells isolated with bronchial brushings and found that the PI3K pathway was upregulated in response to NTCU treatment (Pan et al. 2018). The transcriptomic similarity of NTCU-induced disease with human LUSC has allowed inclusion of NTCU-induced LUSC gene expression in a dataset alongside human pre-invasive samples to help define molecular subtypes of pre-malignant LUSC lesions (Beane et al. 2019).

Importantly, the NTCU-induced carcinogenesis model allows the study of both the pre-invasive and invasive stages of LUSC, which could be influential in further understanding the dynamic molecular changes that occur throughout LUSC development. In addition, the fact that NTCU-induced disease develops alongside an intact functional immune compartment allows the possibility of further understanding the influence of the immune microenvironment on disease development. These features present NTCU-induced LUSC as a potentially powerful pre-clinical model for the development of targeted interventional therapies.

However, better understanding of whether the NTCU model molecularly reflects LUSC development in patients is required to establish its use as a pre-clinical model. The common PIK3CA/AKT2 genomic changes in pre-invasive
LUSC, described in the previous chapter, highlight the PI3K pathway as an important signalling pathway to be investigated. In addition to disease cell intrinsic signalling, recent studies have demonstrated the influence of the immune microenvironment on LUSC development. This presents both as important features that the NTCU model could recapitulate and be used to assess targeted therapies for.

My work described in this chapter begins to evaluate the role of disease-intrinsic PI3K signalling and the immune microenvironment in NTCU-induced carcinogenesis.
4.2. Aims

- Assess the involvement of overactive PI3K pathway activity in NTCU-induced disease.

- Track the changes in the T-cell immune microenvironment that occur with NTCU-induced disease.
4.3. Results

The NTCU-induced model of lung squamous cell carcinoma has been previously optimised within the Janes laboratory. For 12-weeks, NTCU dissolved in acetone is topically applied to the shaved backs of female FVB/n mice, twice weekly. Mice are then monitored for up to a further 12 weeks in which NTCU-induced pre-invasive lesions longitudinally progress to invasive disease (Succony et al. 2022, Zakaria et al. 2021). At 24 weeks most but not all mice have developed invasive disease (Figure 4.1A) (Succony et al. 2022).

NTCU-induced pre-invasive and invasive disease recapitulate human pre-invasive and invasive LUSC histologically. Similarly to human pre-invasive disease, NTCU-induced pre-invasive lesions can be separated histologically into three grades: flat atypia, low-grade and high-grade. Flat atypia is defined as a single cell layer with flattened enlarged nuclei and increased nuclear-cytoplasmic ratio. Low-grade lesions are comprised of a well-ordered multilayered epithelium. High-grade lesions are defined as a multi-layered disorganized epithelium with enlarged nuclei. Invasive disease develops within the alveolar space, having broken through the basement membrane (Figure 4.1B) (Succony et al. 2022).

Both pre-invasive and invasive NTCU-induced disease express keratin 5 (KRT5). This is in contrary to healthy murine lungs, where expression of KRT5 is limited to the trachea and main-stem bronchi and does not extend to intrapulmonary regions (Rock et al. 2010). Therefore, in this study I have used intrapulmonary KRT5 expression to identify NTCU-induced disease.

For the following work, I made use of both pre-existing lung blocks from NTCU-treated mice, courtesy of Dr Sandra Gómez-Lópeze and blocks that I generated myself.
Figure 4.1 - NTCU treatment workflow and resulting pre-invasive and invasive disease. (A) Previously optimised workflow of NTCU treatment, including an initial 12-week treatment period and a following 12-week observation period. (B) Grey headed panel displays histologically normal epithelium from an age-matched mouse not exposed to NTCU. Aqua headed panels display examples of pre-invasive and invasive disease histology triggered by NTCU treatment.
4.3.1. PI3K signalling in NTCU-induced disease

In an effort to better understand if PI3K signalling is important for NTCU-induced carcinogenesis, I employed immunostaining of NTCU-induced disease for nodes of activated PI3K signalling pathway, pAKT and pS6. I chose these read-outs of activated PI3K signalling in the absence of reliable antibodies for p110α immunostaining.

4.3.1.1. pS6 staining

Ribosomal protein S6 (S6) is involved in protein synthesis and is activated by phosphorylation downstream of PI3K signalling. Upon activation of p110α by stimulated growth receptors, phosphorylated AKT relieves inhibition of mammalian target of rapamycin complex 1 (mTORC1). This in turn activates p70 S6 kinases which phosphorylate S6 at positions Ser235/236 (pS6), initiating protein synthesis (Vanhaesebroeck et al. 2010).

Due to a wide availability of effective antibodies for pS6 immunostaining, I started by assessing the presence of pS6 in NTCU-induced lesions. pS6 staining colocalised with NTCU-induced pre-invasive and invasive lesions (Figure 4.2). This was consistent across multiple mice, but detectable pS6 staining was not seen in all lesions. This could indicate that PI3K signalling is active in a subset of NTCU-induced lesions.

However, pS6 is not exclusively induced by activated PI3K signalling. Since pS6 is downstream of mTORC1, any PI3K-independent activation of mTORC1 will also result in pS6.
Figure 4.2 - pS6 staining of NTCU-induced disease. Pre-invasive and invasive disease has been identified by KRT5 in green with pS6 displayed in magenta. DAPI nuclei marker is in blue. Images representative of n>3 mice.
4.3.1.2. pAKT staining

To more directly investigate whether PI3K signalling is induced during NTCU-carcinogenesis I stained for AKT phosphorylated at position Ser473 (pAKT).

Upon activation of p110α by stimulated receptors, PIP2 is converted to PIP3. The PH domain of AKT binds to PIP3, which induces confirmational changes that expose sites Thr308 and Ser473. This allows phosphorylation at site Thr308 by PDK1 partially activating AKT. Full activation of AKT is achieved after an additional phosphorylation at position Ser473. Activated AKT is the main downstream node which disseminates PI3K signalling (Vanhaesebroeck et al. 2010).

pAKT staining was evident in some NTCU-induced pre-invasive lesions. However this was variable between mice and lesion grades. Additionally, pAKT seemed to also be present at similar levels in KRT5 negative epithelium (Figure 4.3). Hence, this staining does not directly suggest a clear role of activated pAKT.

With pS6 not being exclusively activated by p110α and the variability of pAKT staining, I cannot conclude that PI3K activity is associated with NTCU-induced carcinogenesis.
Figure 4.3 - pAKT staining of NTCU treated lungs. Pre-invasive and invasive disease has been identified by KRT5 in magenta with pAKT displayed in yellow. DAPI nuclei marker is in blue. Images representative of n=3 mice.
4.3.2. Longitudinal analysis of the immune microenvironment of NTCU-induced disease.

The contribution of the immune microenvironment in the development of NTCU-induced disease has not been widely studied. One study has noted the accumulation of CD4+ and CD8+ cells as lymphoid aggregates close to main bronchi and blood vessels in the lungs of NIH Swiss mice post NTCU-treatment but the role or influence of these accumulations were not investigated (Riolobos et al. 2019).

As previously discussed, the T-cell immune microenvironment has been shown to be influential in human pre-invasive disease progression, with low infiltration of cytotoxic CD8+ T-cells associated with progression to invasion (Pennycuick et al. 2020). To investigate the T-cell microenvironment in the NTCU model, I have conducted immunostaining analyses for CD4, FOXP3 and CD8 on lung tissues collected at different time-points from NTCU-treatment initiation. This has allowed me to quantify the localisation of CD4+FOXP3- T-helper cells, CD4+FOXP3+ T-regulatory cells and CD8+ cytotoxic T-cells within the lung.

Comparison of T-cell composition and localisation in NTCU-treated mice and age-matched controls has allowed me to investigate the NTCU-induced changes in immune microenvironment. For the longitudinal analysis of NTCU-induced disease, I have assessed four time-points throughout the NTCU workflow: 11-weeks, 15-weeks, 18-weeks and 24-weeks post-first NTCU treatment (Figure 4.4A).

4.3.2.1. Histology of NTCU time-points

These four timepoints represent different stages of disease development with different degrees of KRT5+ coverage along the intrapulmonary airways and varying grades of pre-invasive disease (Figure 4.4B). To streamline analysis,
low-grade pre-invasive lesions included both histologically characterised flat-atypia and low-grade pre-invasive lesions.

At 11-weeks post first NTCU treatment, lungs have very little to no intrapulmonary KRT5+ lesions. Whereas at 15-weeks, lungs have developed low-grade pre-invasive lesions in the most proximal regions of the lung. At 18-weeks, pre-invasive lesions cover a vast extent of the bronchial airway surface, with lesion grades ranging from low- to high-grade. At 24-weeks post NTCU treatment commencement, in addition to the vast coverage of the bronchial airway with pre-invasive disease, ~70% of mice have developed invasive disease (Figure 4.4B).
Figure 4.4 - Timepoints for longitudinal analysis of the T-cell immune microenvironment. (A) NTCU and age-matched controls were harvested at 11, 15, 18 and 24 weeks after first NTCU treatment. (B) Each time-point shows distinct histological disease extents. The range of disease lesions present at each timepoint is displayed with KRT5 in green and DAPI in blue. Scale bar indicates 100μm.
4.3.2.2. Lung T-cell changes throughout timepoints

At each timepoint, I quantified CD4+FOXP3- T-helper, CD4+FOXP3+T-regulatory and CD8+ cytotoxic T-cell subsets, in both the lung parenchyma and in proximity to the bronchial tree. Figure 4.5 displays representative images of T-cell staining with examples of quantification areas.

![Figure 4.5 - T-cell immunofluorescence staining examples and immunofluorescence quantification strategy.](image)

(A) Representative images of T-cell staining in the lung. Demonstrating the nuclear localisation of FOXP3 staining (magenta) and membranous CD4 (white) and CD8 (magenta). (B) Examples of quantification areas with distances highlighted with white dashed lines on merged images. Yellow boxes denote inserts at higher magnification to display staining localisation. Scale bars indicate 100μm on all panels.
4.3.2.3. Parenchyma

Quantifying the T-cell changes in the parenchyma has allowed me to appreciate the broader T-cell changes in the lung throughout NTCU-induced disease development.

At 11-weeks, compared to age-matched controls, the proportion of T-regulatory cells was increased and the fraction of CD8+ T-cells were reduced in the lung parenchyma of NTCU-treated mice (p=0.0003 and p=0.0357 respectively) (Figure 4.6B/C). This could indicate a more immunosuppressive environment with less immunosurveillance that allows initiation of intrapulmonary disease development.

The only difference in the proportions of T-cell subsets in the parenchyma at 15-weeks is a reduction in CD4+FOXP3-, when compared to age-matched controls (p=0.0003) (Figure 4.6A). Demonstrating that the differences in both CD8 and T-regulatory subsets seen at the 11-week timepoint are no longer present.

The 18-week time-point showed no differences in the proportions of T-cell subsets in the parenchyma between NTCU-treated mice and age-matched controls. However, the analyses were underpowered to make any conclusions about both the CD4+ and FOXP3+ cells due to technical difficulties with sectioning artefacts, particularly affecting the parenchyma areas (Figure 4.6A/B/C).

At 24-weeks, the proportions of CD4+FOXP3+ T-regulatory cells in the parenchyma were significantly higher with NTCU treatment than in age-matched controls (p=0.0019) suggesting, again, a more immunosuppressive environment (Figure 4.6B).
When looking exclusively at the NTCU-treated groups, I found very few differences in the proportions of T-cell subsets in the parenchyma among the different timepoints, and no significant trends in changes of T-cell subsets (Figure 4.6D/E/F).

Overall, longitudinal analysis of T-cell parenchyma localisation after NTCU treatment showed very few differences however, comparison to age-matched controls showed increases in regulatory T-cells at the 11- and 24-week timepoints. These changes could favour disease progression by creating a more immunosuppressive environment at these disease stages.
Figure 4.6 - Proportion of T-cell subsets in lung parenchyma. T-cell subsets were quantified as a proportion of total DAPI+ cells. 2-5 areas of parenchyma were quantified from each mouse, 2-6 mice for each group. (A-C) show comparison of the proportions of T-cell subsets in the parenchyma of NTCU-treated mice with age-matched non-treated controls at 11-, 15-, 18- and 24-week time-points. (A) CD4+FOXP3+ T-helper cells. (B) CD4+FOXP3+ T-regulatory cells. (C) CD8+ cytotoxic T-cells. Each point represents a distinct area of parenchyma quantified, n=2-6 mice for each group, mean ±SEM plotted. Statistical significance was tested with a Mann-Whitney test. (D-F) T-cell proportions in the lung parenchyma of NTCU-treated mice at different time-points from treatment initiation. Each point represents a distinct area of parenchyma quantified, n=2-6 mice for each group, mean ±SEM plotted. Statistical significance was tested with Kruskal-Wallis test and Dunn’s multiple comparison tests (* p<0.05, ** p<0.01, *** p<0.001, ns not significant).
4.3.2.4. Association with the bronchial tree

I hypothesised that if changes in the T-cell immune compartment are influencing disease development, this would most likely occur in proximity to the bronchial tree, where disease develops. To assess this, I quantified the proportions of the T-cell subsets in proximity (<100μm) to the bronchial tree (Figure 4.7).

At the 11-week timepoint, when there is little to no intrapulmonary KRT5+ disease, there were no differences in the proportions of T-helper, T-regulatory or cytotoxic T-cells within proximity of the bronchial tree between the NTCU treatment groups and age-matched non-treated controls.

After intrapulmonary disease has started to develop, at the 15-week timepoint, there were significantly more CD4+FOXP3+ T-regulatory cells in proximity to the bronchial tree of NTCU-treated mice when compared to age-matched controls (p=0.0017). Whereas the proportions of both CD4+ T-helpers and CD8+ cytotoxic T-cells showed no statistically significant difference between the treatment and control groups. This shift was also consistent at the 18-week timepoint; when both low- and high-grade pre-invasive lesions have developed (p=0.0099).

At the 24-week timepoint, the proportions of CD4+ T-helpers, CD4+FOXP3+ T-regulatory cells and CD8+ cytotoxic T-cells in proximity to the bronchial tree were all increased following NTCU-treatment, relative to age-matched controls (p=0.0032, p<0.0001 and p=0.0269 respectively). At this time-point, NTCU-induced pre-invasive lesions cover most of the bronchial surface and most mice have developed invasive disease.

As the presence of both CD8+ T-cells and CD4+FOXP3+ regulatory T-cells near the bronchial tree increased at the 24-week time-point, I plotted the CD4+FOXP3+:CD8+ ratio as a readout of whether the bronchial tree
microenvironment is more or less immunosuppressive. The CD4+FOXP3+:CD8+ ratio increased with NTCU treatment at the 24-week timepoint compared to age-matched controls (p=0.0238). This demonstrates that, despite the observed increase in CD8+ T-cells, the T-cell environment of the bronchial tree was more immunosuppressive after NTCU treatment (Figure 4.8D).

The increase in the association of regulatory T-cells to the bronchial tree, compared to age-matched controls, at weeks 15, 18 and 24 could create a more immunosuppressive environment favouring the progression of NTCU-induced disease. The observation that T-regulatory association to the bronchial tree was only increased after the 15-week time-point, could indicate that the T-regulatory association occurred only after intrapulmonary disease is well established.

Overall, longitudinal analysis of T-cell bronchial tree localisation after NTCU treatment showed little change throughout the time-points. However, there was a significant increase in the proportion of CD4+FOXP3+ T-regulatory cells when comparing the 11- to the 24-week time-point (Figure 4.8A/B/C). This indicated that overall T-regulatory cells accumulate in proximity to the bronchial tree with progression of NTCU-induced disease.
Figure 4. T-cell subsets within 100μm of the bronchial tree in NTCU-treated mice and age-matched non-treated controls. Proportion of total cells that were CD8+ or CD4+FoxP3-. CD4+FoxP3+ or CD8+ T-cells localised to the bronchial tree. n=5 mice per group, mean ±SEM plotted. Statistical significance was tested with a Mann-Whitney test (* p<0.05, ** p<0.01, **** p<0.0001, ns not significant).
Figure 4.8 - Changes in the association of T-cells to the bronchial tree throughout NTCU-induced disease development. (A-C) The changes in T-cells within 100um of the bronchial tree in NTCU-treated mice at different time-points after NTCU-treatment start. n=2-6 mice for each group, mean ±SEM plotted. Statistical significance was tested with Kruskal-Wallis test and Dunn’s multiple comparison tests. (D) Ratio of CD4+FOXP3+ :CD8+ cells in proximity to the bronchial tree at the 24-week timepoint. n=2-6 mice for each group, mean ±SEM plotted. Statistical significance was tested with a Mann-Whitney test (* p<0.05, ** p<0.01, *** p<0.001, ns not significant).
4.3.2.5. KRT5- epithelium vs KRT5+ lesions

To determine whether the observed changes in the proportions of T-cell subsets in proximity to the bronchial tree was localised to KRT5+ areas of the bronchial tree, I separated the bronchial tree into KRT5+ lesions and KRT5- epithelium. This was possible for the 15, 18 and 24-week time-points.

The association of T-regulatory cells to the bronchial tree was comparable between KRT5+ and KRT5- airway at all time-points examined (Figure 4.9A). The presence of T-regulatory cells within the proximity of the bronchial tree, regardless of its KRT5 expression status, suggested that the whole bronchial tree potentially is in a T- regulatory mediated immunosuppressive environment. With no specificity to localise to disease.

In contrast, the increased number of CD4+FOXP3- T-helper cells at the 24-week time-point was preferentially associated with the KRT5+ diseased bronchial tree regions than KRT5- regions (p=0.032) (Figure 4.9B). This could indicate this cell subset has a disease specific reaction or role, however this has not been investigated.

Whereas, the observed increased proportion of CD8+ T-cells within the vicinity of the bronchial tree at 24-weeks after NTCU start was not specific to KRT5+ regions (Figure 4.9C).
Figure 4.9 - T-cell subsets in proximity to the bronchial tree separated by KRT5 expression. T-cell subsets were quantified as a proportion of total DAPI+ cells, 100μm from the bronchial tree basement membrane and separated by KRT5 expression in the adjacent epithelium. ≥3 areas were quantified from each mouse, 3-6 mice for each group. Statistical significance was tested with a Mann-Whitney test (** p<0.01, ns not significant).
4.3.2.6. Association of T-cells to pre-invasive lesions of different grades.

While broadly, T-cell localisation to the bronchial tree was comparable between diseased and non-diseased regions of the epithelium. T-cell changes could be preferentially associated to particular pre-invasive disease stages. To investigate this, I subdivided the KRT5+ epithelium according to pre-invasive lesion grade and analysed T-cell localisation with the following categories: KRT5 negative, low- and high-grade. For this analysis I focused on the 24-week time-point which displays the full range of pre-invasive disease lesions and age-matched non-NTCU treated controls.

At 24-weeks, I found that the KRT5- epithelium and both KRT5+ low- and high-grade lesions in NTCU-treated mice have higher association of CD4+FOXP3+ T-cells than the age-matched epithelium from non-NTCU treated controls (p=0.0030, p<0.0001 and p<0.0001 respectively). However, there were no differences of T-regulatory cell distribution between KRT5+ pre-invasive lesions of different grade (Figure 4.10B).

CD4+FOXP3- T-cell association to the bronchial tree was only significantly higher in areas of low-grade pre-invasive disease than in normal epithelium in age-matched controls (p=0.0029) (Figure 4.10A). The association of CD8+ T-cells to the bronchial tree was comparable across pre-invasive lesion grades and to that seen in the age-matched controls (Figure 4.10C).

The observation that T-cell association was not significantly different between lesion grades further illustrated that the association of CD4+FOXP3+ regulatory T-cells, CD4+ T-cells and CD8+ T-cells was generalised to the entire bronchial tree and not specific to pre-invasive disease in general or by pre-invasive grade.
**Figure 4.10 - Proportion of T-cell subsets in proximity to the bronchial tree separated by KRT5 expression and lesion grade.** T-cell subsets were quantified as a proportion of total DAPI+ cells, 100μm from the bronchial tree basement membrane and separated by histological grade. (A) CD4+FOXP3-. (B) CD4+FOXP3+. (C) CD8+. 6 mice for each group. Statistical significance was tested with a Kruskal-Wallis test (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, ns not significant).
4.3.2.7. Immunofluorescence staining summary of T-cell localisation in age-matched and NTCU-treated lungs.

Figure 4.11 displays representative images of the T-cell changes quantified when comparing lungs from NTCU-treated mice at the 24-week timepoint with age-matched control mice.

Figure 4.11 – Representative images of T-cell immunofluorescence staining, demonstrating T-cell localisation in lungs of NTCU-treated (24-weeks) and age-matched (non-NTCU control) mice. CD4+ (grey) FOXP3+ (magenta) T-regulatory cells and CD8+ (magenta) T-cells are indicated with yellow and orange arrows respectively. When compared to the age-matched non-NTCU controls, NTCU treated lungs at the 24-week timepoint have more T-regulatory cells in the lung parenchyma and in proximity to the bronchial tree. In contrast CD8+ T-cells are only increased in proximity to the bronchial tree with NTCU treatment. Scale bars indicate 100μm in all panels.
4.3.3. scRNAseq of epithelial and immune populations following NTCU treatment

This work demonstrates some of the changes regarding the presence of T-cells in the lung immune microenvironment that occur as NTCU-induced disease develops. However, it does not address the functionality of these T-cell subsets, any changes in the wider immune composition, such as the myeloid compartment or the epithelial-immune interaction.

To start to address this in addition to getting a better understanding of the transcriptional profile of NTCU-induced disease, I have employed a scRNAseq strategy. To achieve this, I have used FACS to isolate EPCAM+ epithelial cells and CD45+ immune cells from whole lungs of NTCU-treated mice (NTCU-treated by Sandra Gómez-López). A 1:1 CD45+:EPCAM+ cell mixture was then submitted for scRNAseq at the UCL Cancer Institute scRNAseq core facility (Figure 4.12B). 5' targeted cDNA libraries were generated with additional T-cell receptor (TCR) amplification. Both gene expression and TCR libraries have been sent to our collaborators within Peter Campbells group at the Wellcome Sanger Institute for sequencing. I have prepared samples for scRNAseq at both the 15 and 24-week NTCU timepoints to capture the evolution of the immune/epithelial changes that occur with NTCU-induced disease progression. I am currently awaiting the sequencing results of these libraries.

Assessment of both the epithelial and immune compartment transcriptionally could elucidate how the immune-epithelial interaction is altered throughout NTCU-induced disease development. This will allow me to evaluate more comprehensively how NTCU-induced disease compares to human LUSC and therefore its relevance as a pre-clinical model.

TCR sequencing of the immune cells captured by scRNAseq should demonstrate how TCR clonality changes with NTCU treatment. This is
important in elucidating cancer specific T-cell evolution and if there is evidence of T-cell specific exhaustion after NTCU treatment.

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<th>Timepoint</th>
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<td>24-weeks</td>
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Figure 4.12 - scRNAseq workflow. (A) scRNAseq experimental design, with number of mice of each time-point prepared and submitted. (B) Sample preparation workflow for scRNAseq. Perfused mouse lungs were microdissected into individual lobes and digested to single cells. Cells were then stained for flow cytometry and EPCAM+ and CD45+ cell populations were isolated using FACS. A 1:1 EPCAM+,:CD45+ mixture was submitted for scRNAseq processing.
4.4. Discussion

The work described in this chapter, aimed to investigate the role of PI3Kα signalling and the immune compartment in NTCU-induced disease. This could then allow me to evaluate the potential of the model to be used to test PI3Kα signalling and immune targeted therapies.

The work included in this chapter does not demonstrate a clear role of dysregulated PI3Kα signalling in NTCU-induced carcinogenesis. Immunostaining for activated downstream PI3K pathway nodes has shown heterogeneity of signalling between mice, and between lesions within the same mouse. However, the presence of activated pAKT in some pre-invasive lesions could indicate that a subset of NTCU-induced disease is reliant on PI3K signalling.

For this study, I chose immunostaining to be able to preserve histological information. However, other methods could have focused on isolating either RNA or protein from NTCU-treated lung tissue to get a crude understanding of whether PI3Kα signalling is upregulated or activated compared to age-matched controls. I will get further understanding of the contribution of PI3K signalling in NTCU-induced disease development with the results of the scRNAseq experiment. If PI3Kα signalling is activated in the KRT5+ epithelial compartment during NTCU carcinogenesis, this would indicate that PI3Kα signalling is involved in NTCU-induced disease pathogenesis.

Longitudinal analysis of the T-cell subsets in the lung have demonstrated that there are higher proportions of regulatory T-cells after NTCU treatment both in the parenchyma and in proximity to the bronchial tree. This accumulation of T-regulatory cells to the bronchial tree is irrespective of KRT5 expression and pre-invasive lesion grade and begins at the 15-week timepoint. This raises questions as to what signals are attracting these T-cells to the bronchial tree. One hypothesis could be antigen presenting epithelial cells. To address this, I
could immunostain for MHC complexes. I could also get an idea of the changes in antigen presenting mechanisms from the upcoming scRNAseq data.

Both the observed higher proportions of regulatory T-cells in lungs from NTCU-treated mice and the increased ratio of CD4+FOXP3+:CD8+T-cells in proximity to the bronchial tree, at the 24-week post NTCU treatment time-point, could promote a more immunosuppressive immune-microenvironment in the lung that could favour disease development and progression to invasion. This T-cell immune microenvironment demonstrates some of the immune features seen in human pre-invasive disease.

Although I have assessed T-cell localisation in the lungs following NTCU treatment, my results so far do not address T-cell functionality or the contribution of other immune subtypes and the immune-epithelial interactions that underpin the dynamic immune localisation changes. The future scRNAseq data should help to address this. Since I used CD45 as a marker of immune cells, I should capture additional immune populations including neutrophils, another immune subtype shown to be influential in LUSC (Kargl et al. 2019). Immune transcriptional data will allow me to assess functionality of the immune-microenvironment to better understand their influence on, and interaction with, the epithelial compartment. This could uncover a mechanism that triggers the T-cell localisation changes illustrated in this study.

In addition, the inclusion of cohorts at two time-points along the NTCU treatment protocol within the scRNAseq experiment will allow longitudinal tracking of changes in the wider immune environment and indicate how epithelial transcriptional profiles change with disease progression. Given the progressive nature of the disease, individuals at each time-point would be at distinct disease stages and the epithelial populations will therefore likely display different transcriptional profiles. At the 15-week time-point, intrapulmonary KRT5 expression is established but does not extend distally.
This means that the epithelial compartment should contain both NTCU-induced KRT5 expressing pre-invasive disease, and the KRT5 negative epithelium that has been often overlooked. Greater insight into this compartment could shed light on whether it influences disease progression. It may be possible that the distal colonisation by KRT5+ cells, is triggered by a pro-regenerative environment in response to damage of resident epithelial cells.

At the 24-week time-point however, most of the bronchial airway is covered by KRT5+ disease. This disease represents the full range of pre-invasive lesions and could also include invasive disease. The scRNAseq studies should inform if the transcriptional profile of the KRT5+ epithelial compartment segregates into distinct groups, mirroring the histology. Although as this is a dissociative technique we would not be able to associate a transcriptional profile with a particular disease stage.

Comparison of the KRT5+ compartment between time-points will shed light on whether signalling reliance changes throughout disease progression. This could for instance indicate a transcriptional profile favouring migration at the 15-week time-point, but instead at the 24-week time-point a transcriptional profile that favours a more progressive/invasive phenotype.

Overall, tracking the transcriptional changes in both the immune and epithelial cell populations by scRNAseq, should give invaluable insight into how both cell-intrinsic and immune-mediated mechanisms interact to promote NTCU-induced carcinogenesis and I look forward to working with our collaborators to analyse the data, once available in the coming months.

So far, this work highlights the suitability of the NTCU model as an effective pre-clinical model to test T-cell immune targeted therapies for LUSC, and indicates that PI3Kα inhibitors may be effective in a subset of mice as
interventional therapies to try to modulate disease progression to invasion. The next chapter describes the application of both a PI3K\(\alpha\) and T-regulatory cell targeted immunotherapy to intervene in NTCU-induced disease development.
4.5. Summary

- The role of dysregulated PI3K signalling in NTCU-induced carcinogenesis remains unclear, with PI3K staining suggesting heterogeneity between individuals.

- The T-cell composition of the lung changes after NTCU treatment and with disease development, with higher levels of T-regulatory cells in the lung parenchyma and in proximity to the bronchial tree.

- The increasingly immunosuppressive environment of the bronchial tree following NTCU treatment could promote disease development.

- Interventional therapies could be tested to further evaluate the role of the both the PI3K and immune-microenvironment in NTCU-induced disease.
5. Pharmacological inhibition of PI3Kα and PI3Kδ as intervention therapies to NTCU-induced lung squamous cell carcinoma

5.1. Background

My work described in the previous chapter, demonstrates that a subset of NTCU-induced disease may rely on PI3K signalling and that disease development occurs in a potentially immunosuppressive T-cell microenvironment, with increased regulatory T-cells in proximity to disease. Both of which are important features of human LUSC development (Pennycuick et al. 2019, Mendez and Ramirez 2013). This presents the application of both PI3Kα and T-regulatory targeted therapies to potentially successfully modulate NTCU-induced disease. In this chapter, I describe my efforts to pharmacologically intervene in NTCU-induced disease development by targeting PI3K signalling.

Due to the extensive involvement of oncogenic PI3K signalling in a wide range of cancers, previously discussed, PI3K targeted therapies have been widely assessed in a variety of oncogenic contexts and primarily target PI3Kα and PI3Kδ. Isoform specific PI3K inhibitors have been developed by exploiting isoform residue and structural differences (Vanhaesebroeck et al. 2021).

It was originally thought that targeting PI3Kα would induce death of PI3Kα signalling reliant cancer cells. However, many PI3Kα targeted therapies have not demonstrated a direct cytotoxic anticancer effect and instead largely induce cancer cell dormancy (Morris, Tissenbaum and Ruvkun 1996). However, the PI3Kα targeted therapy Alpelisib was FDA approved in 2019 for the treatment of PIK3CA mutant, hormone receptor (HR) positive, human epidermal growth factor receptor 2 (HER2) negative breast cancer (Narayan
et al. 2021). In this context, PI3Kα inhibition has been shown to increase the dependency of cancer cells on oestrogen. This occurs through increased oestrogen receptor transcription, through multiple mechanisms, but only in the context of low PI3Kα signalling (Shibue and Weinberg 2017). Current PI3Kα targeted clinical trials are investigating the application of PI3Kα inhibitors as combination therapies in other hormone driven cancer subtypes namely other subtypes of breast cancer and ovarian cancer (Verret et al. 2019, Konstantinopoulos et al. 2019).

Despite LUSC not being a hormone driven cancer, due to the dynamic nature of preinvasive LUSC, I will investigate whether PI3Kα inhibition could tip the balance towards preinvasive disease regression to prevent invasive disease development.

PI3Kδ targeted therapies have also shown success in the treatment of cancers despite, PIK3CD mutations and amplifications being rarely found in human cancers (Fruman et al. 2017). PI3Kδ is mostly expressed in leukocytes and is important in the development and activation of T- and B-cells (So and Fruman 2012). Pharmacological PI3Kδ inhibition, in most cases, is therefore not used to target cancer cell intrinsic signalling, but the immune microenvironment tumours develop within.

PI3Kδ inhibitors have been predominately applied to haematological cancers and have shown clinical efficacy by modulating a variety of pro-oncogenic signalling/stimuli. For instance in the treatment of chronic lymphocytic leukaemia (CLL), PI3Kδ inhibition is thought to predominately exploit the reliance of B-cells on PI3Kδ signalling, thus targeting the essential B-cell receptor signalling (Niemann and Wiestner 2013). In addition, it has been suggested that PI3Kδ inhibition also targets the leukaemia supporting stromal cells to interrupt pro-oncogenic signals (Aydin et al. 2020). In the case of CLL, this results in lymphocytosis which releases leukaemic cells from protective niches. This along with the dampened tumour supportive signals leaves them
susceptible to additional therapies e.g. chemotherapy. Another indirect mechanism of the clinical efficacy of PI3Kδ inhibition has been shown to be modulation of the host tumour immune response by alleviating regulatory T-cell immunosuppression (Lim and Okkenhaug 2019). This is thought to play a role in the clinical efficacy of PI3Kδ inhibition in haematological cancers and has been shown to be targetable in solid tumour models.

Although there are very few instances of PI3Kδ-reliant solid tumours, systemic loss of PI3Kδ kinase activity has shown to have a cancer protective effect. PI3Kp110δ<sup>D910A</sup> mice, which have endogenous kinase inactivity of p110δ have been shown to be more resistant to B16 melanoma, displaying reduced tumour incidence and metastases. Enhanced tumour protective effect was also consistent with lewis lung carcinoma, 4T1 mammary and EL4 thymoma cells (Ali et al. 2014).

In addition, pharmacological PI3Kδ inhibition has been shown to be able to induce tumour regression or slow tumour growth in the treatment of 4T1 mammary tumours (Lauder et al. 2020) and in the KPC genetic model of pancreatic ductal carcinoma (Ali et al. 2014). This has been attributed to PI3Kδ inhibition disruption of tumour-induced immune tolerance by unleashing CD8+ cytotoxic T-cells via inhibition of regulatory T-cells (Ali et al. 2014). This presents the possibility that PI3Kδ inhibition in the NTCU-model could disrupt disease development by modulating the T-regulatory cell dominant microenvironment.

In this chapter, I detail the application of two PI3K isoform selective inhibitors: BYL719 targeting PI3Kα and PI-3065 targeting PI3Kδ to the NTCU-induced murine model of lung squamous cell carcinoma, previously optimised in the Janes laboratory and which I have further characterised in this work. This could inform their relevance as interventional therapeutics in the treatment of bronchial dysplasia to prevent human LUSC clinically.
5.2. Aims

- To apply interventional therapies targeting PI3K signalling to an NTCU-induced murine model of lung squamous cell carcinoma

- To demonstrate the *in vivo* pharmacological activity of administered PI3Kα and PI3Kδ inhibitors

- To evaluate both NTCU-induced pre-invasive and invasive lesion development in inhibitor-treated mice compared with vehicle-treated individuals
5.3. Results

5.3.1. PI3K inhibitors as interventional therapies

To investigate the interventional efficacy of BYL719 and PI-3065, both inhibitors were administered via the diet at 10mg/kg and 75mg/kg respectively, available ad libitum (Figure 5.1). The point of intervention was chosen as 15-weeks post NTCU treatment initiation, 3-weeks post NTCU treatment completion. At this point, my studies indicate that intra-pulmonary KRT5 expression has been initiated with some presence of proximal low-grade preinvasive lesions, as assessed by KRT5 immunostaining (Chapter 4). This time-point could represent the point at which patients present in the clinic; after long-term exposure to carcinogens (e.g. cigarette smoke) with some instances of bronchial pre-invasive lesions. Therefore, application of interventional therapies at this point could preclinically evaluate their efficacy in modulating pre-invasive lesion fate to prevent LUSC development.

![Figure 5.1](image)

**Figure 5.1 - Application of interventional PI3K inhibitors to NTCU-induced carcinogenesis.** NTCU was applied on the back of shaved female FVB/n mice, twice weekly for 12 weeks followed by an observational period of a further 12 weeks, during which NTCU-induced lesions progress to higher grades and in ~70% of cases, invasive disease. At 15-weeks the NTCU-treated cohort was switched to diet containing either vehicle, 10mg/kg BYL719 or 75mg/kg PI-3065. All mice were euthanised at 24-weeks for tissue collection.
5.3.2. Weight change associated with NTCU and inhibitor treatment

Previous studies have demonstrated that upon NTCU treatment, mice weight remains low throughout both treatment and observation periods and does not reach that of age-matched controls (Succony et al. 2022, Riolobos et al. 2019). To establish whether inhibitor exposure further impacted body weight, mice were weighed weekly. As expected NTCU treatment, resulted in lower body weight than age-matched controls. This trend was maintained regardless of inhibitor treatment group, throughout observational period up until harvest (Figure 5.2).

Either inhibitor treatment, at chosen doses, was not associated with increased weight loss than the vehicle only NTCU-treated group. Age-matched diet only controls that did not receive NTCU, also did not show significant weight loss associated with prolonged inhibitor treatment either (Figure 5.2). This is indicative that, at the doses tested, prolonged exposure to either BYL719 or PI-3065 was well tolerated, both in the context of health and disease. The lack of increase in body weight, over that of vehicle-treated NTCU mice, indicates that any potential health benefit of either inhibitor treatment did not manifest in an increased weight.
Figure 5.2 - Average relative weight change of mice from inhibitor intervention point (15 weeks post NTCU). NTCU treatment groups (A) and diet only treatment groups. Mixed-effects analysis with multiple comparisons: time effect *** (p=0.0006), time x treatment: not significant. (B). Data points represent the average ± SEM, n=5-12 mice per group. Mixed-effects analysis: time effect **** (p<0.0001), treatment: not significant.
5.3.3. Diet consumption

To compare diet consumption between treatment groups remaining diet per cage was weighed weekly. Approximate diet consumption was similar between treatment groups. The consumption rate remained relatively stable within treatment group across the 9 weeks. A non-significant trend towards decreased food consumption as the experiment progressed was observed in all groups (Figure 5.3). This could be due to decreasing fitness as NTCU-induced disease develops.

![Figure 5.3 - Approximate average grams of diet consumed by each NTCU treated mouse per day, each week after diet change at 15-weeks.](image)

Diet was weighed and replenished weekly. Average ± SEM plotted for each diet (n=3-4 cages, 2-3 individuals for each cage). Statistical analysis by mixed-effects analysis, time effect: ns, inhibitor effect ns. ns= not significant.
5.3.4. Side-effects and adverse reactions

NTCU treatment is associated with a number of side-effects and adverse reactions as advanced disease develops (Riolobos et al. 2019). These adverse reactions were tracked throughout the study to monitor animal welfare and track if specific adverse reactions arose or were exacerbated by inhibitor treatment.

Transient symptoms of disease were noted in several mice including, laboured breathing and shortness of breath, as expected with NTCU treatment. Throughout the course of the experiment however, advanced disease symptoms were widespread and not particularly associated with either BYL719 or PI-3065 treatment groups.

Instances of mice reaching premature humane end-point were low, with only one NTCU-treated mouse (1/36) reaching humane end-point at 20-weeks due to sustained weight-loss above 10% of its total body weight. Upon harvest, it was noticed that the mouse was emaciated and showed signs of respiratory distress i.e. laboured breathing and sluggish behaviour. Histological analysis, via H&E and KRT5 immunostaining, showed that this mouse had extensive invasive squamous cell carcinoma throughout the lungs (Figure 5.4A). This mouse had been treated with BYL719 containing diet for the previous 5 weeks. There was no association with reaching premature end-point due to advanced disease with inhibitor treatment (p=0.473, Chi-square test).

Development of transient dermatitis at NTCU application site has been previously documented (Riolobos et al. 2019). 2/36 NTCU-treated mice displayed dermatitis on NTCU application site at week 11 for 2 weeks. One additional vehicle-treated mouse, had dermatitis and swelling on both front paws at 15-weeks. All dermatitis sites were treated with pink wash for 2 weeks daily, as per veterinary advice. All mice made a full recovery with dermatitis clearing. However, one did not grow fur back at dermatitis/NTCU application
Instances of dermatitis after NTCU treatment were low and were not induced or exacerbated by inhibitor treatment.

Upon lung extraction, one vehicle treated mouse (1/36) displayed a white solid mass associated with the right upper lobe (Figure 5.4B). Upon histological analysis and discussion with pathologist David Moore, it was concluded that this was likely to be an extreme case of obstructive pneumonitis with complete blockage of the bronchi preventing inflation of the lower right lobes and triggering inflammation (Figure 5.4C). Pneumonia development has previously been identified as a common side effect of NTCU treatment (Riolobos et al. 2019). Instances of obstructive pneumonitis were assessed using a H&E in half of the cohort, which were PFA-fixed and paraffin embedded. 50% (9/18 mice) had instances of obstructive pneumonitis, which was often physically associated with advanced invasive disease. Obstructive pneumonitis was observed across all treatment groups but particularly associated with both vehicle and BYL719 treated mice with 4/6 having obstructive pneumonitis compared to 1/6 PI-3065 treated mice. The presence of obstructive pneumonitis could not be assessed using the same methods in the other half of the cohort, whose tissues were PAXgene-fixed and OCT-embedded, as the tissue architecture of the lung parenchyma was not as equally preserved in these samples.

Throughout the exposure period to the PI3K inhibitors, no adverse side-effects were documented above that of NTCU treatment side-effects. Similarly, age-matched controls treated with either inhibitor or vehicle containing diets showed no noticeable symptoms throughout the treatment or at harvest. Therefore, I conclude that both inhibitor doses tested were well tolerated in a healthy and NTCU-induced disease context despite continued exposure for 9 weeks.
Figure 5.4 - Adverse reactions to NTCU. (A) One mouse reached humane end-point at 20 weeks, histological analysis of the lungs revealed widespread LUSC. KRT5 immunohistochemistry staining in brown shown NTCU-induced disease with matching H&E staining. (B) One instance of a white lump found on right upper lobe at tissue harvest. (C) H&E staining of sections of the white lump shows widespread inflammation.
5.3.5. Pharmacologically active inhibitor administrations

To ensure inhibitor administrations at the doses supplied were biologically available and pharmacologically active, I used PK analysis and a number of known systemic effects of PI3Kα and PI3Kδ inhibition.

5.3.5.1. Pharmacokinetic analysis of plasma samples

For both NTCU-treated cohorts and diet-only-treated cohorts, I isolated plasma from blood obtained from cardiac punctures at time of sacrifice. Although this only provides a single time-point, plasma samples were sent to Sygnature Discovery for pharmacokinetic (PK) analysis to appreciate the in vivo exposure of the mice to either PI-3065 or BYL719.

After long-term exposure to PI-3065, the average plasma levels of PI-3065 were 508.47ng/mL and 398.63ng/mL in NTCU-treated and diet-only cohorts respectively. This demonstrates that in both cohorts mice were exposed to PI-3065 systemically.

After long-term exposure to BYL719, the average plasma levels of BYL719 were 47.21ng/mL in the NTCU-treated cohort but were much lower in the diet-only treated cohort. In fact, 70% of the mice, in the diet-only treated cohort, had plasma levels of BYL719 below that of the detection limit of 5ng/mL. The other 30% had an average of 7.14ng/mL BYL719 plasma concentration. As I set up the diet only cohorts after completion of the NTCU cohorts, these lower BYL719 plasma levels seen in the diet-only cohort could have been due to this delay, allowing time for drug diet degradation. This limits any use of the BYL719 diet-only cohort but, importantly for this study the NTCU cohort had sufficient systemic exposure to BYL719 as indicated by plasma levels.
5.3.5.2. *In vivo* pharmacological activity of PI-3065

PI3Kδ inhibition is associated with characteristic systemic effects including a reduction in spleen weight and changes in splenic lymphocyte composition, including reduced regulatory T-cells and depletion of marginal zone B-cells (Okkenhaug et al. 2002).

Gross spleen weight after macroscopic dissection, relative to individual mouse weight, was significantly reduced with PI-3065 treatment in the context of no prior NTCU treatment ($p=0.0035$). However, this trend did not reach significance in the context of NTCU treatment (Figure 5.5A).

Flow cytometry of isolated splenic lymphocytes, both in the context of NTCU and without, have shown that PI-3065 treatment results in lower levels of T-regulatory cells ($p=0.0041$, $p=0.0030$ respectively) (Figure 5.5B) and almost complete depletion of marginal zone B-cells ($p<0.0001$, $p<0.0001$ respectively) (Figure 5.5C) when compared to the vehicle-treated groups. These splenic immune changes were not seen in BYL719-treated mice. These splenic changes demonstrate the biological activity of the PI-3065 compound within my experimental cohort.
Figure 5.5 - Systemic splenic effects of PI-3065 treatment. Upon harvest, individual spleens were cleaned and weighed before digestion into single cells. Dissociated spleen cells were analysed with flow cytometry. (A) Spleen weight relative to individual body weight, comparing vehicle- and PI-3065-treated mice. (B) Proportion of marginal zone B-cells (CD21+CD23-) of total B-cells (B220+CD19+) with representative FACS plots. (C) Proportion of CD4+FOXP3+CD25+ regulatory T-cells of CD4+ T-cells. Representative FACS plots displayed. Statistical analysis was performed using a One-way ANOVA with Dunnett/Tukey’s multiple comparison test n= 5-12 for each group (* p<0.05, ** p<0.01, **** p<0.0001, ns not significant).
5.3.5.3. **In vivo pharmacological activity of BYL-719**

Due to the role of PI3Kα signalling in metabolism, particularly in response to insulin, PI3Kα inhibition *in vivo* can impact metabolism. To appreciate if I can see changes in metabolism in response to BYL719 I measured blood glucose levels and performed insulin tolerance tests (ITT).

In response to PI3Kα inhibition, blood glucose levels can transiently increase due to an impaired insulin signalling mediated response. To investigate if I could see this systemic effect of PI3Kα inhibition in this study, I measured blood glucose levels daily, before and after BYL719 administration. There is a transient increase in blood glucose levels after BYL719 treatment in a subset of mice. This increase in blood glucose levels are not present in any of the vehicle treated mice (Figure 5.6A). However, this is not consistent between all BYL719 treated mice, and comparison of the average change across mice does not reach statistical significance. This variability could be due to varying feeding habits or individual metabolism.

In addition, I also conducted ITTs to investigate the ability of mice to respond to insulin after long-term exposure to BYL719. ITTs periodically track blood glucose levels in response to insulin exposure via injection to compare insulin responsivity. With PI3Kα inhibition it is thought that mice could become insulin resistant, demonstrated by a slower response to insulin, because of the impaired PI3Kα mediated insulin signalling (Hopkins et al. 2018). The ITT showed no difference between vehicle and BYL719 treated groups (Figure 5.6B). However, the application of ITT’s to metabolic studies are tricky and very time and context dependent. For instance, prolonged exposure to PI3Kα inhibition can actually result in more insulin sensitive mice by insulin feedback mechanisms via pancreas insulin overproduction (Hopkins et al. 2018). I conducted this ITT on day 8 after diet change, so mice had not been exposed for the full 9-weeks.
Unfortunately, I only performed these tests in the diet-only treated cohorts as I didn’t want to interfere with NTCU-induced disease development, for instance by injecting high concentrations of insulin and potentially favouring disease growth. Since completing these studies the PK results showed that these mice had very low systemic exposure of BYL719. Although these studies, were completed, 8 weeks prior to the end-point, at which point the exposure to BYL719 is low, I cannot demonstrate that these mice were exposed to sufficient levels of BYL719 at this time-point. Therefore, this questions the relevance of these results and could explain why I saw very little clear BYL719 effect.
Figure 5.6 - Systemic effects of BYL719 exposure in diet-only cohorts. (A) Blood glucose, expressed as fold change from day 0, before switch to either vehicle diet or BYL719 (D= days). Each line represents an individual mouse. Average blood glucose fold change for each treatment group plotted. Two-way ANOVA, treatment effect not significant. n=10 mice for each group. (B) ITT blood glucose measurements plotted. Average blood glucose measurement for each time-point plotted as a fold change from time 0 (immediately prior to insulin injection). Two-way ANOVA, time factor (p<0.0001), treatment effect not significant. n=10 mice for each group. Mean ±SEM.
5.3.6. NTCU-induced disease modulation by interventional PI3K targeted therapies

Susceptibility to NTCU is strain, sex and dosing regimen dependent. Female FVB/n mice have been reported to display intermediate NTCU susceptibility (Riolobos et al. 2019). Previous work with the Janes lab, utilising the NTCU dosing regimen described in this study, demonstrate that all mice will develop pre-invasive disease but not all will display invasive disease at the 24-week timepoint (Succony et al. 2022)(Sandra Gómez-López). To investigate if either PI3Kα or PI3Kδ inhibition had modulated NTCU-induced disease development, I quantified both pre-invasive and invasive disease across all mice that reached the terminal endpoint (24-weeks).

5.3.6.1. Pre-invasive disease modulation

As discussed in the previous chapter, healthy murine airways, lack intrapulmonary KRT5 expression as basal cells expressing KRT5 are mostly restricted to the trachea and extrapulmonary bronchi (Rock et al. 2010, Hogan et al. 2014). Therefore, any KRT5-expressing intrapulmonary epithelium can be considered abnormal and, following histological examination, classified into various grades of pre-invasive disease.

Ongoing work in the Janes laboratory has shown that upon NTCU treatment, KRT5+ cells progressively populate the intrapulmonary airways. To determine whether PI3K inhibition may affect the distal expansion of KRT5+ cells stimulated by NTCU treatment, I measured the percentage of total intrapulmonary epithelium expressing KRT5. There was no statistical difference in the percentage of total intrapulmonary epithelium expressing KRT5, between vehicle- and inhibitor-treated groups (Figure 5.7A). This demonstrates that either inhibitor treatment has no effect on the distal expansion of KRT5 expressing cells stimulated by NTCU treatment.
To assess if NTCU-induced pre-invasive lesion development is altered in response to inhibitor treatments, all intrapulmonary KRT5-expressing lesions were histologically graded. All treatment groups showed very similar proportions of flat atypia, low- and high-grade pre-invasive lesions (Figure 5.7B), suggesting that PI3Kα and PI3Kδ inhibition at these doses have very little effect on development of pre-invasive lesions of any grade.

**Figure 5.7 - The effect of interventional PI3K inhibitors on NTCU induced pre-invasive disease development.** Paraffin- and OCT-embedded lungs were sectioned to reveal the bronchial tree. A section was immunostained for KRT5 to identify the presence of pre-invasive disease. (A) The intrapulmonary KRT5+ epithelium was measured and expressed as a percentage of total exposed epithelium. Kruskal Wallis test with Dunns multiple comparison test. (B) The KRT5+ intrapulmonary epithelium was histologically graded into flat atypia, low- and high-grade pre-invasive lesions. The proportions of each category for each treatment group were compared using individual Kruskal Wallis tests with Dunnetts multiple comparison tests. Graphs display values for individual animals with average ± SEM, n=11-12 for each treatment group.
5.3.6.2. Invasive disease modulation

To determine if invasive disease occurrence was modulated by inhibitor treatment, I measured invasive disease using KRT5 immunostaining. If cells expressing KRT5 had broken the basement membrane and invaded the parenchyma, this was classified as invasive disease and quantified. Individual tumours were distinguished when separated by >200\(\mu\)m of normal tissue.

To assess whether either PI-3065 or BYL719 treatment altered progression to invasion, I began by comparing the proportion of mice that developed NTCU-induced invasive disease with and without inhibitor treatment. Figure 5.8A displays the proportion of mice across treatment groups that displayed invasive disease by KRT5 immunostaining. Both the BYL719 and vehicle treatment groups displayed invasive disease in 83% of mice. However, when compared to vehicle, PI-3065 treatment reduced incidence of invasive disease by 50%, with 41% of mice bearing tumours.

To assess whether the mice that had developed invasive disease, had altered invasive disease progression/growth I also measured, total, average and individual tumour size and number. There were no statistically significant changes in total invasive area with either inhibitor treatment when compared with the vehicle treated group. Although, there was a marked trend towards reduced total invasive area following PI-3065 treatment (p=0.1372). This did not reach significance, likely due to the large variance in invasive area in the vehicle treated group (Figure 5.8B).

The average number of distinct tumours did not significantly change with either inhibitor treatment (Figure 5.8C). Of note however, one BYL719-treated mouse had 38 tumours; almost triple the highest number of tumours in vehicle treated mice (13 tumours). Among the mice with invasive disease, the average tumour area of both BYL719- and PI-3065-inhibitor treated mice was not statistically significantly lower than that of vehicle treated (Figure 5.8D).
However, when comparing the individual tumour sizes of all tumours found in each treatment group, PI-3065 treatment, reduced individual tumour size (from 11603μm$^2$ in vehicle treated mice to 1478μm$^2$) ($p=0.0106$). A smaller reduction in tumour size seemed to be consistent in the BYL719 cohort but this did not reach statistical significance.

PI-3065 treatment prevented the formation of the largest tumours, <10,000μm$^2$, seen in the vehicle treated group (Figure 5.8E). Figure 5.8F displays the relative frequency of tumour sizes across treatment groups, which further demonstrates the lack of tumours above 10,000μm$^2$ in the PI-3065 treated group. In fact, 80% tumours are <2,500μm$^2$ compared to 58% of tumours in the vehicle treated group, further demonstrating the PI-3065 treatment results in smaller NTCU-induced tumours.
### Table: Number of mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>With invasive disease</th>
<th>Without invasive disease</th>
<th>Total</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>2</td>
<td>12</td>
<td>83</td>
</tr>
<tr>
<td>BYL719</td>
<td>10</td>
<td>2</td>
<td>12</td>
<td>83</td>
</tr>
<tr>
<td>PI-3065</td>
<td>5</td>
<td>7</td>
<td>12</td>
<td>41</td>
</tr>
</tbody>
</table>

### Figures:

#### B. Total invasive area
- ns

#### C. Number of tumours
- ns

#### D. Average Tumour Area per mouse
- ns

#### E. Individual Tumour Size
- ns
Figure 5.8 - The effect of interventional PI3K inhibitors on NTCU-induced invasive disease. Invasive disease was quantified from KRT5 immunostaining of at least one tissue section. The presence of KRT5 expressing cells that had broken through the basement membrane into the alveolar space was classified as invasive disease. (A) Contingency table of NTCU inhibitor treated groups with and without invasive disease. Statistical tests performed using Chi-square test, found that the occurrence of individuals with invasive disease was significant reduced in PI-3065 treated mice when compared to vehicle (p=0.035). (B) Total area of invasive disease per mouse separated by treatment group (n=11-12 for each group). (C) Number of distinct tumours (>200μm apart) in each mouse, (n=11-12 for each group). (D) Average tumour area (total invasive area/number of tumours) plotted for mice with invasive disease (n=5-10 for each group). (E) All tumour sizes plotted, separated by treatment group. Average ± SEM plotted, statistical analysis performed using a Kruskal-Wallis test. (F) Histogram displaying the relative distribution of tumour sizes in each treatment group. (* p<0.01, ns not significant).
Finding a subset of mice that after PI-3065 treatment did not develop invasive disease presented the possibility that the response to PI3Kδ inhibition varied with individual response. To better understand if this difference extends to pre-invasive disease modulation, I separated the PI-3065-treated group into ‘responders’, mice with no invasive disease and ‘non-responders’, mice that still developed invasive disease, albeit smaller areas than vehicle-treated mice.

When comparing the pre-invasive disease composition of responders to non-responders, PI-3065 responders had less KRT5+ expressing intrapulmonary epithelium, (46.3% and 61.6% respectively) (p=0.0177), reduced presence of flat atypia (38.7% vs 51.6%) (p=0.0303) and a non-significant trend towards less high-grade lesions. The presence of low-grade lesions was comparable between the two groups (Figure 5.9A).

Figure 5.9B illustrates the distribution of KRT5-negative epithelium and pre-invasive epithelial lesions of different grades as pie-charts, demonstrating that responders had higher proportions of KRT5-negative intrapulmonary epithelium than flat atypia. In contrast, non-responders had the inverse, with more flat atypia than KRT5-negative epithelium. This indicates that in the responding subset of mice, PI3Kδ inhibition can limit the expansion of intrapulmonary KRT5+ cells. This decrease in KRT5-expressing epithelium is mainly accounted for by a decrease in the presence of flat atypia.
Figure 5.9 - Pre-invasive disease proportions separated by PI-3065 response. PI-3065 treated mice were separated into two groups: with invasive disease, termed Non-responders and those with no invasive disease, termed Responders. (A) The proportion of intrapulmonary epithelium that is KRT5+, KRT5- and graded as flat atypia, low or high. Average ± SEM plotted, n=5-7 mice per group. Statistical analysis performed using Mann-Whitney-test (* p<0.01, ns not significant). (B) Pie-charts illustrating proportions of the total exposed epithelium separated by KRT5 expression and pre-invasive lesion histological grade for the PI-3065 Non-responders and Responders.
5.3.7. Hyperactivation of the insulin pathway

The severity of invasive disease in BYL719-treated mice varied amongst the group. A subset of the treatment group, 2/12 displayed enhanced/accelerated invasive disease above that seen in the vehicle-treated cohort. This includes one mouse that reached humane end-point at 20 weeks with extreme invasive disease (Figure 5.4) and another that had 38 distinct tumours.

One possible hypothesis that may explain why a subset of BYL719 exposed NTCU-treated mice displayed accelerated/advanced disease could be hyperactivation of the PI3K pathway, stimulated by insulin feedback mechanisms. This hyperactivation of the pathway could favour the development of more advanced disease by being pro-tumour, cell growth and survival (Hopkins et al. 2018).

To probe whether BYL719 treatment has stimulated a subset of mice with hyperactivation of the PI3Kα pathway through insulin feedback mechanisms, an insulin ELISA was used to quantify the levels of insulin in cardiac puncture blood obtained at time of harvest. Average plasma insulin levels remained constant despite BYL719 treatment, with and without NTCU treatment (Figure 5.10). This gives no indication that insulin levels are higher due to insulin feedback mechanisms. The BYL719-treated outlier, with high plasma insulin levels, did not have invasive disease at time of harvest. I therefore have no evidence that advanced disease is associated with high insulin levels through compensatory insulin feedback mechanisms.
Figure 5.10 - Plasma insulin levels at time of harvest. Plasma was isolated from cardiac puncture blood was taken at time of harvest. Plasma insulin levels compared between BYL719- and vehicle-treated mice in the context of NTCU and non-NTCU, average ± SEM, n=5-12 mice for each group.
5.4. Discussion

This chapter demonstrates the assessment of PI3K inhibitors as potential interventional therapeutics to an NTCU-induced, pre-clinical murine model of LUSC. As part of this study, I have measured inhibitor associated adverse side effects, demonstrated in vivo pharmacological activity and importantly, assessed both pre-invasive and invasive disease modulation. This study could be a valuable tool for future evaluation of interventional therapeutics in NTCU-induced LUSC.

Of the two intervention inhibitors tested, both were well tolerated by NTCU-treated mice, with few associated adverse reactions, despite long term exposure. I have demonstrated the systemic bioavailability of both PI-3065 and BYL719 by PK studies and have demonstrated in vivo activity by investigating systemic readouts.

I have quantified disease modulation by interventional PI3Kα inhibition by BYL719 and PI3Kδ inhibition by PI-3065. This study does not indicate PI3Kα as a promising future interventional therapeutic for LUSC, at least at the dose and regimen tested as there was no evidence of disease modulation. This could have been due to the varied PI3Kα signalling reliance of NTCU-induced carcinogenesis as demonstrated in the previous chapter.

PI-3065 however, demonstrated a durable therapeutic benefit resulting in lower NTCU-induced tumour incidence and in mice with invasive disease, tumours were fewer and smaller. These promising results highlight the importance of future studies to further optimise the PI-3065 dose and regime to maximise therapeutic benefit. It would first be important to investigate whether PI-3065 treatment, with the current dose and regimen, can altogether prevent invasive disease development in responding mice and quantify any associated effect on overall survival and lifespan extension. This could be achieved by harvesting mice as and when they reach a defined humane end-
point. Since lung pre-invasive disease coverage still extended and lesions have progressed despite PI-3065, although to a reduced extent in a subset of mice, I suspect that invasive disease development would still eventually occur, if left long enough. However, this could still significantly prolong survival. Studying the effect of PI-3065 treatment on lifespan and overall survival will help evaluate the clinical potential of the application of PI3Kδ inhibition to the treatment of human LUSC.

Additionally, it would be interesting to apply PI-3065 as a prophylactic therapeutic (before/during NTCU treatment) to assess whether rebalancing the immune composition prior to NTCU treatment could have a more profound effect on pre-invasive disease development and subsequently invasive disease. Although, not necessarily as clinically relevant this could further investigate the power of the T-cell immune microenvironment in NTCU-induced carcinogenesis.

Comparing responses to PI-3065 treatment, has demonstrated that treated individuals in this study can be separated into two groups. Responding individuals, that have not developed invasive disease at 24-weeks and display lower proportions of pre-invasive disease, indicated by less KRT5 expressing epithelium, and non-responding tumour-bearing mice. This lower pre-invasive disease in responding mice can be attributed in particular, to less flat atypia. This could suggest that PI-3065 modulates disease progression by modulating the expansion of KRT5 expressing cells distally, which is mostly classified as flat atypia, as opposed to modulating pre-invasive progression of flat atypia to low- and high-grade. However this could be further investigated, more in-depth in a larger study alongside investigating the underlying mechanism that determines this differential degree of response.

Some studies have demonstrated enhanced therapeutic efficacy when PI3Kδ inhibition is applied as a combination therapy, often with other immunomodulatory drugs (Lauder et al. 2020). This presents a potential future
area of study assessing the use of combinatorial therapeutic strategies to see if interventional efficacy can be enhanced. These strategies could be informed by better understanding the mechanisms by which some mice have developed invasive and disease and some have not in this study.

The efficacy of an immunomodulatory therapeutic in modulating NTCU-induced disease further demonstrates the involvement of the immune microenvironment in NTCU-induced tumour formation. This both highlights the model for further immune characterisation and as an important pre-clinical model to study LUSC-immune interaction.

The observed therapeutic benefit of interventional PI3Kδ inhibition in this chapter, raises questions as to what immune changes, both systemic and local, are associated with disease modulation. Investigation of the associated immune changes with PI-3065 treatment are the focus of the next chapter.
5.5. Summary

- PI3K inhibitors were applied as interventional therapies to the NTCU-induced model of lung squamous cell carcinoma.

- Both BYL719 and PI-3065, at the tested dose and treatment duration, caused very few adverse reactions in both healthy and NTCU-treated mice.

- BYL719 treatment showed little therapeutic value in the context of the NTCU model, with no effect on pre-invasive disease development or invasive disease incidence.

- PI-3065 treatment showed little effect on pre-invasive disease development but reduced tumour incidence and tumour size in the context of NTCU-induced carcinogenesis.
6. Assessing immune modulation by PI3Kδ inhibition in NTCU-induced lung carcinogenesis

6.1. Background

Recent pre-clinical studies in mice have applied PI3Kδ inhibitors to solid tumours. PI3Kδ inhibition has been shown to slow tumour growth and even trigger cancer regression in some cases (Ali et al. 2014, Lauder et al. 2020). These tumour responses to PI3Kδ inhibition have been attributed to a reduction in the immunosuppressive T-cell tumour microenvironment with a shift to increased tumour immunosurveillance.

The ability of PI3Kδ inhibition to modulate tumour responses has been largely linked to a reduction in the number of tumour-infiltrating T-regulatory cells and resulting enhanced infiltration and activation of cytotoxic T-cells. These immunostimulatory effects have been documented both in preclinical murine models (Lauder et al. 2020, Ali et al. 2014) and most recently in a window-trial of head and neck cancer (Eschweiler et al. 2022).

PI3Kδ inhibition has been shown to preferentially relieve T-regulatory mediated immunosuppression in tumours as T-regulatory cells are more sensitive to PI3Kδ inhibition than CD4+ and CD8+ T-lymphocytes (Ali et al. 2014). While this sensitivity of T-regulatory cells to PI3Kδ inhibition is not currently understood, but T-regs are more dependent on PI3K signalling. PI3Kδ inhibition results in discordant transmission of PI3K in dividing T-cells, biasing activated T-cells to give rise to effector and self-renewing phenotypes (Nish et al. 2017). These cell types are able to mount a more robust tumour T-cell response.
It has been demonstrated that tumour response to PI3Kδ inhibition is dependent on the abrogation of PI3Kδ kinase activity specifically in T-regulatory cells. Adoptive T-cell transfer experiments have demonstrated that wild-type T-regulatory cells transferred into mice expressing a kinase-dead version of p110δ restore EL4 lymphoma growth, with fewer tumour infiltrating CD8+ T-cells (Ali et al. 2014).

In addition to depleting tumour-infiltrating T-regulatory cells it has been shown that PI3Kδ inhibition reduces the immunosuppressive function of the remaining regulatory T-cells. Following PI3Kδ inhibition, T-regulatory cells have altered function, with decreased IL-10 production (Patton et al. 2006). This reduction both in infiltration and immunosuppressive function of T-regulatory cells lowers their immunosuppressive influence on CD4+ and CD8+ T-cells (Chellappa et al. 2019), facilitating a more anti-tumour immune microenvironment.

It has also been suggested that self-renewal capacity and metabolic fitness of tumour-infiltrating cells is higher after PI3Kδ inhibition with improved mitochondrial function (Lauder et al. 2020). This increase in metabolic fitness of tumour infiltrating CD8+ T-cells in response to PI3Kδ inhibition has been linked to tumour response. With tumours that showed an enhanced response to PI3Kδ inhibition having more CD8+ T-cells expressing increased Glut1, which suggests these cells could support enhanced metabolic activity by additional glycolysis (Lauder et al. 2020).

However, PI3Kδ inhibition may also partially have a negative effect on tumour immunity. Selective loss of p110δ kinase activity in regulatory T-cells leads to an enhanced anti-tumour response. It has been proposed that this could be due to some negative impact of p110δ loss on the functionality of CD8+ T-cells. It has been demonstrated that this could be by disrupting CD8+ T-cell differentiation to cytotoxic T-lymphocytes. This has been demonstrated by lower CD8+ T-cells mediated cell killing and lower expression of cytotoxic
mediators \textit{in vitro} with p110\(\delta\) loss of function (Ali et al. 2014). However, ultimately in the context of reduced tumour infiltrating T-regulatory cells, CD8+ T-cells are able to mediate tumour response despite this negative effect of PI3K\(\delta\) inhibition.

In this chapter, I aimed to do quantitative assessment of the immune changes induced by PI-3065 treatment in the NTCU-induced mouse model of lung squamous cell carcinoma. I analysed both systemic immune alterations, by quantifying the immune composition of the spleen, and local immune changes in proximity to NTCU-induced disease in the lung. Additionally, I investigated whether there is a correlation between PI-3065 modulation of disease progression and T-cell immunological responses.
6.2. Aims

- Identify the splenic lymphocyte changes that occur with PI-3065 treatment in the context of NTCU-induced disease

- Measure the overall lung T-cell changes that occur with PI-3065 treatment in the NTCU-induced model of LUSC

- Quantify the changes in the immune microenvironment of NTCU-induced disease lesions following PI-3065 treatment

- Assess varying immunological responses in PI-3065-treated mice with and without NTCU-induced invasive disease.
6.3. Results

6.3.1. Systemic spleen changes

Systemic splenic changes have been shown to occur in response to tumour development (Hiam-Galvez, Allen and Spitzer 2021) and after PI3Kδ inhibition (Okkenhaug et al. 2002). It has been shown that NTCU-treated mice retain the ability to mount a robust and specific immune response with spleen immune composition effects varied across strain and dosing regimens (Riolobos et al. 2019). Using flow cytometry, I have begun to characterise the changes that occur in splenic lymphocytes after PI3065-treatment with and without NTCU treatment.

6.3.1.1. Proportions of splenic lymphocytes

Looking exclusively at the lymphocyte populations of the spleen, after NTCU treatment alone the proportion of TCRβ+ T-cells decreased (p=0.0045) and the proportion of B220+CD19+ B-cells increased (p<0.0001) (Figure 6.1). This trend was maintained when absolute spleen B-cell numbers were assessed (p=0.0232) but there was no significant difference in T-cell numbers (Appendix Figure 8.4). T-lymphocyte CD4+ and CD8+ subpopulations were unchanged both in proportions and absolute numbers, unlike the reduction in CD4+CD25+FOXP3+ T-regulatory cell proportions (Chapter 5, Figure 5.5, p<0.0001). There were no statistically significant changes in proportions or absolute numbers of CD21+CD23+ follicular B-cells and CD21-CD23- B1 B-cells after NTCU treatment (Figure 6.1, Appendix Figure 8.4).

The overall proportions of splenic T and B-lymphocytes were unchanged with PI-3065 treatment, both in NTCU-treated mice and age-matched controls (when compared to respective vehicle treated mice). The proportion of T-cells that were either CD4+ or CD8+ were also unchanged with PI-3065 treatment in both contexts (Figure 6.1). However, as demonstrated in Chapter 5 (Figure 5.5), PI-3065 treatment decreased the splenic proportions of
CD4+CD25+FOXP3+ T-regulatory cells both in age-matched controls and NTCU-treated mice (p=0.0041, p=0.0030).

Although the overall proportion of B-cells were unchanged with PI-3065 treatment compared to vehicle, B-cells sub-populations were altered. As shown in Chapter 5 (Figure 5.5), PI-3065 almost completely depleted CD21+CD23- marginal zone B-cells. The resulting splenic B-lymphocyte population after PI-3065 treatment had higher proportions of CD21+CD23+ follicular B-cells (both in the context of NTCU treatment and not) and decreased proportions of CD21-CD23- B1 B-cells (with NTCU treatment) (p<0.0001, p<0.0001) (Figure 6.1). However, to assess whether this was due to direct effects on these populations I assessed absolute spleen numbers. The number of follicular B-cells was unchanged with PI-3065 treatment but the number of splenic B1 B-cells significantly decreased (p=0.0019) (Appendix Figure 8.4). Therefore there is no impact of PI-3065 treatment on the number of splenic follicular B-cells and the subsequent increase in the proportions of follicular B-cells is due to the near loss of marginal zone B-cells and reduction in B1 B-cells (with NTCU treatment).
Figure 6.1 - Splenic lymphocyte proportions assessed by flow cytometry. Proportions of TCRβ+ T-lymphocytes, CD4+ and CD8+ T-cells. Proportions of B220+CD19+ B-lymphocytes, CD23+CD21+ Follicular B-cells and CD23-CD21- B1 B-cells. Each point represents the splenic proportions in individual mice, 5-12 mice per group plotted ± SEM. Statistical significance was tested with One-way ANOVA with Tukey’s multiple comparison tests or Kruskal-Wallis with Dunns multiple comparisons tests (** p<0.01, **** p<0.0001, ns not significant).
6.3.1.2. Proportion of T-lymphocytes expressing activation markers

Since PI-3065 treatment has been applied in this study to primarily modulate the T-cell immune microenvironment, I looked at the activation of splenic T-lymphocytes.

With NTCU treatment alone, there were no changes in the proportion of splenic T-cells expressing the activation marker CD44. However, after PI-3065 treatment NTCU-treated mice showed lower proportions of both activated/memory CD44+CD4+ and CD44+CD8+ T-cells (p<0.0001 and p=0.0192, respectively). These reductions were not seen in non-NTCU treated age-matched controls. The CD44+ expression of T-regulatory cells was unchanged with PI-3065 treatment (Figure 6.2).

CD69 is considered an early marker of activation on T-cells and is an important co-stimulatory molecule for T-cell activation and proliferation (Cibrián and Sánchez-Madrid 2017). NTCU treatment increased the proportion of CD8+ T-cells expressing CD69+ (p=0.0002), but had no effect on CD69 expression of CD4+ T-cells. The proportions expressing CD69 for both subsets were unchanged by PI-3065 treatment, both in age-matched controls and in the context of NTCU treatment (Figure 6.2).

However, the proportion of T-regulatory cells that express CD69 increased with NTCU treatment (p<0.0001) (Figure 6.2). Expression of CD69 on the surface of T-regulatory cells has been linked to a more immunosuppressive T-regulatory functionality (Yu et al. 2018). This could indicate that the splenic T-regulatory cells after NTCU treatment were primed for a more immunosuppressive function. In agreement with its reported activity in targeting T-reg function (Patton et al. 2006), PI-3065 treatment reduced the proportion of T-regulatory cells that express CD69 in the context of NTCU treatment only. Thus PI-3065 treatment counteracts the increased proportions of CD69+ T-regulatory cells associated with NTCU treatment, this potentially
reduced the immunosuppressive role of the remaining regulatory T-cells (p=0.0001) (Figure 6.2).

**Figure 6.2 - Activation of splenic T-lymphocyte proportions assessed by flow cytometry.** Proportions of CD4+ T-cells, CD8+ T-cells and CD4+CD25+FOXP3+ T-cells that are either CD44+ or CD69+. Each point represents the splenic proportions of individual mice, 5-12 mice per group plotted ± SEM. Statistical significance was tested with One-way ANOVA with Tukey’s multiple comparison tests or Kruskal-Wallis with Dunn’s multiple comparisons tests (* p<0.05, *** p<0.001, **** p<0.0001, ns not significant).
6.3.1.3. Proliferation of T-cell subsets within the spleen

To assess whether NTCU or PI-3065 treatment resulted in increased proliferation of T-cells in the spleen, I used Ki-67 staining. There were no statistically significant differences in the proliferation of CD4+, CD8+ or CD4+FOXP3+CD25+ T-cells when comparing NTCU treated mice to vehicle only controls (Figure 6.3). Additionally, there were no significant differences in the proliferation of T-cell subsets in the spleen with PI-3065 treatment in NTCU treated mice or age-matched controls.

![Figure 6.3 - Proliferation of splenic T-lymphocyte proportions assessed by flow cytometry.](image)

Figure 6.3 - Proliferation of splenic T-lymphocyte proportions assessed by flow cytometry. Proportions of CD4+ T-cells, CD8+ T-cells and CD4+CD25+FOXP3+ T-cells that are Ki-67+. Each point represents the splenic proportions of individual mice, 5-12 mice for each group plotted ± SEM. Statistical significance was tested with One-way ANOVA with Tukey’s multiple comparison tests or Kruskal-Wallis with Dunns multiple comparisons tests (ns not significant).
6.3.2. T-cell localisation in the lung after PI-3065 treatment

I hypothesised that the NTCU-induced disease modulation by PI-3065 treatment described in the chapter 5, was mediated by an altered lung T-cell immune microenvironment. To assess this, I have quantified the localisation of T-cell subsets both in the lung parenchyma and within proximity to the bronchial tree in mice with and without PI-3065 treatment.

6.3.2.1. Parenchyma

To assess whether PI-3065 treatment has changed the overall T-cell composition of the lungs, not specific to the bronchial tree, I first assessed the T-cell composition of the parenchyma (Figure 6.4).

![Figure 6.4](image)

**Figure 6.4 - Proportion of T-cell subsets in lung parenchyma with NTCU treatment and interventional PI-3065 treatment.** T-cell subsets were quantified as a proportion of total DAPI+ cells. 4-5 0.25mm² regions were quantified from each mouse. 3-6 mice for each group. Statistical significance was tested with Kruskal-Wallis test and Dunn’s multiple comparison tests (* p<0.05, ** p<0.01, ns not significant).
Neither NTCU of PI-3065 treatment had a significant effect on the proportions of CD4+FOXP3- T-helper cells and cytotoxic CD8+ T-cells in the lung parenchyma. However, PI-3065 treatment significantly decreased the fraction of CD4+FOXP3+ T-regulatory cells in the parenchyma of NTCU-treated mice (p=0.0428) to levels comparable to those seen in non-NTCU treated, age-matched controls (Figure 6.4). This demonstrates that PI-3065 treatment was able to inhibit the increased T-regulatory cells associated with NTCU treatment, in the lung parenchyma.

6.3.2.2. T-cell association with the bronchial tree

As described in Chapter 4, NTCU-induced disease development was associated with an increased localisation of T-cells to within proximity of the bronchial tree, particularly at the 24-week time-point. To assess whether PI-3065 treatment has modulated this T-cell localisation to the bronchial tree, the percentage of total DAPI+ cells that are either CD4+FOXP3- T-helper, CD4+FOXP3+ T-regulatory and CD8+ T-cells were quantified within 100 μm of the basement membrane, using immunostaining.

Although I have demonstrated that localisation of T-cells to the bronchial tree increases irrespective of KRT5 expression or pre-invasive grade, with NTCU treatment (Chapter 4). I separated the bronchial tree by KRT5 expression and pre-invasive grade for this analysis, to appreciate the influence of PI-3065-induced T-cell environment changes at each disease stage.

6.3.2.3. Non-KRT5 expressing epithelium

I began by assessing T-cell localisation to non-KRT5 expressing epithelium. With PI-3065 treatment there were fewer CD4+FOXP3+ T-regulatory cells associated with non-KRT5 expressing epithelium than with vehicle treatment (p<0.0001). This reduction with PI-3065 resulted in regulatory T-cell proportions that were comparable to those observed in epithelium in non-NTCU treated mice, completely counteracting the increase with NTCU
treatment alone (p=0.0015). There was no difference in the association of CD4+ and CD8+ T-cells with non-KRT5 expressing epithelium among the three groups (Figure 6.5).

**Figure 6.5 - Presence of T-cell subsets in proximity to lung epithelium not expressing KRT5.** T-cell subsets were quantified as a proportion of total DAPI+ cells, 100μm from epithelium not expressing KRT5. 3-5 areas of non-KRT5 epithelium quantified from individual mice, 3-6 mice for each group. Statistical significance was tested with Kruskal-Wallis test and Dunn’s multiple comparison tests (** p<0.01, **** p<0.0001, ns not significant).
6.3.2.4. Association with pre-invasive disease

When comparing the association of T-cells to low-grade lesions, PI-3065-treated mice had fewer CD4+FOXP3- (p=0.0001), CD4+FOXP3+ (p=0.0002) and CD8+ T-cells (p<0.0001) in proximity to low-grade lesions than vehicle-treated mice (Figure 6.6).

This was also the case for high-grade pre-invasive disease. When compared to high-grade lesions in vehicle-treated mice, high-grade lesions in PI-3065-treated individuals had lower numbers of all three T-cell types: CD4+FOXP3- (p=0.0313), CD4+FOXP3+ (p=0.0065) and CD8+ (p=0.0443) (Figure 6.7).
Comparison of the changes in T-cell localisation in proximity to non-KRT5 expressing epithelium with that of pre-invasive grades demonstrate that PI-3065 treatment reduced the localisation of all three T-cell subsets to pre-invasive disease but only CD4+FOXP3+ localisation in KRT5-epithelium. This potentially demonstrated a varied PI-3065-response of CD4+FOXP3- and CD8+ localisation dependent on disease status. Why this occurred has not be investigated but could be linked to regional differences within the lung.

With PI-3065 treatment, irrespective of pre-invasive grade there were fewer T-helper, regulatory T-cells and cytotoxic T-cells, in proximity to NTCU-induced pre-invasive lesions, regardless of grade. The reduced-presence of T-regulatory cells suggests that the pre-invasive lesions are in a less immunosuppressive environment. However, the accompanying reduction in cytotoxic T-cells makes it difficult to conclude whether the environment after PI-3065 treatment favours immunosurveillance.
Comparison of T-cell localisation to healthy epithelium

Since T-cell localisation to within proximity of NTCU-induced pre-invasive disease decreased with PI-3065, I compared this reduced localisation with the T-cell localisation to normal healthy lung epithelium.

There were no statistical differences between T-cell localisation to NTCU-induced pre-invasive disease after PI-3065 treatment and normal healthy epithelium (Figure 6.8). This demonstrated that the localisation of T-cells more closely represented that seen in healthy non-diseased lungs after PI-3065 treatment. This is in contrast to the enrichment of CD4+FOXP3- T-helper and CD4+FOXP3+ T-reg in the proximity of NTCU-induced low- and high-grade pre-invasive lesions with NTCU treatment alone (Figure 4.9).

Figure 6.8 - T-cell subsets to within 100μm of normal epithelium and NTCU-induced low- and high-grade lesions. T-cell subsets were quantified as a proportion of total DAPI+ cells, 100μm from the associated basement membrane. 3-5 areas were quantified from individual mice, 3-6 mice for each group. Statistical significance was tested with a Kruskal-Wallis test (ns not significant).
6.3.2.5. Association of T-cells to invasive disease

To understand if PI-3065 treatment altered the T-cell microenvironment of invasive disease I quantified the T-cell composition as a proportion of total cells within a 100μm radius surrounding tumours (Chapter 4, Figure 4.5B) and compared PI-3065-treated with vehicle-treated.

The proportion of total cells identified as T-helper cells, surrounding invasive disease was unchanged with PI-3065 treatment. The proportion of T-regulatory cells in proximity to invasive disease decreased (p=0.0139), whereas the fraction of CD8+ T-cells increased with PI-3065 treatment (p=0.0042) (Figure 6.9). This demonstrated that PI-3065 treatment had influenced T-cell-tumour localisation, potentially creating a less immunosuppressive more cytotoxic tumour microenvironment.
6.3.2.6. Immunofluorescence staining summary of T-cell localisation in proximity to disease, with and without interventional PI-3065 treatment.

Figure 6.10 displays representative images of the T-cell changes quantified in proximity to NTCU-induced disease with and without interventional PI-3065.

Figure 6.10 – Representative images of T-cell staining in proximity to NTCU-induced disease within the lung, with and without interventional PI-3065 treatment. CD4+FOXP3+ T-regulatory cells are indicated by yellow arrows and CD8+ T-cells highlighted by orange arrows. CD4+FOXP3+ regulatory T-cells are decreased in proximity to both the bronchial tree and tumour. CD8+ T-cells increase in proximity to NTCU-induced tumours with interventional PI-3065 treatment. Scale bars indicate 100μm on all panels.
6.3.2.7. Ratio of CD4+FOXP3+:CD8 T-cells

As PI-3065 treatment changed both the association of T-regulatory and CD8+ T-cells to NTCU-induced disease, to approximate whether the lesion immune microenvironments were less immunosuppressive and more cytotoxic, the ratio of CD4+FOXP3+:CD8+ T-cells was examined.

The average ratios of CD4+FOXP3+:CD8+ T-cells for both low- and high-grade lesions were unchanged with PI-3065 treatment when compared with lesions in vehicle-treated mice. However, when looking at the tumour microenvironments, the ratio of CD4+FOXP3+:CD8+ T-cells decreased with PI-3065 treatment when compared to vehicle treatment (p=0.0357). This suggests that tumours but not pre-invasive lesions in PI-3065 treated mice were in a more cytotoxic, less immunosuppressive environment than those in vehicle-treated mice (Figure 6.11).

![Figure 6.11](image)

**Figure 6.11** - The ratio of CD4+FOXP3+ T-cells to CD8+ T-cells in the immune microenvironment of NTCU-induced pre-invasive and invasive disease, with and without PI-3065 treatment. The average proportion of total cells that are CD4+FOXP3+ or CD8+ T-cells for each lesion grade in each mouse was plotted. Average values for regions in 3-6 mice plotted, mean ±SEM indicated. Statistical significance was tested with a Mann-Whitney test (*) p<0.05, ns not significant.)
6.3.2.8. Comparison of PI-3065 treated mice with and without invasive disease

Chapter 5 has demonstrated how PI-3065-treated mice can be split into two groups: one including mice that have developed invasive disease and one with no invasive disease. To understand if this difference in disease development was associated with variations in T-cell localisation in the lung, I have compared T-cell localisation between mice with and without invasive disease.

There were no differences in the association of T-cell subsets to non-KRT5 epithelium, low- or high-grade pre-invasive disease between mice with and without invasive disease after PI-3065 treatment. In fact, the only statistically significant difference in T-cell localisation between responding and non-responding mice was found in the parenchyma. With responding mice having higher proportions of CD4+FOXP3- T-cells (p=0.0014) and lower numbers of CD8+ T-cells (p<0.0001) (Figure 6.12). This was unexpected, as I expected higher cytotoxic CD8+ T-cells in responding mice that facilitate a more cytotoxic immune microenvironment. However, the presence of invasive disease in itself could have influenced the immune microenvironment of the lung and this could have affected this comparison. It could also be due to a difference in T-cell functionality and contributing factors from the wider immune compartment that determine PI-3065 response.

This analysis has been completed on three responding and three non-responding mice, which I had available for immunostaining. However, completing this analysis on the rest of the cohort will be important to make conclusions from this data.
Figure 6.12. T-cell localisation in PI-3065 responders vs non-responders. The proportion of total cells that were CD4+FOXP3+, CD4+FOXP3−, or CD8+ T-cells either in the parenchyma, localised to Non-KRT5 epithelium or low and high-grade pre-invasive lesions separated by PL-3065 response. n=3 mice for each group. Mean ±SEM plotted. Statistical significance was tested with a Mann-Whitney test (\( p<0.01 \), ** \( p<0.0001 \), ns not significant).
To more broadly understand the immune microenvironment changes that occur after PI-3065 treatment, spatial transcriptomics will be performed in collaboration with the Wellcome Sanger Institute (Moritz Przybilla, Peter Campbell).

For an initial assessment, two vehicle-treated and two PI-3065-treated lungs have been sectioned onto VISIUM spatial transcriptomic capture slides (by collaborators). Figure 6.13 displays the captured areas of each block for spatial transcriptomics. The vehicle-treated blocks display extensive NTCU-induced disease, including multiple areas of pre-invasive and invasive disease. The matched anatomical lung regions of PI-3065-treated mice display no invasive disease but pre-invasive disease lines the exposed bronchial tree. VISIUM spatial transcriptomics has a resolution of around 50μm. So although it will not be able to give us single-cell resolution it should give an insight into the wider immune composition of the NTCU-treated lung and how this changes with PI-3065 treatment.

We are currently awaiting the transcriptomic data for analysis.
Figure 6.13 - **Target areas for spatial transcriptomic analysis.** H&E stained sections of 2 vehicle-treated and 2 PI-3065-responding NTCU-treated mice submitted for VISIUM 10x spatial transcriptomics. Arrows indicate areas of invasive disease. Target regions, highlighted are 6mm², to match VISIUM capture areas.
6.4. Discussion

I have demonstrated that PI-3065 treatment decreases the presence of CD4+FOXP3+ regulatory T-cells both in the spleen and the lung of NTCU-treated mice. These reductions in T-regulatory cells after PI-3065 treatment are consistent with other studies that have applied PI-3065 to pre-clinical models of solid tumours (Ali et al. 2014, Lauder et al. 2020).

Previous investigations of the immune cell populations in NTCU-treated mice show very little splenic changes (Riolobos et al. 2019). My work shows that with NTCU treatment spleen cell composition shifts to lower proportions of both T-cells and regulatory T-cells. Although this study also assessed NTCU-induced splenic effects in FVB/N mice splenic differences may be due to dosing regimens. The mechanism and significance of these changes have not been assessed. Exposure to NTCU treatment had no effect on activation or proliferation of T-cell subsets but did result in higher activated proportions of both CD8+ and T-regulatory cells, indicating activation of these subsets.

PI-3065 treatment following NTCU administration significantly reduced the proportions of T-regulatory cells expressing CD69, whilst having no effect on the CD8+CD69+ fraction. This indicates that PI3Kδ inhibition by PI-3065 treatment has reduced the pool of splenic activated T-regulatory cells in this LUSC model.

In mice expressing a kinase-dead version of p110δ, 4T1 tumour inoculation results in expansion of activated/memory CD44+CD4+ and CD44+CD8+ T-cells in the spleen. This is not seen in mice with functional p110δ, and demonstrates that in the absence of p110δ signalling mice were able to mount a CD4+ and CD8+ immune response to tumour inoculation (Ali et al. 2014). This was not seen with PI-3065 treatment in this study, with no change in the overall CD44+ T-cell proportions with and without NTCU treatment. Indeed, PI-3065 treatment reduced the proportions of activated/memory CD44+CD4+
and CD44+CD8+ T-cells following NTCU treatment. However, the significance of the changes in the splenic populations on the immune composition of the lung and therefore disease has not been investigated.

My data shows that the NTCU-induced association of T-regulatory, T-helper and cytotoxic CD8+ T-cells to pre-invasive disease decreased after PI-3065 treatment. However, the ratio of T-reg to cytotoxic T-cells is unchanged in proximity to pre-invasive disease, potentially indicating that the pre-invasive T-cell microenvironment is not completely switched from immunosuppressive to immunosurveillance. This could account for there being no differences in the development of pre-invasive disease with PI-3065 treatment compared to vehicle-treated mice.

The T-cell microenvironment of NTCU-induced tumours is also changed with PI-3065 treatment, with a reduction of regulatory T-cells and an increase in cytotoxic CD8+ T-cell proportions. This results in a significant decrease in the average CD4+FOXP3+:CD8+ ratio after PI-3065 treatment, indicating a PI-3065-induced shift in the tumour microenvironment to favour immunosurveillance. This PI-3065-treated tumour microenvironment, with enhanced immunosurveillance, could favour slower tumour growth, resulting in the development of smaller tumours as described in Chapter 5.

I have demonstrated that within the half of the PI-3065-treated cohort I analysed, there are no significant differences in the T-cell composition of the immune microenvironment of pre-invasive disease when comparing responding and non-responding mice. The only significant changes were documented as higher T-helper cell and lower cytotoxic T-cell proportions in the parenchyma in responding mice. Analysis of the other half of the treatment cohort, should allow validation of these results. If and how these changes may influence disease progression could be investigated by further phenotyping their functionality.
In a 4T1 breast cancer model it has been demonstrated that functionality of the remaining tumour regulatory T-cell pool after PI3Kδ inhibition is associated with tumour treatment response. A more proliferative T-regulatory cell immune phenotype was documented in tumours that displayed dampened tumour PI3Kδ inhibitor response, with higher T-regulatory cell expression of Ki-67 and of co-inhibitory receptor LAG3 (Lauder et al. 2020). This presents the possibility that the varied disease modulation capability of PI3Kδ inhibition, seen in this study, could be due to diverse T-regulatory cell function.

In the same breast cancer study, it was demonstrated that these proliferative LAG3 expressing T-regulatory cells can be targeted by anti-LAG3 antibody therapy in combination with PI3Kδ inhibition to reduce tumour burden. This further highlights the importance of phenotyping the T-regulatory cells in the NTCU-treated lung after PI3Kδ inhibition. This could elucidate the difference between responding and non-responding mice and identify effective combinatorial strategies to enhance PI3Kδ efficacy (Lauder et al. 2020). For instance, if remaining T-regulatory cells after PI-3065 treatment are expressing LAG3 in the NTCU model, subsequent LAG3 antibodies could be tested in combination with PI-3065.

In addition to modulating T-cell mediated anticancer activity, PI3Kδ inhibition has been shown to decrease activity of cancer promoting myeloid-derived suppressor cells (Ali et al. 2014) and cancer-associated macrophages in some contexts (Lim et al. 2018). However subsequent work has suggested that this reduction of myeloid-derived suppressor cells is a consequence of reduced tumour growth rather than PI3Kδ inhibition directly. My work has not addressed whether these cells play a role in NTCU-induced carcinogenesis and if macrophages have been modulated by PI-3065 treatment in this context.

As part of this work we have employed spatial transcriptomics to better understand the wider microenvironment of NTCU-induced disease and how
this is modulated by PI3Kδ inhibition. This strategy could identify additional influential microenvironment features in NTCU-induced carcinogenesis and if they have been modulated by PI-3065 treatment.

If successful this strategy could be further implemented to compare the wider immune microenvironment of PI-3065 responding and non-responding mice. This strategy would provide both transcriptional data to profile both the composition and functionality of the immune microenvironment whilst preserving spatial information. However, this will depend on the resolution that we can achieve to be able to discern immune populations. Alternatively, lung immune cells could be digested from PI-3065 treated mice and profiled via FACS including markers to discern functionality. Additionally, multi-plexed fluorescent imaging could be used to more comprehensively quantify the immune microenvironment after PI-3065 treatment. This would be more high-throughput than the immunostaining strategy used in this study whilst preserving the spatial immune information.

Overall, I have demonstrated both immune changes in the spleen and lung associated with PI3Kδ inhibition in the context of NTCU-induced disease. Analyses of lung T-cell localisation demonstrate fewer regulatory T-cells in all lung regions and a resulting shift to a more cytotoxic tumour T-cell immune microenvironment. This immune microenvironment modulation could facilitate the observed tumour initiation prevention in responding mice and slower tumour growth in mice that still develop disease.
6.5. Summary

- PI-3065 treatment alters both the splenic and lung T-cell composition.

- PI-3065 treatment results in lower association of CD4+, CD4+FOXP3+ and CD8+ T-cells with NTCU-induced pre-invasive low and high-grade lesions.

- With interventional PI-3065 treatment, NTCU-induced tumours develop in a less immunosuppressive environment, with decreased association of CD4+ and CD4+FOXP3+ T-cells and increased CD8+ T-cells.

- There was no difference in association of T-cells with pre-invasive disease in PI-3065 treated mice that have (non-responding to treatment) and have not (responding to treatment) developed invasive disease.
7. Discussion

LUSC is a major contributor of cancer morbidity worldwide. Successful therapeutic targeting of the observable pre-invasive phase of LUSC development could significantly reduce patient suffering and morbidity. However, efforts so far have been largely clinically unsuccessful. The development of future efficacious interventional therapies will centre on exploiting improved understanding of the intrinsic and extrinsic factors that influence pre-invasive disease progression to invasion and will require effective pre-clinical models.

Frequently documented genetic and transcriptomic changes in PIK3CA both in pre-invasive and invasive LUSC, alongside mechanistic studies, have implicated PI3Kα signalling in the development and maintenance of LUSC. In addition, recent studies have implicated various immunomodulatory mechanisms in determining the fate of pre-invasive LUSC disease.

In this study, I have optimised methods to generate an in vitro system to investigate the role of dysregulated PI3K signalling in the transformation of human bronchial epithelial cells. I have also evaluated the recapitulation of human LUSC features, including immune interactions and PI3K signalling reliance in a pre-clinical murine LUSC model. Application of interventional therapies to this pre-clinical model has allowed me to evaluate the potential of PI3K targeted therapeutics to influence disease fate by modulating cell signalling and immune microenvironment.

7.1.1. Summary

Although in the time frame of this project, I have been unable to assess the impact that PIK3CA alterations recurrently identified in LUSC exert on HBEC phenotype, I have optimised gene-editing of immortalised HBECs to model in vitro early dysregulation of PI3K signalling in the airway epithelium. This HBEC
modification strategy could be applied to investigate additional candidate genes and identify potential therapeutics to intervene in LUSC development.

I have investigated the role of PI3K signalling in NTCU-induced carcinogenesis and tested a PI3Kα targeted therapy (BYL719) as an interventional treatment for the progression of pre-invasive disease to invasive LUSC. BYL719 showed no interventional therapeutic value at the dose and regimen tested. Further work would be needed to determine if this lack of efficacy is because NTCU-induced lesion development and progression is PI3Kα signalling-independent. My efforts using immunostaining for PI3K signalling nodes have not clearly implicated PI3Kα signalling in NTCU-induced lesion development. However, the transcriptional profiles of the epithelial fraction of the scRNAseq experiment will shed further light on this. In addition, as part of a wider characterisation of the NTCU-induced pre-clinical model, the Janes lab in collaboration with Peter Campbell’s group at the Wellcome Sanger Institute, are currently investigating the genomic profile of NTCU-induced disease. Both transcriptomic and genomic investigations, will help to definitively elucidate the role of PI3Kα signalling in NTCU-induced carcinogenesis.

I have demonstrated that NTCU-induced carcinogenesis develops in a dynamic immunosuppressive environment, characterised by increased regulatory T-cells. This reflects at least one of the T-cell mediated immunosuppressive mechanisms that determine the fate of human LUSC. I have demonstrated that application of a PI3Kδ inhibitor (PI-3065) as an interventional therapy can modulate the progression of NTCU-induced pre-invasive disease to invasive tumours, resulting in lower tumour incidence and tumour burden. This modulation is coupled with a switch in the T-cell immune microenvironment, with less regulatory T-cells in proximity to disease (Figure 7.1). These promising results present the possibility that PI3Kδ inhibition could be used as an interventional therapy to human LUSC development.
This study demonstrates how the NTCU model can be used to assess interventional therapies for LUSC. However, there are limitations to the NTCU model. For instance, NTCU-induced disease develops over many months with very little reliable way to monitor disease progression throughout, limiting its potential in being used to screen large quantities of interventional therapeutics. Alternative simpler *in vivo* models could be used prior to the NTCU model to narrow down potential therapeutic candidates. This could include, xenograft models with the candidate therapy given prophylactically before cell or tumour engraftment. Although this wouldn't truly recapitulate progression from pre-invasive disease this model would provide a quicker way to screen interventional candidate efficacy and highlight promising therapies to be investigated using the NTCU model where the lung microenvironment is recapitulated. This is a potential area for future study and could even exploit cell lines derived from NTCU induced disease.
7.1.2. Immediate future directions

To build on this study, future studies could evaluate if interventional PI3Kδ treatment confers a survival benefit by preventing NTCU-induced tumour development long-term. This, along with optimisation of intervention points and dose, could further evaluate the therapeutic potential of PI3Kδ inhibition in modulating LUSC disease development.

In this study I have investigated the influence of the T-cell immune compartment on NTCU-induced disease development, however, the influence of the wider immune compartment has not yet been characterised. Both the scRNAseq and spatial transcriptomic results could give a wider understanding of the immune changes associated with NTCU-induced carcinogenesis. This could then further inform, more in-depth quantification of the immune compartment utilising multiple fluorescence imaging (MFI) techniques.

Understanding the immune compartment throughout NTCU-induced carcinogenesis is particularly important as other immune populations have been shown to be influential in LUSC, including neutrophils (Kargl et al. 2019). If the neutrophil compartment in this model reflects neutrophil disease influence as seen in human LUSC, it could lead to the pre-clinical evaluation of dual PI3Kδ/β inhibitors as an interventional LUSC therapy. As PI3Kβ has been shown to be involved in neutrophil activation (Kulkarni et al. 2011).

Application of PI3Kδ inhibitors in solid tumour models have also demonstrated that additional alleviation of immunosuppression can occur independently of T-regulatory cells, via modulation of other immune cell-types, including cancer-associated macrophages (Goulielmaki et al. 2018). The contribution of potential changes in these populations has not yet been addressed in this context. However, the spatial transcriptomic results, depending on resolution could indicate additional changes in these populations in PI3Kδ treated mice.
In this study, interventional PI3Kδ inhibition showed limited modulation of NTCU-induced development of pre-invasive disease but a significant reduction in tumour development. This raises questions as to why alleviated T-reg mediated immunosuppression only manifests as a reduction in tumour burden. Could this be due to lower mutational burden of pre-invasive disease, subsequent lower neo-antigen load and therefore less immune activation? Or could this be more influenced by the varying disease stage niches, with varied immune cell availability and immunomodulatory signals?

It would be interesting to better understand if PI3Kδ immune modulation has in fact influenced disease evolution genomically by immune cell clearance. Future studies could use the PAXgene-fixed PI-3065 treated blocks generated in this study to investigate the influence on genomic burden using laser-capture microdissection. This strategy is currently being used in parallel studies to genomically characterise the NTCU model.

7.1.3. Considerations for the application of PI3Kδ inhibitors as interventional therapies for LUSC development

This study suggests that PI3Kδ inhibition could be a potential promising interventional therapeutic to prevent LUSC development in high-risk patients. However, if this is to be possible, any adverse reactions would need to be minimised. This is particularly important as patients are otherwise healthy, with very few to no associated symptoms and critically, no certainty that they will develop LUSC. Therefore, the threshold for acceptable impact on a patient’s quality of life would be extremely low.

Immune related side-effects of PI3Kδ inhibition have been well documented in a range of therapeutic contexts in humans. A recent phase II clinical trial of PI3Kδ inhibition in head and neck cancer patients (Eschweiler et al. 2022) showed significant occurrence of immune related adverse effects, including diarrhoea and skin rashes, resulting in treatment discontinuation in 12/21
patients. Thus, limiting therapeutic efficacy. However, an intermittent dosing strategy has shown promise in a mouse model that develops similar immune-related adverse events including colitis, with reduced colonic related immune related adverse effects, whilst maintaining tumour growth reduction (Eschweiler et al. 2022).

With the lung being the target organ with LUSC, PI3Kδ inhibitors could be administered via inhalation. This could avoid systemic immune changes and minimise immune-related side effects. Current clinical trials are assessing the clinical efficacy and safety of inhaled PI3Kδ inhibitors, for the treatment of respiratory conditions including chronic obstructive pulmonary disorder (COPD)(NCT03345407, NCT04032535). Nemiralisib, a highly PI3Kδ selective, inhibitor has shown no clinical benefit in a phase II clinical trial for the treatment of COPD, but importantly also showed no connected severe adverse reactions, with only a few instances of post-inhalation cough and one instance of bronchospasm (Fahy et al. 2021). The capability of PI3Kδ inhibitors to modulate the immune composition of the lung when administered by inhalation, has not yet been widely assessed.

The presence of multiple immunosuppressive mechanisms in pre-invasive LUSC presents the possibility of combining PI3Kδ inhibition with other immune targeted therapies to further influence disease fate. For instance, the expression of immune checkpoints in pre-invasive disease could be targeted in combination with PI3Kδ inhibition. PD-1/PD-L1 targeted therapy in combination with PI3Kδ inhibition is currently being assessed in clinical trials for advanced and metastatic solid tumours (NCT02646748, NCT03589651). To successfully apply these therapies in combination for pre-invasive disease intervention, therapy timing will be critical. In combination with checkpoint inhibitors, PI3Kδ inhibition has been shown to antagonise immune checkpoint inhibition. This is attributed to the reduced cytotoxic CD8+ T-cell function associated with PI3Kδ inhibition, on which check-point efficacy relies (Lim et al. 2018). However, if applied sequentially, PI3Kδ inhibition could enhance

This work demonstrates the evaluation of interventional treatments for LUSC using a pre-clinical model, induced by NTCU. I have highlighted the potential of PI3Kδ inhibition to modulate the immunosuppressive environment of NTCU-induced carcinogenesis and intervene in LUSC development. These results, framed within the context of ongoing clinical trial outcomes, will inform the potential of PI3Kδ inhibition as an interventional treatment for human pre-invasive LUSC. As discussed, successful application will require treatment efficacy to be achieved without compromising patient quality of life. If this can be achieved, PI3Kδ inhibition could be applied alone or in combination to achieve interventional therapeutic efficacy in LUSC development, which could significantly improve patient suffering and mortality.
8. Appendix

**Entry vectors:**

A. Entry vector containing mCherry, linker, P2A, and AKT2, generated by PCR amplification of P2A, link, AKT2 and mcherry with linker. Combination vector was generated via restriction digest, utilising the BspEI site in linker region (highlighted).

B. mCherry entry vector, generated by PCR amplification of mcherry.

C. Entry vector containing tGFP, linker, P2A, and PIK3CA, generated by PCR amplification of link, P2A, PIK3CA and combination with an already available linker, tGFP entry vector. Combination vector was generated via restriction digest, utilising the BspEI site in linker region (highlighted).

**Figure 8.1- Generated entry vectors for gateway cloning.** (A) Entry vector containing, mCherry, linker, P2A, and AKT2, generated by PCR amplification of P2A, link, AKT2 and mcherry with linker. Combination vector was generated via restriction digest, utilising the BspEI site in linker region (highlighted). (B) mCherry entry vector, generated by PCR amplification of mcherry. (C) Entry vector containing, tGFP, linker, P2A, and PIK3CA, generated by PCR amplification of link, P2A, PIK3CA and combination with an already available linker, tGFP entry vector. Combination vector was generated via restriction digest, utilising the BspEI site in linker region (highlighted).
**Destination vectors:**

**A.**

![Diagram of destination vector](Created with SnapGene®)

**B.**

![Diagram of another destination vector](Created with SnapGene®)

**Figure 8.2- Generated destination vectors for gateway cloning.** (A) The CAG promoter was replaced with EF1α promoter. EF1α promoter was PCR amplified and incorporated with restriction enzymes displayed. (B) The antibiotic selection cassette of the destination vector was switched from neomycin to blasticidin via restriction enzyme digest.
**shRNA Lenti-viral vectors:**

A.

B.

**Figure 8.3- Generated lenti-viral plasmids.** (A) The antibiotic selection cassette was switched from blasticidin to hygromycin via restriction enzyme digest. (B) This was also completed for the respective empty vector plasmid.
Figure 8.4- Splenic lymphocytes assessed by flow cytometry (absolute numbers). Numbers of TCRβ+ T-lymphocytes, CD4+ and CD8+ T-cells. Numbers of B220+CD19+ B-lymphocytes, CD23+CD21+ Follicular B-cells and CD23-CD21- B1 B-cells. Each point represents the number of splenic cells in individual mice, 5-12 mice per group plotted ± SEM. Statistical significance was tested with One-way ANOVA with Tukey’s multiple comparison tests or Kruskal-Wallis with Dunns multiple comparisons tests (* p<0.05, ** p<0.01, ns not significant).
9. Bibliography


