Deciphering the Immune Landscape in Renal Cell Carcinoma and in Anti-PD-1 Therapy

Emine Hatipoglu

Student Number: [Redacted]

University College London

Cancer Immunology Unit
Department of Haematology
University College London

PhD supervisors: Professor Sergio A. Quezada and Professor Charles Swanton

A thesis submitted for the degree of Doctor of Philosophy

2021
DECLARATION

I, Emine Hatipoglu, 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.

Date: 05 September 2021

Signature: [Redacted]
ABSTRACT

Antigen recognition and T-cell mediated cytotoxicity are major tenets of cancer immunology that are not fully understood in clear-cell renal cell carcinoma (ccRCC). We evaluated multiregional treatment naïve nephrectomy samples from 27 patients as well as blood samples from 21 and normal kidney tissue from 11 patients from the TRACERx Renal (TRAcking Cancer Evolution through therapy [Rx]) study via high dimensional flow cytometry. Results showed that the T cells in the tumour, normal kidney and blood have different phenotypes and differentiation patterns. A predominantly exhausted CD8 cell phenotype with expression of PD-1, TIM-3, Eomes, CD38 and CD39 was seen in the tumour immune microenvironment. ADAPTeR is a phase II study evaluating nivolumab (anti-PD1 antibody) in patients with treatment-naïve metastatic ccRCC. Immunophenotyping by using high dimensional flow cytometry and multiplex immunofluorescence in addition to T cell receptor (TCR) sequencing was performed on 93 pre- and post-treatment, multi-region tumour and peripheral blood samples from 15 patients. We showed that an increased Granzyme B production in the CD8 cells and higher B cell infiltration at baseline were associated with response to Nivolumab. TCR sequencing analysis showed that maintenance of expanded TCR clones during the anti-PD1 treatment which were present pre-treatment and increased clustering of TCR clonotypes are associated with response to therapy. Comparing a responder patient with a non-responder by using single cell RNA Sequencing (SC RNA Seq) showed a more dysfunctional phenotype in the responder. In addition, Nivolumab bound CD8 cells in the responder also had higher Granzyme B and TCF7 expression suggesting a more cytotoxic and progenitor-like phenotype is associated with response. This study provides important data that needs to be validated in a bigger cohort to identify biomarkers of response to anti-PD-1 therapy in ccRCC.
IMPACT STATEMENT

In this thesis, the immune landscape in clear cell renal cell carcinoma at baseline and on anti-PD1 therapy has been described. Despite its use in multiple cancers over several years, the mechanism of action of anti-PD-1 therapy and markers of response and resistance to treatment are not fully understood. This project attempts to analyse the markers of response and resistance to anti-PD-1 therapy in depth despite small sample size.

High dimensional flow cytometry analysis of treatment naïve samples showed that T cells in tumour, tumour adjacent normal tissue and peripheral blood had different phenotypes and differentiation patterns. Peripheral blood had a high CD8 Temra population with high levels of CD57 and Granzyme B expression. On the other hand, TILs had high levels of CD8 tissue resident cells (TRM) which had higher expression of co-inhibitory checkpoints including PD-1, TIM-3, CTLA-4, transcription factor Eomes as well CD38 and CD39 suggesting a dysfunctional phenotype in the tumour. In addition, PD-1+ and PD-1- CD8 cells also had different phenotypes in the TILs with a more dysfunctional phenotype in the PD-1+ CD8 cells.

Within the context of anti-PD-1 treatment (Nivolumab), T cell and myeloid cell infiltration did not predict response to Nivolumab treatment. However, an increase in Granzyme B production in the CD8s and B cell infiltration at baseline were associated with response to Nivolumab. As a novel finding, we showed that anti-PD-1 treatment did not cause expansion of new TCR clones with Nivolumab. Instead, maintenance of TCR clones which were present at baseline and maintained through therapy was associated with response to treatment. In addition, maintained expanded clones showed more clustering in the responders which suggest they respond to the same maintained antigen. Another key finding was that the peripheral blood was not representative of the tumour in terms of TCR repertoire.

As another novel part of the study, we performed an in-depth analysis of Nivolumab-bound T cells by using SC RNA Seq and TCR Sequencing which has not been reported in the literature before. This analysis was done only on two patients (one responder and one non-responder) and a more dysfunctional phenotype in overall and Nivolumab bound CD8 cells with higher TCF7 and Granzyme B expression was seen in the responder. This suggests a progenitor-like exhausted T cell state with the ability to preserve cytotoxic function. An upregulation of pathways involved in T cell activation was seen in Nivolumab bound T cells regardless of response to treatment. In addition, Nivolumab bound CD8 cells showed a higher level of TCR expansion and cytotoxic phenotype with more clustering in the responder patient compared to the non-responder patient. These findings suggest a maintained progenitor-like state of dysfunctional CD8s and ability to get activated via antigenic stimulus can be important
in response to Nivolumab. However, the information obtained from these patients is descriptive and hypothesis generating and cannot be used to make any conclusions. Therefore, these findings should be confirmed in a validation cohort.
ACKNOWLEDGEMENTS

I am very thankful to my supervisors Prof. Sergio Quezada and Prof. Charlie Swanton for their endless support, encouragement, and guidance during the PhD. This PhD would not be possible without them. I owe a special thank you to Sergio who has not only taught me a lot about immunology and science but also always believed in me, gave the space to be independent and creative while encouraging me to achieve my true potential.

I am indebted to Prof. Karl Peggs and Prof. Benny Chain for his continuous mentorship and support during my PhD. Their vision, patience and guidance have truly made an impact on my PhD journey.

I have learned a lot from Dr. Pablo Becker and he has been a true role model in terms of his scientific skills. I am also extremely thankful for his continuous support and help during the past few years.

I owe a special thanks to my dear friends who have always been there for me unconditionally: Kroopa Joshi, Isabelle Solomon, Mariana Werner Sunderland, Dafne Franz and Anna Sledzinska. I am also very grateful to all members of the Quezada lab.

And finally, huge thanks to my parents Ali and Nurcihan Hatipoglu and my brother Fehim Hatipoglu for always believing in me, for their endless love, enthusiasm, positivity and for always giving me perspective during my PhD.
Table of Contents

DECLARATION ......................................................................................................................... 1
ABSTRACT ................................................................................................................................. 2
IMPACT STATEMENT .................................................................................................................. 3
LIST OF FIGURES ..................................................................................................................... 9
LIST OF TABLES .......................................................................................................................... 11
ABBREVIATIONS ...................................................................................................................... 12

1. BACKGROUND ...................................................................................................................... 14

1.1. Immune System in Homeostasis ......................................................................................... 14
1.2. Cancer and Immune Escape ............................................................................................... 15
1.3. Immunotherapy in Cancer ................................................................................................. 20
  1.3.1 Immunoglobulin Superfamily – CD28 family ................................................................. 22
  1.3.1.1 Cytotoxic T Lymphocyte-Associated antigen 4 (CTLA-4) ........................................ 22
  1.3.1.2 PD-1 ............................................................................................................................. 23
  1.3.1.3 Inducible T cell Co-stimulatory Inhibitor (ICOS) ....................................................... 24
  1.3.2 Immunoglobulin Superfamily – TIM family ................................................................. 25
  1.3.2.1 T Cell Immunoglobulin and Mucin Domain 3 (TIM-3) ............................................. 25
  1.3.3 Tumour Necrosis Factor Receptor Superfamily (TNFRSF) ......................................... 26
  1.3.3.1 4-1BB ......................................................................................................................... 26
  1.3.3.2 CD27 .......................................................................................................................... 27

1.4. Renal Cell Carcinoma ......................................................................................................... 27
  1.4.1. Epidemiology ............................................................................................................... 27
  1.4.2. Histological subtypes ................................................................................................... 29
  1.4.3. Treatment of Renal Cell Cancer ................................................................................... 31
  1.4.3.1. Local Disease ............................................................................................................. 31
  1.4.3.2. Metastatic Disease ................................................................................................... 31
  1.4.3.2.1. Targeted Therapies in Metastatic RCC ................................................................. 31
  1.4.3.2.2. Immune Landscape in RCC and Role of Immunotherapy .................................. 33

2 MATERIALS and METHODS ................................................................................................. 38
  2.1 Flow Cytometry ................................................................................................................ 38
  2.2 Multiplex Immunofluorescence and Immunohistochemistry Staining and Image Analysis ................................................................................................................................. 44
  2.3 T Cell Receptor Sequencing ............................................................................................. 47
  2.4 Single Cell RNA Sequencing ............................................................................................ 49
3 CHARACTERISING THE IMMUNE LANDSCAPE IN TRACERx RENAL COHORT
3.1 CHAPTER INTRODUCTION
3.2 RESULTS
3.2.1 Main characteristics of the lymphocytes in TRACERx Renal Cohort
3.2.2 CD8 cells have a different phenotype in different tissue types
3.2.3 PD-1+ and PD-1- CD8 cell have different expression profiles
3.2.4 CD4 effector cells have different T cell subsets in different tissue types
3.2.5 PD-1+ and PD-1- CD4 effectors cells have different phenotypes
3.2.5.1 Phenotype of Tregs in TILs, normal tissue and PBMC
3.3 CHAPTER DISCUSSION
4 DECIPHERING THE EFFECT OF ANTI-PD-1 THERAPY IN RENAL CELL CARCINOMA: ADAPTeR
4.1 CHAPTER INTRODUCTION
4.2 RESULTS
4.2.1 Overview of the ADAPTeR Study
4.2.2 T cell infiltration at baseline does not predict response to anti-PD-1 treatment
4.2.3 Level of Granzyme B expression predicts response to anti-PD-1 treatment
4.2.4 TCR sequencing demonstrates maintained clonal expansion in responders
4.3 CHAPTER DISCUSSION
5 UNDERSTANDING THE EFFECT OF ANTI-PD-1 THERAPY ON PBMC IN RENAL CELL CARCINOMA
5.1 CHAPTER INTRODUCTION
5.2 RESULTS
5.2.1 CD8 T cell phenotypes pre- and post-anti-PD-1 therapy in the Peripheral blood
5.2.2 CD4 T cell phenotypes pre- and post-anti-PD-1 therapy in the peripheral blood
5.2.3 Myeloid subsets pre- and post-anti-PD-1 therapy in the peripheral blood
5.3 CHAPTER DISCUSSION
LIST OF FIGURES

FIGURE 1.1 HALLMARKS OF CANCER AND THERAPIES TARGETING THEM ...........................................16
FIGURE 1.2 STEPS INVOLVED IN THE CANCER-IMMUNITY CYCLE ................................................18
FIGURE 1.3 SUMMARY OF CANCER IMMUNOEDITING ....................................................................19
FIGURE 1.4 HISTOLOGY OF THE MOST COMMON RCC SUBTYPES ................................................30
FIGURE 1.5 TREATMENT PATHWAY FOR METASTATIC RCC PATIENTS .............................................37

FIGURE 2.1 GATING STRATEGY FOR FLOW CYTOMETRY ANALYSIS ...................................................49
FIGURE 2.2 BIOINFORMATICS PIPELINE FOR TCR SEQ ANALYSIS ...............................................49

FIGURE 3.1 COMPARISON OF T CELL SUBSETS IN TUMOUR, NORMAL TISSUE AND PBMC IN CCRCC. .................................................................................................................................55
FIGURE 3.2 COMPARISON OF CD8 T CELL PHENOTYPES IN TUMOUR, NORMAL TISSUE AND PBMC IN CCRCC. ........................................................................................................................58
FIGURE 3.3 CD8 CELLS HAVE DIFFERENT DIFFERENTIATION SUBSETS IN DIFFERENT TISSUE TYPES IN CCRCC. .............................................................................................................60
FIGURE 3.4 EXPRESSION PROFILE IN CD8 TEMRA AND TRM CELLS .................................................61
FIGURE 3.5 EXPRESSION PROFILE IN PD1+CD8 AND PD-1+CD8 CELLS .............................................64
FIGURE 3.6 COMPARISON OF EXPRESSION PROFILE IN PD1+CD8 AND PD-1+CD8 CELLS IN TILS ........................................................................................................................................65
FIGURE 3.7 COMPARISON OF EXPRESSION PROFILE IN PD1+CD8 AND PD-1+CD8 CELLS IN NORMAL TISSUE ................................................................................................................................66
FIGURE 3.8 COMPARISON OF EXPRESSION PROFILE IN PD1+CD8 AND PD-1+CD8 CELLS IN PBMC ........................................................................................................................................67
FIGURE 3.9 COMPARISON OF CO-EXPRESSION PROFILE IN CD8 CELLS IN TUMOUR, NORMAL TISSUE AND PBMC ........................................................................................................................................68
FIGURE 3.10 COMPARISON OF CD4 EFFECTOR DIFFERENTIATION SUBSETS IN DIFFERENT TISSUE TYPES .........................................................................................................................................70
FIGURE 3.11 COMPARISON OF THE EXPRESSION PATTERN ON CD4 EFFECTOR T CELLS IN TUMOUR, NORMAL TISSUE AND PBMC IN CCRCC. ........................................................................71
FIGURE 3.12 EXPRESSION PROFILE IN PD1+ CD4 EFFECTOR AND PD-1+ CD4 EFFECTOR CELLS ...............................................................................................................................................73
FIGURE 3.13 COMPARISON OF EXPRESSION PROFILE IN PD1+CD4 EFFECTOR AND PD-1+ CD4 EFFECTOR CELLS IN TILS ........................................................................................................74
FIGURE 3.14 COMPARISON OF EXPRESSION PROFILE IN PD1+CD4 EFFECTOR AND PD-1+ CD4 EFFECTOR CELLS IN NORMAL TISSUE ................................................................................75
FIGURE 3.15 COMPARISON OF EXPRESSION PROFILE IN PD1+CD4 EFFECTOR AND PD-1+ CD4 EFFECTOR CELLS IN PBMC ..........................................................................................................76
FIGURE 3.16 COMPARISON OF EXPRESSION PROFILE IN TREGS ................................................................78

FIGURE 4.1 WORKFLOW FOR MIF AND IHC ANALYSIS AND DETAILS OF THE SAMPLES USED. ..............................................................................................................................88
FIGURE 4.2 PATHOLOGY VIEW OF THE MARKERS USED FOR MIF STAINING. ........................................89
FIGURE 4.3 IMMUNE INFILTRATION AT BASELINE .................................................................................91
FIGURE 4.4 IMMUNE INFILTRATION ON TREATMENT ..........................................................................92
FIGURE 4.5 DENSITY OF T CELLS SUBSETS OUT OF TOTAL CELLS ....................................................93
FIGURE 4.6 PATHOLOGY VIEW FOR B CELLS, PLASMA CELLS AND PD-1+ CELLS ................................94
FIGURE 4.7 GRANZYME B EXPRESSION IN IMMUNE SUNSETS AT BASELINE AND ON TREATMENT .................................................................................................................................96
FIGURE 4.8 MHCII AND NFATC1 EXPRESSION ON T CELLS IS SHOWN ............................................97
FIGURE 4.9 TCR REPertoire SIMILARITY IN THE TUMOUR SAMPLES ...................................................99
FIGURE 4.10 TCR REPertoire CLONALITY IN THE TUMOUR AND PERIPHERAL BLOOD .......................100
FIGURE 4.11 FREQUENCY OF EXPANDED TCRS PRE AND POST TREATMENT ..................................101
FIGURE 4.12 CLUSTERING OF TCR CLONES IN RESPONDERS AND NON-RESPONDERS .............103
FIGURE 4.13 CLUSTERING EXAMPLES IN SPECIFIC PATIENTS ................................................................104
FIGURE 4.14 TCR REPertoire CLONALITY IN PERIPHERAL BLOOD ......................................................105
LIST OF TABLES

TABLE 2.1 T CELL PANEL 1 FOR TRACERX RENAL COHORT.............................................................39
TABLE 2.2 T CELL PANEL 2 FOR TRACERX RENAL COHORT..........................................................40
TABLE 2.3 T CELL PANEL 1 FOR ADAPTER COHORT.................................................................41
TABLE 2.4 T CELL PANEL 2 FOR THE ADAPTER COHORT.........................................................42
TABLE 2.5 MYELOID CELL PANEL FOR THE ADAPTER COHORT..................................................43
TABLE 2.6 IF STAINING DETAILS FOR THE ADAPTER COHORT..................................................46
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-1BB</td>
<td>CD137</td>
</tr>
<tr>
<td>ADAPTeR</td>
<td>A Study of Anti-PD-1 Therapy as Pre- and Post-Therapy in Metastatic RCC</td>
</tr>
<tr>
<td>ADC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Arg-1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>CCR4</td>
<td>C-C motif chemokine receptor 4</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C motif chemokine receptor 7</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDR3</td>
<td>Complementarity determining region 3</td>
</tr>
<tr>
<td>CPI</td>
<td>Checkpoint inhibitors</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated antigen 4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinases</td>
</tr>
<tr>
<td>EOMES</td>
<td>Eomesodermin</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>FDGF</td>
<td>Fibroblast derived growth factor receptor</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>Gal-9</td>
<td>Galalectin-9</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-Induced TNFR-Related protein</td>
</tr>
<tr>
<td>GzmB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hereditary papillary renal cell carcinoma</td>
</tr>
<tr>
<td>Hobit</td>
<td>Homolog of Blimp-1 in T cell</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible T-cell co-stimulator</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine-pyrrole 2,3-dioxygenase</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITH</td>
<td>Intratumour heterogeneity</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Killer cell lectin-like receptor subfamily G member 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid derived suppressor cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor associated T cell</td>
</tr>
<tr>
<td>NF-KB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>UMAP</td>
<td>Uniform manifold approximation and projection for dimension reduction</td>
</tr>
<tr>
<td>UMI</td>
<td>Unique molecular identifier</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-ADP ribose polymerase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death-1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed death-ligand 1</td>
</tr>
<tr>
<td>PD-L2</td>
<td>Programmed death-ligand 2</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SCRNASeq</td>
<td>Single cell RNA sequencing</td>
</tr>
<tr>
<td>SHP-2SHP-2</td>
<td>Src homology 2- domain containing tyrosine phosphatase 2</td>
</tr>
<tr>
<td>TCM</td>
<td>Central memory T cell</td>
</tr>
<tr>
<td>TEM</td>
<td>Effector memory T cell</td>
</tr>
<tr>
<td>TCF-7</td>
<td>Transcription factor 7</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TIGIT</td>
<td>T cell immunoglobulin and ITIM domain</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour-infiltrating lymphocyte</td>
</tr>
<tr>
<td>TIM-3</td>
<td>T cell immunoglobulin and mucin-domain containing-3</td>
</tr>
<tr>
<td>TLS</td>
<td>Tertiary lymphoid structure</td>
</tr>
<tr>
<td>TME</td>
<td>Tumour microenvironment</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNFRSF</td>
<td>Tumour necrosis factor receptor superfamily</td>
</tr>
<tr>
<td>TRACERx</td>
<td>TRAcking Cancer Evolution through therapy</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TSCM</td>
<td>Stem central memory T cell</td>
</tr>
<tr>
<td>TOX</td>
<td>Thymocyte selection-associated high mobility group box protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
1. BACKGROUND
   1.1. Immune System in Homeostasis

   The immune system plays a critical role in maintaining the homeostatic state in the body by protecting it against pathogens including microbes, viruses and tumours. A balance between an overactive immune system and an underactive immune system is key in protecting the body against autoimmune disorders and infections. The immune system maintains this balance and carries out its key through both the innate immune and the adaptive immune systems. Charles Janeway played an important role in defining how these two systems interact with each other. Innate immunity is the first line of defence in detecting invading pathogens. Innate immune cells such as dendritic cells (DCs), macrophages, neutrophils and natural killer (NK) cells express pattern recognition receptors (PRRs) which are encoded in the germline DNA. These receptors bind the pathogen associated molecular patterns (PAMPs) expressed on the pathogens which allows the innate immune system to recognise the pathogens.\(^1\)\(^2\) Once the PRRs on the innate immune cells recognise and bind the PAMPs, the innate immune system gets activated. PRRs can be transmembrane (i.e. Toll-Like Receptors – TLRs and C-type lectins) and cytosolic (retinoic acid inducible gene -1 like receptors- RLRs, nucleotide-binding and leucine rich repeat containing receptors (NLRs).\(^3\) This information is then communicated to the adaptive immune system via antigen presentation mechanisms. The antigens presenting cells (APCs) present the antigen associated peptide to T cells. More specifically, once the PRR-PAMPs axis is activated, DCs present peptides from the antigen on major histocompatibility complex (MHC) I and MHCII to CD4 T cells and CD8 T cells respectively as well as upregulating co-stimulatory molecules such as CD80/86 which bind to CD28 on the T cells. This process leads to activation of the TCRs which are key players of the adaptive immune system. Another important part of the communication between the innate and the adaptive immune system is the secretion of cytokines such as interleukin(IL)-6, IL-12 and tumour necrosis factor (TNF)-\(\alpha\) which are important in the activation and differentiation of naïve T cells.\(^4\) This process creates a memory in the immune system which allows for recognition of the antigen if the immune system re-encounters it.

   T cells are key players of the immune system in haemostasis and in disease and this thesis specifically focuses on T cell differentiation, function, and regulation. T cells are critical in mediating the adaptive immune system which generates a more targeted response specific for the antigen compared to the initial first line of defence generated by the innate immune system. T cells go through maturation in the thymus. During this
process, T cells differentiate into CD4 and CD8 T cells. CD4 cells act as helper T cells that help with stabilising the interaction with the APCs. On the other hand, CD4 cells that express CD25 and produce TGF-β and IL019 are called regulatory T cells and they are important inhibiting immune activation. During the maturation process, T cells express unique TCRs which are antigen specific. Mature T cells which have never encountered an antigen are called native T cells and in an adult human, there are up to 100 million different clones. Naïve T cells then go through homing which involves them migrating to the secondary lymphoid organs where the antigen presentation process takes place. Several adhesion molecules and chemokines play a role in T cell homing. Selectins and integrins are important adhesion molecules that help T cell with tethering to the endothelial cells and slow down the rolling of T cells in the blood stream. Integrins require activation via the chemokines where are the selectins do not need activation. Chemokines are peptides that are secreted to guide homing of the T cells and they transmit their signals via G coupled proteins. After the homing process, when naïve CD4 and CD8 T cells encounter an antigen, they go through an activation process via engagement of the TCRs and gain an effector function. IL-12 secreted by the APCs differentiate CD4 cells into Th1 CD4 cells which secrete IFN-γ and IL-2. On the other hand, IL-4 differentiates CD4 Th2 cells which produce IL-4, IL-5, IL-9 and IL-13. In CD8 T cells, engagement of the TCR with the antigen and stimulation of the CD28 co-receptor leads to activation of the PI3 kinase pathway which leads to promoted T cell survival and activates target cell killing machinery. In specific, activated CD8 T cells which now have a cytotoxic function produce granules such as granzyme, perforin and granulysin which get released into the target cell and lead to cell death.

1.2. Cancer and Immune Escape

The mechanisms involved in oncogenesis have previously been categorized into 6 main factors by Hanahan and Weinberg. Cancer cells gain the ability to proliferate without being dependent on external growth signalling. Instead, they produce growth factors and integrins that facilitate cell growth and proliferation. The SOS-Ras-Raf-MAP kinase pathway plays an important role in this. In addition, cancer cells become desensitised to antigrowth signals which normally prevent the cells from proliferating. One of the other unique features of the cancer cells is their ability to evade apoptosis which allows them to survive longer. As a result, the cancer cells gain an unlimited replication potential. In order to maintain their oxygen needs, cancer cells then start an angiogenesis process via upregulating the vascular endothelial growth factors and fibroblast growth factors. In
cancer cells, E-cadherin and cell-to-cell adhesion molecules go through modifications and lose their function. This allows the cancer cells to metastasise to other sites. In addition, genomic instability resulting from chromosomal abnormalities, mutations in tumour suppressor genes (i.e. \( p53 \)) and DNA repair enzymes also plays an important role in oncogenesis. More recently, it has also been suggested that a pro-inflammatory microenvironment, especially the innate immune cells, also has a role in activating the oncogenic pathways. In addition, once the oncogenesis process starts, the ability of the tumour cells to evade the immune system via mechanism described later on this chapter allows the cancer cells to survive and form a tumour. Metabolism is another key factor that contributes to oncogenesis. The Warburg effect which refers to the cancer cells entering glycolysis under pseudohypoxic conditions is another pathway which is important in survival of cancer cells. Over time, specific therapies have been developed to target different factors that contribute to oncogenesis. A summary of these is shown in Figure 1.1.  

Figure 1.1 Hallmarks of cancer and therapies targeting them  
(Taken from Hanahan and Weinberg, 2011) The figure shows the hallmarks of cancer and the therapies developed to date to target these hallmarks.
The relationship between cancer and the immune system was first identified in the early 20th century by Paul Ehrlich who stated that the immune system played an important role in protecting the organisms from developing cancer.\textsuperscript{13} Several years later, Burnet and Thomas introduced the concept of immunosurveillance in cancer.\textsuperscript{14} This hypothesis was proven initially in the animal models in the early 2000s. In 2001, Shankaran and colleagues showed that mice deficient in recombination activating gene -2 (RAG-2) which is specific to lymphocytes or the interferon(IFN)-$\gamma$ receptor, developed sarcomas following injection of carcinogenic methycholanthrene. This demonstrated the important role of the immune system in preventing carcinogenesis.\textsuperscript{15} A similar pattern was also reported in humans where immunocompromised patients were found to have higher incidence of cancer.\textsuperscript{16} These findings demonstrated and highlighted the importance of immunosurveillance in cancer.

Cancer cells are recognised and killed by the immune system over several steps of what is referred to as the cancer-immunity cycle. (Figure 1.2). The process starts with proteins with mutated structures that are produced by cancer cells called the neoantigens, which are subsequently recognised by DCs. Following this step, presence of inhibitory or stimulatory signals determine whether a T cell response will be mounted by the immune system. This step is followed by priming of effector T cells when the DCs present the neoantigens to T cells on MHCI and MHCII. Once the T cells are activated following the antigen presentation process, they migrate to the tumour site and infiltrate the tumour. In the tumour microenvironment, T cells bind the tumour cells via the TCR which leads to death of the tumour cells.\textsuperscript{17}
Figure 1.2 Steps involved in the cancer-immunity cycle

(Taken from Chen and Mellman 2013). The diagram shows the stages involved in the cancer-immunity cycle as well as the inhibitory and stimulatory molecules. The inhibitory molecules prevent immune activity while the stimulatory molecules enhance the immune activity.

However, immunocompetent animals and humans also develop cancer suggesting the immunosurveillance mechanisms do not always work and the tumour escapes the immune system through various pathways. This communication between the host immune system and the tumour where they modify and shape each other is called immunoeediting. The cancer immune editing model has three phases: elimination, equilibrium and escape. In the elimination phase, the developing tumour is recognised by innate immune cells that leads to production of IFN-γ and chemokines with subsequent recruitment of NK cells and T cells to tumour microenvironment and death of the tumour cells. In the equilibrium phase, some of the tumour cells survive the elimination phase and stay dormant. The adaptive immune system plays an important role in equilibrium during which it is able to prevent the tumour cells from
growing but not able to clear them. In escape phase, the tumour cells continue to grow despite the presence of immune cells in the tumour microenvironment. The mechanisms of escape are complex and can be due multiple reasons such as the reduction in the tumour antigens, dysfunction of the antigen presenting machinery, loss of MHC proteins and the immunosuppressive effects of the tumour on the immune system (Figure 1.3).¹⁸,¹⁹

Figure 1.3 Summary of cancer immunoediting

(Taken from Desai et al., 2022).²⁰ The figure shows the key steps involved in cancer immunoediting including elimination, equilibrium and immune escape steps. The purpose of immunotherapy is to revert the cancer cells to elimination phase. Different modalities of therapy are used for different degrees of immunogenicity of tumours (hot to hold).
1.3. Immunotherapy in Cancer

Given that immune escape plays an important role in cancer progression, modifying the immune system to recognize and react to tumour cells has been an important aim in the oncology field with immunotherapy having revolutionised the anti-cancer therapeutic approach. Although immunotherapy for cancer is a hot topic at the moment, the first immunotherapy dates back to the late 19th century when Coley used a bacteria toxin to evoke an immune response to treat sarcoma.21 More recently, an improved understanding of the immune regulation has led to advances in cancer immunotherapy. Currently immunotherapies mainly target T cells due to the data demonstrating the role of tumour reactive T cells in tumour control.22 Checkpoint molecules have been identified on T cells and this has been instrumental in developing immune checkpoint blockade (ICB) to treat various cancers (Figure 1.4). The checkpoints consist of several members and each are potential targets for therapeutic antibodies.
Figure 1.4 Co-stimulatory and co-inhibitory receptors on T cells. (Taken from Pardoll, 2012). The figure shows the co-inhibitory (marked with ‘-’ sign) and co-stimulatory receptors (marked with ‘+’ sign) on T cells and associated ligands on the antigen presenting cells are shown in the figure.
1.3.1 Immunoglobulin Superfamily – CD28 family

1.3.1.1 Cytotoxic T Lymphocyte-Associated antigen 4 (CTLA-4)

One of the main milestones in the field was the discovery of CTLA-4 which is a protein that belongs to the immunoglobulin family. It consists of 223 amino acids and is a type 1 transmembrane glycoprotein with a V-like domain. CTLA-4 has a similar structure to CD28 which has a co-stimulatory function in T cells. In addition, similar to CD28, CTLA-4 also binds to CD80 and CD86 ligands which leads to secretion of IL-2 which is important in T cell differentiation and programmed cell death. However, CTLA-4 has much higher affinity for CD80 and CD86 which means that CTLA-4 prevents CD28 from activating T cells by competing with CD28. CD28 activates T cells when the TCR recognises and binds an antigen. However, the interaction between CTLA-4 and CD80/CD86 is greater following TCR engagement. Therefore, CTLA-4 blocks T cell activation by preventing CD28 causing T cell activation. CTLA-4 is mainly expressed in activated CD8 T cells, CD4 effectors and regulatory T cells. Walunas and colleagues showed that CTLA-4 expression was upregulated in activated T cells and it played an important role in downregulating the activation of T cells, mainly by inhibiting the T cell TCR transduction. Allison and colleagues showed that blocking CTLA-4 lead to tumor activity in immunogenic tumours such as B7-colon cancer and fibrosarcoma mouse models which lead to a Nobel Prize in 2018. The same group also demonstrated that combining anti-CTLA-4 treatment with a GM-CSF producing vaccine in poorly immunogenic B16-BL6 melanoma mouse models lead to rejection of primary and metastatic tumors in and CD8 T cell and NK1.1 cell dependent manner. Later on, it was demonstrated that blocking CTLA-4 led to rejection of tumours in mouse models and had anti-tumour effects in different cancers. This has revolutionised treatment of cancer, initially in melanoma where giving ipilimumab (a humanised IgG1 anti-CTLA-4 monoclonal antibody) led to improved overall survival in stage III and IV melanoma.

CTLA-4 blockade can also reduce Treg function contributing to increased effector T cell response either by its blocking activity or by direct depletion of Tregs. In mouse models, it has been demonstrated that anti-CTLA-4 antibodies increase the CD4 effector to Treg ratio and directly kill Tregs via antibody dependent cell cytotoxicity (ADCC). Most recently, data both from mouse models and humans studies showed that anti-CTLA-4 antibodies play a key role in Treg depletion and Fcγ receptors are instrumental for this. In mouse models and human melanoma samples, we demonstrated that baseline TIL infiltration and Fcγ receptor polymorphisms (CD16a-V158F human melanoma samples) associated with response to anti-CTLA4 antibodies. In addition,
increased mutation burden in human melanoma samples which is also associated with higher TIL infiltration was also associated with better clinical outcome to anti-CTLA-4 treatment.\textsuperscript{40} Although our data suggests that anti-CTLA-4 antibodies perform their function by impairing the Treg function, conflicting studies still challenge the role of Treg depletion in the activity of anti CTLa4 antibodies in humans.\textsuperscript{41} This is an important question that needs to be answered and understood at greater depth in order to unveil the mechanism of action of anti-CTLA-4 antibodies including ipilimumab.

1.3.1.2 PD-1

Another important milestone in cancer immunotherapy is the discovery of programmed-death 1 (PD-1) which is another co-inhibitory molecule expressed on activated T cells and its ligand PD-L1.\textsuperscript{42,43} PD-1 gene was discovered by Ishida and his colleagues in 1992 and they showed that activation of this gene lead to programmed cell death in activated mouse 2B4.11 T cells.\textsuperscript{42} PD-1 belongs to the type I transmembrane glycoprotein family and is 55 kDa in size. It has three main domains: Ig variable type extracellular domain, transmembrane domain and the cytoplasmic tail which plays an important part in PD-1 signalling. The cytoplasmic tail consists of immunoreceptor tyrosine based inhibitory motif (ITIM) and immunoreceptor tyrosine based switch motif (ITSM). In contrast to the other CD28 family members such as CTLA-4 and ICOS, the Ig V-type domain of PD-1 has only one interchain disulphide.\textsuperscript{44} PD-1 protein has two ligands, PD-L1 and PD-L2. PD-L1 which is coded by the \textit{PD-L1} gene is located on chromosome 9. It has IgV and and IgC domains on its extracellular aspect and is expressed by antigen presenting cells.\textsuperscript{43,45} PD-1 and PD-L2 are expressed on tumour cells as well as on myeloid cells. In the clinical setting, expression of PD-L1 on tumour cells has been used as a prognostic factor in some cancers.\textsuperscript{23} PD-1 is found on the activated T cell surface as well as other immune cells such as B cells, NK cells, macrophages and DCs. Interaction between PD-1 with its ligands PD-L1 or PD-L2 leads to dephosphorylation of Zap 70 on the T cell receptor via using the tyrosine phosphatase on src homology 2- domain containing tyrosine phosphatase 2 (SHP-2SHP-2).\textsuperscript{46} This inhibits activation of T cells via preventing phosphorylation of Akt and Ras by inhibiting PI3K and MEK/ERK pathways.\textsuperscript{47} PD-1 expression has an important role in down regulating the effector function of T cells by downregulating the expression of transcription factors such as EOMES, TBET and GATA-3 as well as reduced cytokine production.\textsuperscript{48}
Mouse studies following the discovery of PD-1 showed that PD-1 knockout mice had splenomegaly and B cells had increased proliferation although this was not observed in T cells.\(^4^9\) In addition, PD-1 knockout mice also developed autoimmune conditions such as lupus and nephritis and accumulation of IgG3 which suggest that PD-1 plays an important role in inhibiting immune response and maintaining self tolerance.\(^5^0\) In humans, high levels of PD-1 expression was seen in CD8 and CD4 T cells in chronic HIV patients and this was associated with poor clinical outcomes.\(^5^1\) PD-1 is also expressed in high levels in tumour infiltrating lymphocytes (TILs) compared to the T cells in normal tissue and peripheral blood. This increased PD-1 expression was associated with reduced cytokine production and effector function which suggests an exhausted/dysfunctional T cell state.\(^5^2\) Antibodies blocking PD-1 and PDL-1 as well as combination therapies with anti-CTLA-4 and anti-PD-1/PD-L1 antibodies have been shown to have significant anti-tumour effects and led to clinical benefit in multiple tumour types with different stages.\(^5^3\)–\(^5^8\)

1.3.1.3 Inducible T cell Co-stimulatory Inhibitor (ICOS)

ICOS was identified by Hutloff and colleagues in 1999 as a co-stimulatory molecule of the CD28 family. It is a homodimeric protein expressed on the cell surface and is 55-60kDa in size.\(^5^9\) It has FDPPPF motif which allows ICOS to bind to its ligand B7-H2. ICOS ligand B7-H2 has an important role in T cell activation and is also bound by CD28 and CTLA-4. However, ICOS has a much higher affinity for it compared to CD28 and CTLA-4. ICOS also plays an important role in T cell development and cytokine production. Via its YMFM SH2 binding motif, ICOS binds to PI3K which leads to Akt signalling. This then results in production of important cytokines such as IL-4, IL-10 and IL-21 which are important in CD4 T helper 2 (CD4 T\(_{H2}\)) cells and T follicular helper cells (T\(_{FH}\)) cells. ICOS is also important in proliferation and survival of Tregs.\(^6^0\) Although ICOS is expressed in low levels in naïve T cells, TCR engagement leads to a significant increase in ICOS expression. Src kinase Fyn and MAP kinase ERK play an important role in transcriptional regulation of ICOS expression following T cell activation. NFATc2 phosphorylation and nuclear translocation takes place when the phosphatase calcineurin gets activated by Fyn. Binding of NFATc2 and ERK to the ICOS promoter in the nucleus leads to activation of the promoter and ICOS expression.\(^6^1\) In addition to its role in T cell maturation, ICOS is also plays a role in B cell maturation and proliferation via CD40 signalling.\(^6^2\) Mouse studies showed that ICOS\(^-/-\) T helper cells did not get activated following aCD3 stimulation. In addition, ICOS\(^-/-\) mice had impaired T cell effector function as well as reduced IL-4 and IL-13 cytokine production.\(^6^3\) In addition, it
was seen that anti-CTLA-4 treatment in B16/B6 melanoma mouse models leads to an increase in ICOS expression in CD8 and CD4 effector cells. In light of these results, B16/B6 mice with melanoma were given ICOS ligand expressing IVAX vaccine in combination with the anti-CTLA-4 antibody which lead to a synergistic effect in tumour rejection which highlights the potential role of ICOS in combination immunotherapy. The first Phase 1 clinical trial using an agonistic ICOS antibody alone and in combination with anti-PD-1 therapy showed a good safety profile and promising anti-tumour activity. Currently, there are multiple clinical trials running with ICOS agonistic and antagonistic antibodies.

1.3.2 Immunoglobulin Superfamily – TIM family
1.3.2.1 T Cell Immunoglobulin and Mucin Domain 3 (TIM-3)

TIM-3 is a type 1 transmembrane protein and is a member of the TIM family. Similar to other members of the TIM family, it has a variable immunoglobulin domain with four cysteines, an extracellular glycosylated mucin domain and a single transmembrane domain. The cysteine residues form two disulphide bridges which form a binding domain for TIM-3 ligands. There are four main ligands identified for TIM-3 to date: galectin-9 (Gal-9), phosphatidylserine (PtdSer), high mobility group protein B1 (HMGB1), and carcinoembryonic antigen cell adhesion molecule 1 (Ceacam1). In the absence of a ligand, Bat-3 binds the tail of TIM-3 and forms an intracellular complex with Lck which allows for TCR signalling and prevents TIM-3 mediated apoptosis. When the TIM-3 ligands bind the tail of TIM-3, they lead to phosphorylation of Tyrosine 256 and Tyrosine 263 residues. This process removes Bat-3 from the tail and allows it to bind to SH2 domain of the Src kinases such as Fyn and Lck. This in turn leads to inhibition of TCR signalling.

TIM-3 is mainly expressed as an inhibitory receptor on cytotoxic CD8 cells, Th1 CD4 cells and Tregs. It is also expressed in myeloid cells such as macrophages, DC and NK cells. It plays an important function in inhibiting production of IFN-γ, TNF-α and IL-12 production by T cells which are important for effector T cell function and it was shown to be important in maintaining peripheral self tolerance. In humans, an increased expression of TIM-3 was seen in CD8 cells in chronic HIV patients who progressed. These CD8 cells were found to be in a more exhausted/dysfunctional state with reduced ability to produce cytokines and proliferate. In addition, TIM-3 expression on T cells has also been associated with poor clinical outcomes in various cancers including non-small cell lung cancer (NSCLC), colon cancer and cervical cancer. Therefore, TIM-3 is
potentially good immunotherapy target in cancer with several TIM-3 antibodies in trial at the moment.\textsuperscript{71} Phase 1a/b clinical trial with anti-TIM-3 antibody alone and in combination with an anti-PDL-1 antibody in advanced and relapsed solid tumours showed a good safety profile as well as some anti-tumour activity.\textsuperscript{72}

1.3.3 Tumour Necrosis Factor Receptor Superfamily (TNFRSF)

1.3.3.1 4-1BB

4-1BB is receptor that is expressed on the surface of T cells. 4-1BB was initially identified in mice but the human homologue has a similar structure. 4-1BB is expressed in several immune cells including T cells, NK cells while 4-1BB ligand (4-1BBL) is expressed in macrophages, DCs and B cells. It plays an important role in CD8 T cell activation. It has a ligand called 4-1BB-L which is expressed on APCs and binds to 4-1BB with high affinity. 4-1BB signalling leads to activation and proliferation of T cells both with and without CD28 involvement. The TNF receptor associated factors (TRAFs) such as TRAF1, TRAF2 and TRAF3 are required for signalling when 4-1BB binds 4-1BBL. This process consequently upregulates expression of genes involved in cell survival and downregulates apoptotic genes as a result of activation of certain pathways such as MAPK, ERK, NF-κB and AKT. Activation of 4-1BB-4-1BBL interaction in T cells leads to production of pro-inflammatory cytokines and enhances cytotoxic function of CD8 cells via TCR signalling.\textsuperscript{73,74}

Pre-clinical studies in mice showed that 4-1BB is also important in anti-tumour immunity. Treating poorly immunogenic Ag104 sarcoma and P815 mastocytoma mice with an agonistic monoclonal anti-4-1BB antibody led to rejection of tumours in mice as well as an increased cytotoxic activity in CD8 and CD4 T cell.\textsuperscript{75} Following the promising efficacy of anti-4-1BB treatment in mice, several clinical trials have been conducted to test the efficacy of agonistic anti-4-1BB antibodies in solid tumours. A phase 1 clinical trial looking at the safety of urelumab, an agonistic 4-1BB antibody, showed significant adverse events and toxicity, especially hepatotoxicity in patients treated with ≥1 mg/kg. However, 0.1 mg/kg every 3 weeks was shown to be safe.\textsuperscript{76} Another agonistic anti-4-1BB monoclonal antibody is utomilumab. Phase 1 study with utomilumab monotherapy in advanced cancer showed a safe toxicity profile and evidence of anti-tumour activity.\textsuperscript{77} There are other clinical trials looking at different agonistic 4-1BB antibodies in solid tumours at the moment.\textsuperscript{78}
1.3.3.2 CD27

CD27 is a co-stimulatory receptor that belongs to the TNFRSF family. It is a type 1 transmembrane protein with cysteine rich domain which play an important role in forming three disulphide bridges. CD27 signalling mainly happens via TRAF2,3, and 5 which leads to activation of NF-κB pathways. CD27 is mainly expressed in resting CD4 cells, Tregs and CD8 cells as well as NK cells and B cells. Stem-cell memory and central memory cells have high levels of CD27 while effector T cells downregulate CD27 expression. In addition, majority of the effector memory cells express CD27 but the opposite is observed in effector memory cells expressing CD45RA. The ligand for CD27 is CD70. Binding of CD27 to its ligand leads to expansion and differentiation of T cells as well as cytokine production. In CD27−/− cells, it was observed that the CD4 memory T cell function was impaired in addition to a reduced influenza virus specific CD8 T cell count. Varilumab is a human immunoglobulin G1 kappa agonistic anti-CD27 monoclonal antibody. It was used tested in transgenic colorectal, thymoma and lymphoma mouse models. The results showed that varilumab lead to reduction in tumour size as well as activation of CD4 and CD8 cells. Specifically, proliferation of CD8 cells and IFN-γ production was observed. Varilumab was the first therapeutic anti-CD27 antibody tested in clinic in melanoma and renal cell carcinoma patients. The phase 1 trial showed tolerable safety profile. Clinical trials with varilumab and nivolumab combination therapy are still ongoing.

1.4. Renal Cell Carcinoma
1.4.1. Epidemiology

Renal cell cancer (RCC) is the 9th most common tumour worldwide and there are over 300,000 new cases every year. Incidence of RCC is different in different parts of the world. The highest numbers are seen in Europe, North America and Australia. The lowest incidence is in Africa although North Americans with African origin have a high incidence of RCC. RCC is most commonly seen in males with only a third of the cases seen in females. The incidence of the disease increases with age with a mean age of 64 at diagnosis. The mortality from RCC is approximately 175,000 globally which accounts for 1.8% of all cancer related deaths world-wide. The survival rate from RCC depends on the stage of the disease with a spectrum of 95% 5 year survival rate in stage 1 disease and 12% 5 year survival rate in stage 4 disease.

There are several modifiable risk factors associated with RCC. An important one of these factors is smoking. Smoking as well as higher pack years and duration of smoking
are associated with advanced RCC. In addition, active smokers are also found to have more advanced disease with worse prognosis compared to ex-smokers.\textsuperscript{86} Although the exact mechanism is not clear, obesity is another independent risk factor of RCC. The risk is particularly increased for developing clear cell and chromophobe subtypes.\textsuperscript{85} Hypertension is another important risk factor for RCC. In a study performed by the European Prospective Investigation into Cancer and Nutrition (EPIC) in a larger cohort of 296638 individuals, it was seen that hypertension was associated with increased risk of developing RCC whereas there was no association with anti-hypertensive medication use and RCC.\textsuperscript{87}

Inherited genetic factors form another important risk category for RCC. Van Hippel Lindau (VHL) is the one the inherited syndromes which occurs due to the germline mutation in the \textit{VHL gene} which is found on chromosome 3p25. Loss of VHL function prevents ubiquitination and degradation of the transcription factor hypoxia inducible factors, HIF-1α and HIF-2α. This leads to stabilisation of HIF and up regulation of the molecules such as vascular endothelial growth factor (VEGF) pathway.\textsuperscript{88} It is inherited in an autosomal dominant manner and is seen in 1 in 35,000 people. Patients with the condition develop multiple renal cysts or clear cell renal cell carcinoma (cRCC), phaeochromocytoma, haemangioblastomas, retinal angiomas and pancreatic islet tumours which can start developing in early adulthood.\textsuperscript{89} Hereditary papillary renal cell carcinoma (HPRC) is another syndrome which is inherited in an autosomal dominant way and leads to development of multifocal, bilateral type 1 papillary renal cancer. Gain of function mutations in the \textit{MET} oncogene is associated with HPRC. Kidney is the only organ affected in this syndrome and patients usually develop the papillary renal cancer in their 50s or 60s.\textsuperscript{90} The third inherited genetic syndrome is Birt-Hogg-Dube Syndrome which is caused by the somatic mutations in the \textit{FLCN} gene which is on chromosome 17p11.2. It is inherited in an autosomal dominant way and the clinical features include spontaneous pneumothorax, renal cell carcinoma, pulmonary cysts and fibrofolliculoma.\textsuperscript{91} Hereditary leiomyomatosis renal cell cancer (HLRCC) is the syndrome caused by germline mutations in the \textit{FH} gene which codes for the fumarate hydratase enzyme in Krebs cycle. Mutations in this gene leads to accumulation of the fumarate enzyme which is normally converted to malate by fumarate hydratase. High levels of fumarate inhibit the hypoxia inducible factor (HIF) propyl hydroxylase enzymes and lead to pseudohypoxia and expression of HIFs. It’s an autosomal recessive disease and patients develop skin and uterine leiomyomas and type 2 papillary renal cell cancer.\textsuperscript{90,92}

Recent advances in the human genomic studies have allowed for a better understanding of the genomic aetiology of RCC. In a detailed study where whole exome
sequencing (WES) was performed in 95 biopsies from 33 ccRCC patients, it was seen that the chromosome 3p loss in childhood or adolescence is the first trigger for developing ccRCC although the cancer may not develop until late adulthood. In addition, activation of the second VHL allele and mutations in the PBRM1, BAP1, SETD2 and TERT genes have also been identified as driver for developing ccRCC.93 Given the important role of clonal evolution and intratumoural heterogeneity in cancer,94,95 WES and whole genome sequencing (WGS) were performed on 1206 primary tumour samples from 101 ccRCC patients using multiregional sampling. A degree of intratumour heterogeneity (ITH) with different combinations of driver events leading to clonal sweep was observed. In addition, more than 10 subclonal drivers were associated with rapid progression of tumours. Tumours with high ITH had a lower rate of disease progression whereas ccRCC tumours with low ITH and high somatic copy number alterations had more rapid disease progression.96 Genomic analysis of 335 metastasis from 100 patients with ccRCC showed that the 9q and 14q loss are key events that drive tumour metastasis in ccRCC.97

1.4.2. Histological subtypes

Renal cancer has several different histological subtypes. The most common is clear cell carcinoma accounting for approximately 70% of the cases. One of the common features of this subtype is that the cytoplasm of the tumour cells have clear appearance although an eosinophilic cytoplasm can also be seen. It usually originates from the proximal convoluted tubules and is vascular nature. It can be associated with VHL mutations. The second most common histological subtype which make up approximately 10% of the cases is papillary renal carcinoma. The cells usually have a spindle shape appearance. Macroscopically, the tumour can have large cystic and haemorrhagic areas. Papillary renal cancer has two different subtypes: type 1 and type 2. The most distinguishing feature of type 1 papillary renal cancer is the presence of basophilic cells in the basal layer. On the other hand, type 2 papillary cancer the papillae have an eosinophilic cytoplasm. In addition to the differences at a cellular level, type and type 2 papillary renal cancer also have different prognostic trajectories where type 1 papillary cancer is typically lower grade and gets diagnosed at an earlier stage. On the other hand, type 2 papillary renal cancer is usually high grade, has venous invasion and can be bilateral. Chromophobe RCC is another histological subtype which makes 5% of the RCC cases. It is a less aggressive form with better prognosis compared to ccRCC and metastasis is rare. The typical histological features include a reticulated cytoplasm of large tumour cells. Collecting duct carcinoma which is also known as Bellini duct carcinoma is a very rare form of renal cancer (seen in <1% of the cases) but it is extremely aggressive with very
poor prognosis. Desmoplasia around the collecting duct wall is a typical feature of collecting carcinomas. Another very rare form of renal cancer which is seen in younger patients is medullary renal cell carcinoma. It originates from the collecting duct cells and is very aggressive (Figure 1.5).\textsuperscript{98,99} Fuhrman’s histological grading is used as to predict the prognosis of renal cancer.\textsuperscript{100}

![Image of histology of the most common RCC subtypes](image)

**Figure 1.5 Histology of the most common RCC subtypes**

(Taken from Muglia, V and Prando, A, 2015).\textsuperscript{99} The figure shows the microscopic appearance of different histological subtypes of RCC. (A) Clear cell RCC (B) Type 1 Papillary RCC (C) Type 2 Papillary RCC (D) Chromophobe RCC (E) Collecting duct RCC (F) Medullary RCC

In addition to the staging and grading system, International Metastatic RCC Database Consortium (IMDC) prognostic model is also used to predict the survival of patients. IMDC criteria takes into account the following risk factors: anaemia, thrombocytosis, neutrophilia, hypercalcaemia, Karnofsky performance status <80%, and <1 year from diagnosis to treatment. Patients with none of these factors are classified as favourable risk factor patients while patients with one to two risk factors are intermediate risk patients and patients with more than 3 risk factors are in the poor risk category.\textsuperscript{101} Prior to the IMDC risk scoring, the Memorial Sloan Kettering Cancer Centre (MSKCC) risk criteria was developed which took into account Karnofsky performance status (<80%), absence of prior nephrectomy, low haemoglobin, elevated lactate dehydrogenase levels (more than 1.5 times upper limit of normal and raised “corrected” serum calcium (< 10 mg/dL)). Patients with none of these factors were categorised as the ‘favourable risk’ group. Patients with
one or two risk factors were classified as ‘intermediate risk’ and patients with more than two risk factors were in the ‘poor risk’ group. Analysis of data from 670 patients with advanced RCC showed that the three year survival in the favourable risk group was 31% followed by 7% and 0% in the intermediate and poor risk categories respectively.\textsuperscript{102}

1.4.3. Treatment of Renal Cell Cancer
   1.4.3.1. Local Disease

   Management of renal cancer depends on the stage of the cancer. Small renal masses (SRMs) which are smaller than 4 cm (Stage 1a) are usually found in older patients and up to 20% of them are benign. These masses are usually managed by active surveillance if the risk of the surgery outweighs the benefits as they tend to grow very slow and rarely metastasize. Stage 1b tumour which are 4-7 cm in size are usually treated with partial nephrectomy which allows for removing the entire tumour while preserving the kidney. If partial nephrectomy is technically challenging, then patients go through a radical nephrectomy.\textsuperscript{103}

1.4.3.2. Metastatic Disease
   1.4.3.2.1. Targeted Therapies in Metastatic RCC

   Treatment for metastatic RCC is different with different treatment options compared to local disease and has changed significantly over the years. Cytokine therapies including interferon alfa-2a and high dose interleukin-2 (IL-2) were early therapies approved for treatment of metastatic RCC. Durable response was observed in 7-10% of metastatic RCC patients treated with high dose IL-2, lending support to the idea that immunotherapies could be an important element of systemic therapy for RCC.\textsuperscript{104,105} However, significant toxicity including mortality due to treatment lead to discontinuation of high dose IL-2 therapy in RCC.\textsuperscript{106}

   Loss of function mutations in the von Hippel Lindau (\textit{VHL}) gene play a key role in development of ccRCC. ccRCC is the most common type of RCC and biallelic VHL inactivation is seen in up to 80% of patients. This leads to increased HIF expression which consequently leads to upregulation of the vascular endothelial growth factor (VEGF) pathway. The mammalian target of rapamycin (mTOR) pathway is also up regulated in a subset of ccRCC patients.\textsuperscript{107} Identification of these pathways lead to development of targeted therapies to treat metastatic renal cell carcinoma. CALGB 90206 is a phase 3
clinical trial with 732 patients which compared the anti-VEGF monoclonal antibody in combination with IFN-α versus IFN-α as first line therapy in metastatic ccRCC patients. The results showed that the combination therapy lead to better overall survival (OS) compared to IFN-α therapy alone (18.3 months versus 17.4 months) and the hazard ratio was 0.86.\textsuperscript{108} Sorafenib which is a multikinase inhibitor targeting VEGF pathway was compared with placebo as a first line treatment for advanced ccRCC. In this randomised, placebo controlled trial 903 patients were enrolled and the results showed that the median progression free survival (PFS) was better in the Sorafenib arm (5.5 months) compared to the placebo arm (2.0 months). However, overall median OS was inconclusive.\textsuperscript{109} In addition to the VEGF inhibitors, mTOR inhibitors were also used in metastatic RCC. A randomised control phase 3 study compared combination therapy with mTOR inhibitor temsorilimus and IFN-α versus IFN-α alone versus temsorilimus therapy alone in poor risk metastatic RCC patients. The overall survival in the temsorilimus alone group was better compared to the combination therapy group (10.9 versus 8.4 months). Temsorilimus also had a better toxicity profile compared to IFN-α.\textsuperscript{110} Another mTOR inhibitor used in metastatic RCC is everolimus. In patients with metastatic RCC who progressed on VEGF inhibitors, using second line everolimus lead to better median PFS in the everolimus arm (4.9 months) compared to the placebo arm (1.9 months) with Hazard Ratio of 0.33.\textsuperscript{111} A key drug in treatment of metastatic RCC has been Sunitinib which inhibits multiple kinases including VEGF receptor, platelet derived growth factor receptor (PDGFR), KIT, RET, CSF-1R and flt3. In a randomised phase 3 clinical trial with treatment naïve 750 metastatic RCC patients, it was seen that the sunitinib treatment alone had better overall survival compared to IFN-α treatment (26.4 versus 21.8 months) with a Hazard Ratio of 0.821. The objective response rate for sunitinib was 47% compared to IFN-α which was 12%. The most common toxicities associated with Sunitinib were hypertension, diarrhoea, hand and foot syndrome and fatigue.\textsuperscript{112} Pazopanib is another multikinase inhibitor which inhibits FGFR, PDGFR, fibroblast derived growth factor receptor (FDGFR) and c-kit was also shown to have better overall survival in first line setting in treatment naïve and IFN-α treated metastatic RCC. Therefore, a non-inferiority study was performed to compare Sunitinib versus Pazopanib in the first line setting. This randomized phase 3 clinical trial with 1110 patients showed that OS and PFS with Pazopanib was non-inferior to Sunitinib. Both drugs had similar toxicity profiles although Pazopanib lead to more significant hepatotoxicity.\textsuperscript{113} cabozantanib is another tyrosine kinase inhibitor that target VEGFR, MET and AXL receptors. The latter two are receptors that have been associated with resistance to VEGFR therapy. METEOR trial showed that in metastatic renal cell carcinoma patients who have progressed on first VEGFR targeting therapy, cabozantanib was found to lead to a better PFS (7.4 versus 3.8 months) and objective response rate
(21% versus 5%) compared to Everolimus.\textsuperscript{114} CABOSUN, which is a phase 2 clinical trial which compared cabozantinib versus Sunitinib as a first line therapy in metastatic RCC patients, showed that patients with intermediate to poor risk disease who were treated with cabozantinib had better PFS (8.2 months versus 5.2 months) and overall response rate (33% versus 12%) compared to Sunitinib.\textsuperscript{115} The role of combination therapy with antiangiogenic therapy was also tested in the second line setting for metastatic RCC. In a randomised, open labelled phase 2 study, patients with metastatic RCC who progressed on first line antiangiogenic therapy were given either lenvatinib (an antiangiogenic drug targets the VEGFR, VEGFR2, VEGFR3, FGFR1, FGFR2, FGFR3 and FGFR4, PDGFRα, RET, and KIT receptors) alone, everolimus alone or combination of lenvatinib and everolimus. PFS was better in the group treated with combination lenvatinib and everolimus therapy compared to everolimus alone (14.6 months versus 5.5 months) and lenvatinib alone (7.4 months). Lenvatinib alone also had a longer PFS compared to everolimus alone (Hazard Ratio 0.61) However, more grade 3 and 4 toxicities were seen in the combination therapy and lenvatinib alone group compared to everolimus alone.\textsuperscript{116}

However, not all patients respond to these therapies and amongst the responders, secondary resistance is almost inevitable. Thus, some patients continue to receive futile treatment, and others experience relatively short-lived benefit.\textsuperscript{117}

\textbf{1.4.3.2.2. Immune Landscape in RCC and Role of Immunotherapy}

Advances in understanding the role of immune system in cancer and development of new immunotherapies have revolutionised the treatment landscape of cancer. Infiltration of tumours with TILs has been associated with better prognosis in several cancers including ovarian and colorectal cancer.\textsuperscript{118} However, a different profile was seen in RCC. In 221 treatment naïve patients with different histological subtypes and grades, peritumoral TIL infiltration (CD8 an CD4 cells) did not predict prognosis in RCC.\textsuperscript{119} A detailed characterisation of the immune landscape in 73 patients with ccRCC with different stages and 5 non-tumour kidney tissue was performed. The main immune cell subtype seen in ccRCC was T cells which made up 51% of the CD45\textsuperscript{+} immune cell population. This was followed by the myeloid cell (31%), NK cells (9%) and B cells (4%). A significant proportion of the CD8 T cells expressed high levels of PD-1 and these cells were found to co-express TIM-3, CD38, HLA-DR, ICOS but very lows levels of IL-7 which suggests an exhausted phenotype. In the CD4 cells, less PD-1 expression was observed but the PD-1\textsuperscript{+} cells also co-expressed in TIM-3 and CD38. In addition, a high level of CTLA-4 expression was seen in CD4 cells. Expression profile in the normal tissue was different with lower PD-1
expression and mainly CD4 central memory, CD8 and CD4 effector memory cells were seen. In terms of the myeloid compartment, macrophages which are at a differentiation stage from monocytes to macrophages as well as CD169+ anti-tumourogenic macrophages and CD206+ pro-tumourogenic macrophages were seen in the ccRCC microenvironment.120 Similar to the findings by Chevrier et al., Granier and colleagues also identified PD-1+ CD8 cells which co-express TIM-3 suggesting an exhausted phenotype in treatment naïve non-metastatic ccRCC patients. These patients had more aggressive disease with bigger tumour size and higher Fuhrman grade, higher risk of relapse and worse survival compared to the group which did not have the exhausted CD8 phenotype.121 In silico RNA-Seq analysis of biopsies from 6 treatment naïve patients with metastatic ccRCC showed that a high immune infiltration in pre-treatment stage was associated with a better clinical response to Nivolumab (anti-PD-1 therapy). It has been shown that a high tumour mutation burden (TMB) is associated with response to immunotherapy certain cancers such as melanoma and lung cancer. The TMB is intermediate level in RCC in comparison to other cancer types. However, no correlation was seen between response to anti-PD-1 therapy and TMB or any driver mutations in this ccRCC cohort.122 In addition, multiregional TCR Seq analysis in treatment naïve ccRCC patients showed that there was heterogeneity in the TCR clonality within the different regions of the tumour. In two patients who were treated with an mTOR inhibitor prior to the biopsy, the clonality was reduced compared to the treatment naïve patients which suggests that treatment can influence the TCR repertoire.123 However both of these studies have very small sample sizes which is a major limitation that needs to be taken account while interpreting the results. Advances in the SC RNA Seq technology have led to a better understanding of the immune microenvironment at an in depth single cell resolution. SC RNASeq analysis of the immune cells from 13 treatment naïve ccRCC cases with different stage (stage I, II, III and IV) and matched tumour adjacent normal renal tissue showed that CD8 cells acquired a more dysfunctional phenotype in more advanced ccRCC stages with higher expression of PD-1, TIM-3, LAG-3 and EOMES. In addition, a subset of these dysfunctional CD8 cells also expressed CD39 which is a marker expressed in tumour reactive T cells. TCR Seq analysis showed a lower TCR diversity in the TILs compared to the tumour adjacent normal renal tissue. Comparison of the TCR repertoire in different T cells showed a lower TCR diversity in the dysfunctional CD8 cells. Analysis of the myeloid cells showed that the percentage of the pro-inflammatory M1 macrophages was reduced in tumours with more advanced stage disease and there was an increase in the pro-tumourogenic M2 macrophages.124
Considering the change in clinical practise with an increasing role of anti-PD-1 therapy in RCC treatment, a more in-depth understanding of mechanisms of response and resistance to anti-PD-1 therapy is still needed. Immunofluorescence (IF), RNaseq and genomic analysis of the ccRCC samples from 153 anti-PD-1 treated and 66 mTOR inhibitor treated samples showed that CD8 infiltration at baseline did not predict response to anti-PD-1 therapy. In tumours which did not have CD8 infiltration, infiltration with M2 macrophages, NK cells and CD4 memory cells was observed. In addition, although copy number alterations, frameshift indels and non-synonymous mutations were found in the tumours, there was no correlation with response to anti-PD-1 therapy or mTOR inhibitors. On the other hand, PBRM1 mutations lead to lower CD8 infiltration in the tumours. In a small heterogenous cohort of 8 ccRCC patients of which 5 were treated with both antiangiogenic therapy followed by anti-PD-1 therapy and three were treatment naïve, SC RNA Seq analysis was used to understand the immune microenvironment and changes that happen with therapy. In patients who responded to anti-PD-1 therapy, the responder had a terminally exhausted/dysfunctional CD8 phenotype with high PD-1, HAVCR2 (TIM-3) and TIGIT gene expression while these cells were low in 4-1BB expression which is a marker of activation. Moreover, an increase in the cytotoxic genes such GZMB, IFNG and PRF1 was seen following anti-PD-1 treatment. Patients who responded to anti-PD-1 therapy also had higher infiltration of pro-inflammatory M1 type macrophages. Analysis of the tumour cells showed an upregulation of the genes involved in MHCI in the responder which suggests an important role of antigen presenting machinery in response to anti-PD-1 therapy. In a different study SC RNA Seq analysis was performed on 29 samples including tumour samples, tumour adjacent normal tissue and peripheral blood from 6 patients with either stage III or IV disease. Four patients were treated with either anti-PD-1 or combination of anti-PD1 and anti-CTLA-4 treatment. One of those patients was treated with Sunitinib prior to immunotherapy. Two patients did not receive treatment. There was a higher infiltration of CD8 TRM cells in the immunotherapy treated patients compared to treatment naïve patients. They were also present in the normal kidney tissue but the level of infiltration varied depending on how close the normal tissue sample was the tumour. In addition, TCR Seq analysis showed more TCR expansion in the responders following immunotherapy and the expanded clones had a tissue resident CD8 phenotype. In addition, some of the intratumoural expanded TCR clones were also detected in the peripheral blood and the normal kidney tissue. However, these are small cohorts with heterogenous treatment history which needs to be taken into consideration while interpreting the studies. A bigger validation cohort is needed to be able to make definitive conclusions.
A better understanding of the tumour immune microenvironment has had an impact in the clinical setting for RCC too. In 2015, CheckMate 025 which is a randomised open label phase 3 clinical trial with 821 patients with advance ccRCC who had progressed on at least one line of antiangiogenic therapy were treated with either anti-PD-1 therapy (nivolumab) or an mTOR inhibitor (everolimus). Patients who were treated with nivolumab has better median OS (25 months) compared to the patients treated with everolimus (19.6 months). In addition, the toxicity profile was also better with nivolumab. Following this, Checkmate 214 which is a randomised phase 3 clinical trial that compared combination anti-PD-1(nivolumab) and anti-CTLA-4 therapy (ipilimumab) to antiangiogenic therapy (Sunitinib) as first line therapy in 1096 advanced intermediate or poor risk RCC patients. Combination therapy with ipilimumab and nivolumab had better OS (Hazard Ratio 0.66). Given the important role of antiangiogenic therapy in RCC and the recent data on the role of immunotherapy, Keynote 426 phase 3 trial compared combination therapy with anti-PD-1 therapy (pembrolizumab) and antiangiogenic therapy (axitinib) to sunitinib in 861 advanced RCC patients who did not have any previous treatments and had different IMDC risk scores ranging from favourable to poor risk. At 12 months, 89.9% of the patients were alive in the pembrolizumab+axitinib arm while this percentage was 78.3% in the sunitinib arm. The PFS was also better in patients treated with pembrolizumab+axitinib (15.1 months) compared to the patients treated with (11.1 months). Both arms had similar toxicity profiles. In addition, JAVELIN study looked the role of anti-PD-L1 (avelumab) and antiangiogenic (axitinib) therapy compared to Sunitinib in treatment naïve advanced RCC patients, regardless of the PD-L1 expression level in the tumour. 886 patients were involved in the study and out of those, 560 patients had PD-L1 positive (>1% expression) tumours. The results showed that the median PFS was better (13.8 months) in the combination therapy arm compared to the Sunitinib arm (7.2 months). In the patients with PD-L1 positive tumours who were treated with combination avelumab and axitinib therapy, the objective response rate was 55.2% compared to the group treated with sunitinib. The toxicity profile was similar in both groups.

In light of these studies, the European Association of Urology guidelines for renal cell carcinoma recommend combination Ipilimumab and nivollimab treatment as first line therapy for metastatic RCC patients with intermediate to poor risk. For patients with favourable risk, the recommendation is combination therapy with pembrolizumab and axitinib. For patients for whom immunotherapy is contraindicated, an antiangiogenic agent can be used as first line therapy. For patients who progress on first line therapy, they can be treated with an antiangiogenic agent Figure 1.6.
Figure 1.6 Treatment pathway for metastatic RCC patients

(Adopted from Albige et al. 2019). Figure shows the summary of therapies used for metastatic RCC patients. Lines of treatment for favourable, intermediate, and poor risk patients are shown.
2 MATERIALS and METHODS

2.1 Flow Cytometry

Renal tumour resections and normal tissue were cut into small pieces (2-3mm) by using sterile disposable scalpel plus forceps in RPMI (Sigma-Aldrich) with Collagenase I (Sigma-Aldrich) and DNAse I (Roche) and was digested for 1 hour at room temperature using the gentleMACS dissociator (Miltenyi Biotec). The digest was passed through a 70-µm cell strainer by using 5-10 ml of RPMI containing 2% fetal bovine serum (FBS) to obtain a single cell suspension. Lymphocytes were obtained from the single cell suspension by using Ficoll Paque Plus (GE Healthcare) density gradient centrifugation (750g for 10 min). Isolated lymphocytes were washed with RPMI and 2%FBS and cryopreserved in 90% FBS with 10% dimethyl sulfoxide (Sigma–Aldrich). PBMCs were isolated from blood samples collected in Vacutainer EDTA blood collection tubes (BD) using Ficoll Paque Plus (GE Healthcare) density gradient centrifugation and cryopreserved in in 90% FBS with 10% dimethyl sulfoxide (Sigma–Aldrich).

Thawed lymphocytes and PBMCs were washed with 1x phosphate-buffered saline (PBS) and incubated with 5µl of FC block for 20 minutes. The samples were then stained with the antibodies listed below. Antibody mastermixes were prepared in Brilliant Staining Buffer (BD). eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set was used for the intracellular staining. The extracellular staining was done over 30 minutes and the intracellular staining was done over 60 minutes at room temperature. Before acquiring the samples on the flow cytometer, compensation was performed for each marker. Fluorophores can spill over to other channels and compensation allows to correct this spillover. UltraComp eBeads were stained for each fluorophore with the antibody conjugated to the specific fluorophore of interest. Compensation for each marker was then calculated using the BD Symphony and FlowJo compensation matrixes. Isotype controls were not used. The samples were acquired on the BD Symphony flow cytometer. Data was analysed using the FlowJo (version 10). The following flow cytometry panels were used for staining the samples. Flow cytometry panels used for staining are shown below. The gating strategy used in flow cytometry analysis is shown in Figure 2.1.
Table 2.1 T Cell Panel 1 for TRACERx Renal Cohort.

The table shows the antibodies used in one of the high dimensional flow cytometry panels used for the TRACERx Renal cohort with their corresponding fluorophores and clones. This panel has a specific focus on T cell differentiation. Markers in bold (Ki67, EOMES, FoxP3, GzmB and Hobit) are markers stained by using intracellular antibodies.
<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Antibody</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUV395</td>
<td>4-1BB</td>
<td></td>
</tr>
<tr>
<td>BUV496</td>
<td>CD8</td>
<td>RPA-T8</td>
</tr>
<tr>
<td>BUV563</td>
<td>CD45RA</td>
<td>HI100</td>
</tr>
<tr>
<td>BUV661</td>
<td>CD38</td>
<td>HIT2</td>
</tr>
<tr>
<td>BUV737</td>
<td>CD39</td>
<td>TU66</td>
</tr>
<tr>
<td>BUV805</td>
<td>CD3</td>
<td>SK7</td>
</tr>
<tr>
<td>BV421</td>
<td>PD1</td>
<td>EH12.1</td>
</tr>
<tr>
<td>BV480</td>
<td>GITR</td>
<td>V27-580</td>
</tr>
<tr>
<td>BV605</td>
<td>CD57</td>
<td>NK1</td>
</tr>
<tr>
<td>BV650</td>
<td>TIM-3</td>
<td>7D3</td>
</tr>
<tr>
<td>BV786</td>
<td>ICOS</td>
<td>WD1928</td>
</tr>
<tr>
<td>BB515</td>
<td>CD103</td>
<td>Ber-ACT8</td>
</tr>
<tr>
<td>PerCp-Cy5.5</td>
<td>CD27</td>
<td>323</td>
</tr>
<tr>
<td>PE</td>
<td>FoxP3</td>
<td>PCH101</td>
</tr>
<tr>
<td>PE-CF594</td>
<td>CD25</td>
<td>M-A251</td>
</tr>
<tr>
<td>PE-CY7</td>
<td>TIGIT</td>
<td>A15153G</td>
</tr>
<tr>
<td>APC</td>
<td>CTLA4</td>
<td>L3D10</td>
</tr>
<tr>
<td>APC-R700</td>
<td>CD4</td>
<td>SK3</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>Viability</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.2 T Cell Panel 2 for TRACERx Renal Cohort.**

The table shows the antibodies used in the one of the high dimensional flow cytometry panels used for the TRACERx Renal cohort with their corresponding fluorophores and clones. This panel has a specific focus on checkpoints expressed on T cells. Markers in bold (FoxP3, CTLA4) are markers stained by using intracellular antibodies.
<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Antibody</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUV395</td>
<td>Strep</td>
<td>N/A</td>
</tr>
<tr>
<td>BUV496</td>
<td>CD8</td>
<td>RPA-T8</td>
</tr>
<tr>
<td>BUV563</td>
<td>CD45RA</td>
<td>HI100</td>
</tr>
<tr>
<td>BUV615</td>
<td>CD4</td>
<td>SK3</td>
</tr>
<tr>
<td>BUV737</td>
<td>CD38</td>
<td>HIT2</td>
</tr>
<tr>
<td>BUV805</td>
<td>CD3</td>
<td>SK7</td>
</tr>
<tr>
<td>BV421</td>
<td>FoxP3</td>
<td>206D</td>
</tr>
<tr>
<td>BV510</td>
<td>CCR4</td>
<td>L291H4</td>
</tr>
<tr>
<td>BV570</td>
<td>LD yellow</td>
<td></td>
</tr>
<tr>
<td>BV605</td>
<td>CD57</td>
<td>QA17A04</td>
</tr>
<tr>
<td>BV650</td>
<td>Ki67</td>
<td>B56</td>
</tr>
<tr>
<td>BV711</td>
<td>CD39</td>
<td>TU66</td>
</tr>
<tr>
<td>BV750</td>
<td>CCR7</td>
<td>G043H7</td>
</tr>
<tr>
<td>BV785</td>
<td>CD69</td>
<td>FN50</td>
</tr>
<tr>
<td>BB515</td>
<td>CD103</td>
<td>Ber-ACT8</td>
</tr>
<tr>
<td>PerCp-Cy5.5</td>
<td>CXCR5</td>
<td>RF8B2</td>
</tr>
<tr>
<td>PE</td>
<td>TCF7</td>
<td>7F11A10</td>
</tr>
<tr>
<td>PE-CF594</td>
<td>GzmB</td>
<td>GB11</td>
</tr>
<tr>
<td>PE-Cy5</td>
<td>CD25</td>
<td>M-A251</td>
</tr>
<tr>
<td>PE-CY7</td>
<td>PD-1</td>
<td>EH12.2H7</td>
</tr>
<tr>
<td>APC</td>
<td>TOX</td>
<td>REA473</td>
</tr>
<tr>
<td>AF700</td>
<td>HLA-DR</td>
<td>LN3</td>
</tr>
<tr>
<td>Biotin</td>
<td>IgG4</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3 T cell Panel 1 for ADAPTeR Cohort.

The table shows the antibodies used in the one of the high dimensional flow cytometry panels used for the ADAPTeR cohort with their corresponding fluorophores and clones. This panel has a specific focus on T cell differentiation. Markers in bold (FoxP3, TCF7, GzmB and TOX) are markers stained by using intracellular antibodies.
<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Antibody</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUV395</td>
<td>Strep</td>
<td>N/A</td>
</tr>
<tr>
<td>BUV496</td>
<td>CD8</td>
<td>RPA-T8</td>
</tr>
<tr>
<td>BUV563</td>
<td>CD45RA</td>
<td>HI100</td>
</tr>
<tr>
<td>BUV615</td>
<td>CD4</td>
<td>SK3</td>
</tr>
<tr>
<td>BUV661</td>
<td>4-1BB</td>
<td>4B4-1</td>
</tr>
<tr>
<td>BUV737</td>
<td>CD38</td>
<td>HIT2</td>
</tr>
<tr>
<td>BUV805</td>
<td>CD3</td>
<td>SK7</td>
</tr>
<tr>
<td>BV421</td>
<td>FOXP3</td>
<td>206D</td>
</tr>
<tr>
<td>BV510</td>
<td>CD226</td>
<td>11A8</td>
</tr>
<tr>
<td>BV570</td>
<td>LD yellow</td>
<td></td>
</tr>
<tr>
<td>BV605</td>
<td>CD57</td>
<td>QA17A04</td>
</tr>
<tr>
<td>BV650</td>
<td>TIM-3</td>
<td>7D3</td>
</tr>
<tr>
<td>Superbright 702</td>
<td>KLRG1</td>
<td>13F12F2</td>
</tr>
<tr>
<td>BV750</td>
<td>CD27</td>
<td>O323</td>
</tr>
<tr>
<td>BV785</td>
<td>ICOS</td>
<td>C398.4A</td>
</tr>
<tr>
<td>BB515</td>
<td>CD103</td>
<td>Ber-ACT8</td>
</tr>
<tr>
<td>PerCp-eFlour710</td>
<td>EOMES</td>
<td>WD1928</td>
</tr>
<tr>
<td>PE</td>
<td>Fas</td>
<td>DX2</td>
</tr>
<tr>
<td>PE-CF594</td>
<td>GrzmB</td>
<td>GB11</td>
</tr>
<tr>
<td>PE-Cy5</td>
<td>CD25</td>
<td>M-A251</td>
</tr>
<tr>
<td>PE-CY7</td>
<td>PD-1</td>
<td>EH12.2H7</td>
</tr>
<tr>
<td>APC</td>
<td>CTLA-4</td>
<td>L3D10</td>
</tr>
<tr>
<td>APC/Fire-750</td>
<td>GITR</td>
<td>108-17</td>
</tr>
<tr>
<td>Biotin</td>
<td>IgG4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.4 T Cell Panel 2 for the ADAPTeR Cohort.**

The table shows the antibodies used in one of the high dimensional flow cytometry panels used for the TRACERx Renal cohort with their corresponding fluorophores and clones. This panel has a specific focus on checkpoints expressed on T cells. Markers in bold (FoxP3, EOMES, GzmB and CTLA-4) are markers stained by using intracellular antibodies.
<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Antibody</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUV395</td>
<td>CD45</td>
<td>HI30</td>
</tr>
<tr>
<td>BUV496</td>
<td>CD16</td>
<td>3G8</td>
</tr>
<tr>
<td>BUV563</td>
<td>CD56</td>
<td>NCAM16.2</td>
</tr>
<tr>
<td>BUV615</td>
<td>CD19</td>
<td>SJ25C1</td>
</tr>
<tr>
<td>BUV661</td>
<td>CD15</td>
<td>W6D3</td>
</tr>
<tr>
<td>BUV737</td>
<td>CD38</td>
<td>HIT2</td>
</tr>
<tr>
<td>BUV805</td>
<td>CD3</td>
<td>SK7</td>
</tr>
<tr>
<td>AF405</td>
<td>CD32b</td>
<td>N297Q-006-G11</td>
</tr>
<tr>
<td>BV480</td>
<td>CD11c</td>
<td>3.9</td>
</tr>
<tr>
<td>BV570</td>
<td>LD yellow</td>
<td></td>
</tr>
<tr>
<td>BV605</td>
<td>CD73</td>
<td>AD2</td>
</tr>
<tr>
<td>BV650</td>
<td>CD64</td>
<td>10.1</td>
</tr>
<tr>
<td>BV711</td>
<td>CD33</td>
<td>WM53</td>
</tr>
<tr>
<td>BV750</td>
<td>CD14</td>
<td>63D3</td>
</tr>
<tr>
<td>BV786</td>
<td>CD40</td>
<td>5C3</td>
</tr>
<tr>
<td>AF488</td>
<td>CD32a</td>
<td>N297Q-002-E008</td>
</tr>
<tr>
<td>PerCp-Cy5.5</td>
<td>CD68</td>
<td>Y1/82A</td>
</tr>
<tr>
<td>PE</td>
<td>Arg-1</td>
<td>14D2C43</td>
</tr>
<tr>
<td>PE-CF594</td>
<td>CD86</td>
<td>IT2.2</td>
</tr>
<tr>
<td>PE-Cy5</td>
<td>CD206</td>
<td>15.2</td>
</tr>
<tr>
<td>PE-CY7</td>
<td>IDO</td>
<td>Eyedio</td>
</tr>
<tr>
<td>APC</td>
<td>CLEC9a</td>
<td>8F9</td>
</tr>
<tr>
<td>AF700</td>
<td>HLA-DR</td>
<td>LN3</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>CD11b</td>
<td>ICRF44</td>
</tr>
</tbody>
</table>

**Table 2.5 Myeloid cell panel for the ADAPTeR Cohort.**

The table shows the antibodies used in one of the high-dimensional flow cytometry panels used for the ADAPTeR cohort with their corresponding fluorophores and clones. This panel has a specific focus on myeloid cells. Markers in bold (CD68, Arg-1, IDO) are markers stained by using intracellular antibodies.
Figure 2.1 Gating strategy for flow cytometry analysis

The main gating strategy for flow cytometry analysis is shown above. As a first step, lymphocytes are identified. This is followed by single cell and live cell gating. Then, CD3 cells are identified out of the live cells. CD8 and CD4 cells are gated out of live CD3 cells.

2.2 Multiplex Immunofluorescence and Immunohistochemistry Staining and Image Analysis

FFPE locks were cut in 2 micron thick slides and slides were baked for 60 minutes. The slides were stained using the antibodies listed below and opal fluorophores. Leica Bond III machine was used for the immunofluorescence staining. Images of the stained slides were acquired by using the Vectra 3 automated quantitative pathology imaging system (PerkinElmer). Matching H&E image of each slide was reviewed by a pathologist and areas to annotate on the immunofluorescent images for analysis were identified. Necrotic and stromal areas as well as non-tumour areas were excluded. All slides for patient ADR009 was excluded from the analysis as the tissue was necrotic. Up to 20 multispectral image (MSI) was acquired per slide depending on the size of the tumour. Representative MSI from different slide were used while training the algorithms for each marker. Scoring of each slide was performed using the inForm software on Vectra. The quality and accuracy of the scoring was checked by two clinicians one of whom was a pathologist. MSIs with poor tissue quality were excluded from the analysis. Merged data obtained by using the inForm software was analysed using the phenoptrReports tool (Akoya Biosciences) on R. Mann-Whitney U test was used for statistical analysis of the
data. Optimisation for the IF as well as the IHC staining were performed by the Prof. Teresa Marafiotti and her team (Ayse Akarca, William Yang and Ignazio Puccio).

For immunohistochemistry, FFPE tissue sections of clear-cell renal cell carcinoma and normal tonsil tissues were subjected to H&E and multiplex immunostaining. The primary antibodies used for multiplex immunolabeling are as follows: CD19 (rabbit monoclonal, SP291, 1:10 dilution), CD138 (mouse monoclonal, MI15, 1:100 dilution), PD1 (mouse monoclonal, NAT105/E3, 1:2 dilution).
<table>
<thead>
<tr>
<th>Antibody 1</th>
<th>Antibody 2</th>
<th>Antibody 3</th>
<th>Antibody 4</th>
<th>Antibody 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
<td>CD8</td>
<td>CD4</td>
<td>FoxP3</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Mouse</td>
<td>Mouse</td>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Dilution</td>
<td>1:100</td>
<td>1:50</td>
<td>1:80</td>
<td></td>
</tr>
<tr>
<td>Incubation Time</td>
<td>30 min</td>
<td>30 min</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>Incubation Temperature</td>
<td>Room Temperature</td>
<td>Room Temperature</td>
<td>Room Temperature</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>Opal Fluorophore</td>
<td>620</td>
<td>540</td>
<td>570</td>
<td></td>
</tr>
<tr>
<td>Opal Dilution</td>
<td>1:150</td>
<td>1:100</td>
<td>1:150</td>
<td></td>
</tr>
<tr>
<td>Opal Incubation Time</td>
<td>20 min</td>
<td>20 min</td>
<td>20 min</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody 1</th>
<th>Antibody 2</th>
<th>Antibody 3</th>
<th>Antibody 4</th>
<th>Antibody 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
<td>CD8</td>
<td>PAX8</td>
<td>MHC-II</td>
<td>CD4</td>
</tr>
<tr>
<td>Species</td>
<td>Mouse</td>
<td>Mouse</td>
<td>Mouse</td>
<td>Mouse</td>
</tr>
<tr>
<td>Dilution</td>
<td>1:100</td>
<td>1:200</td>
<td>1:1000</td>
<td>1:50</td>
</tr>
<tr>
<td>Incubation Time</td>
<td>15 min</td>
<td>30 min</td>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Incubation Temperature</td>
<td>Room Temperature</td>
<td>Room Temperature</td>
<td>Room Temperature</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>Opal Fluorophore</td>
<td>620</td>
<td>690</td>
<td>540</td>
<td>520</td>
</tr>
<tr>
<td>Opal Dilution</td>
<td>1:150</td>
<td>1:50</td>
<td>1:100</td>
<td>1:50</td>
</tr>
<tr>
<td>Opal Incubation Time</td>
<td>20 min</td>
<td>20 min</td>
<td>20 min</td>
<td>20 min</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody 1</th>
<th>Antibody 2</th>
<th>Antibody 3</th>
<th>Antibody 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
<td>CD8</td>
<td>Granzyme B</td>
<td>CD3</td>
</tr>
<tr>
<td>Species</td>
<td>Mouse</td>
<td>Mouse</td>
<td>Mouse</td>
</tr>
<tr>
<td>Dilution</td>
<td>1:100</td>
<td>1:80</td>
<td>1:100</td>
</tr>
<tr>
<td>Incubation Time</td>
<td>30 min</td>
<td>30 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Incubation Temperature</td>
<td>Room Temperature</td>
<td>Room Temperature</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>Opal Fluorophore</td>
<td>540</td>
<td>620</td>
<td>520</td>
</tr>
<tr>
<td>Opal Dilution</td>
<td>1:150</td>
<td>1:150</td>
<td>1:50</td>
</tr>
<tr>
<td>Opal Incubation Time</td>
<td>20 min</td>
<td>20 min</td>
<td>20 min</td>
</tr>
</tbody>
</table>

**Table 2.6 IF staining details for the ADAPTeR Cohort.**

The tables show the antibodies used and the staining conditions used for the IF staining for the ADAPTeR cohort.
2.3 T Cell Receptor Sequencing

TCR α-chain and β-chain sequencing was performed by utilizing whole RNA extracted from tissue samples or from cryopreserved PBMC samples, by using a quantitative experimental and computational TCR sequencing pipeline described previously.\textsuperscript{132–135} The bespoke protocol involves using unique molecular identifiers attached to each cDNA TCR molecule that which allows for correction for PCR and sequencing errors.

1000ng of RNA from tumour samples and 500ng of RNA from the PBMC samples was used for the TCR experiments. The RNA was initially treated with DNase. 1μl of RQ1 DNase (Promega) and 1ul reaction buffer was added to 8 ul of RNA and incubated in 37°C for 30 minutes. After the incubation, 1ul of Promega DNase stop buffer was added to the sample and the mix was incubated at 65°C for 10 minutes. After the DNAse treatment, the reverse transcription step was performed. 1.5ul of αRC2 (10uM) which is an oligonucleotide for the α chain of the TCR and and βRC2 (10uM) which is an oligonucleotide for the β chain of the TCR, 1.5ul of dNTPs (10mM) and 4 ul of water was added to the product from the DNAse step and incubated at 65°C for 5 minutes and then kept on ice for 1 minute. The second step of the reverse transcription process involved adding 1.5μl of SuperScript IV reverse transcriptase (RT) 1.5ul DTT (0.1M), Promega 1.5μl of RNasin (RNase inhibitor) and 6μl of SSIV buffer (5x) and the mix was incubated at 55°C for 20 minutes, then at 80°C for 10 minutes. The cDNA from the reverse transcriptase was purified by using AMPure XP Magnetic beads.

Ligation step was carried out following the cDNA purification. This allows for ligating the unique molecular identifiers (UMIs) which allows for identifying errors during the PCR amplification. During the ligation step, 6 ul of T4 RNA ligase buffer, 6 ul of BSA/HCC(1mg/ml/10mM), 2 ul ATP (10mM), 2 ul M13 Ligation Oligo 6N_i8.1_6N_M13_2 (10μM), 4 ul T4 RNA ligase and 20 ul of PEG8000 (50%) was used for the ligation mix. The ligation mix was added to the cDNA and was incubated at 16°C for 20 hours followed by 65°C for 10 minutes. The ligation product was purified by using AMPure XP Magnetic beads to remove the ligation oligonucleotide.

Following the purification of ligation step, first PCR step was performed by using 10ul of 5xHF buffer, 2.5 ul αRC1 (10μM), 2.5 ul βRC1.1+1.2 (10μM), 2.5 ul SP2-M13 (10μM), 1 ul dNTP (10mM) and 0.5 ul of Phusion Polymerase. This mastermix was added to the 31 ul of the purified ligation product. SP2 primer anneals to the sequence by the ligation oligonucleotide. αRC1 primer binds to the constant region of the TCRα gene and βRC1.1+1.2 primer binds to the constant region of the TCRβ gene. PCR amplification was carried out
using the following conditions: denaturation at 98°C for 3 minutes, denaturation at 98°C, 15 seconds, annealing at 69°C for 30 seconds, extension at 72°C for 40 seconds for 4 cycles; followed by the final extension step at 72°C 5 minutes. The PCR product was purified by using AMPure XP Magnetic beads. Following the purification step, PCR2 which uses P5 and P7 sequencing adaptors, SP1 primer and two sequencing indexes which allows sequencing different samples in the same run. 10 ul of the 5xHF buffer, 2.5 ul SP1-P5 (10uM), 1 ul dNTP (10mM) and 0.5 ul of Phusion polymerase were used and added to 31ul of the PCR1 product. A Total of 6 cycles was carried out using the same conditions used for PCR1. The PCR2 product was purified using the AMPure XP Magnetic beads. The PCR product was amplified further by using quantitative PCR (qPCR). The qPCR mastermix consisted of 10ul 5xHF, 5 ul of Sybr green, 1.25 ul of the dNTP (10mM), 1 ul Rox dye , 2.5 ul P5s (10uM), 2.5 ul P7 (10uM) and 0.5 ul Phusion polymerase and was added to 22.75 ul of the purified PCR2 product. The conditions used for PCR1 and PCR2 were used for the qPCR and the samples were ran for 8-12 cycles depending on when the samples reached the threshold. The qPCR product was purified using AMPure XP Magnetic beads. The PCR product was then quantified at using the Agilent High Sensitivity DS1000 ScreenTape System on the 2200 Tapestation. Samples which had gone through successful library preparation had a peak with a size of approximately 650 bp. The final libraries were prepared by using 4 nM of each sample and samples were run using the Illumina NextSeq protocol.

The wet lab experiments for TCR sequencing were performed by myself and bioinformatics analysis was done by Marc Robert de Massay (PhD student in the Quezada/Pegg lab).

The bioinformatic analysis of the TCR data has several steps. FASTQ files were analysed using Phyton scripts and the Decombinator software. The Decombinator recognises tags in the sequencing data. In the first step, the Demultiplexor script allows for detecting the UMIs and the TCR sequence from the FASTQ files generated as a result of the Illumina NextSeq run. In the second step, the Decombinator script allows for detecting recombined TCR sequences by identifying the V,J and D sequences for the TCR chains. In the third step, the Collapsinator script uses the files generated by the Decombinator and used UMIs to correct for any sequencing errors. Finally, the CDR3 translator translates the information from all the previous steps into an amino acid sequence with specific structure of the TCR chains (Figure 2.2).
Figure 2. 2 Bioinformatics pipeline for TCR Seq analysis

(Taken from Oakes et al., 2017). The diagram shows the demultiplexor, decombator, collapsinator and CDR3 translator analysis steps of the FASTQ files generated as a result of the Illumina NextSeq run.

The tools used for TCR identification, error correction and CDR3 extraction is freely available at [https://github.com/innate2adaptive/Decombinator](https://github.com/innate2adaptive/Decombinator).

2.4 Single Cell RNA Sequencing

TILs from ADR01 and AD013 were stained with CD3 (PE, SK7 clone), IgG4 (Biotinylated) and Streptavidin (BV650) antibodies for flow cytometry. Stained cells were FACS sorted as CD3+IgG4⁻ (40,000 cells) and CD3+IgG4⁺ (20,000 cells) for ADR001 and CD3+IgG4⁻ (50,000 cells) and CD3+IgG4⁺ (90,000 cells) for ADR013. FACS sorted cells were single cell sorted using the 10X Genomic machine. The sorted cells were processed using the 10X Genomic Chromium Next GEM Single Cell 5’ Reagents Kit V2 (dual index) for 5’gene expression library construction and V(D)J library construction. The samples were sequenced on the NextSeq using the High Output Kit v2.5 (150 Cycles).

FASTQ files containing gene expression (GEX) and VDJ were demultiplexed using cellranger mkfastq (10x Genomics). GEX reads were aligned to GRCh38 and counted.
using cellranger count, VDJ reads were aligned to cellranger’s GRCh38 VDJ reference dataset using cellranger vdj. Expression matrices were analysed using the Seurat package.\textsuperscript{136} To remove technical variation in the data, TCR, ribosomal and heat-shock protein genes were removed from the analysis, also cells with mitochondrial reads making up >10% total read content were removed. 8382 CD3\textsuperscript{−}IgG4\textsuperscript{−} and 10083 CD3\textsuperscript{−}IgG4\textsuperscript{+} cells in ADR013; and 4648 CD3\textsuperscript{−}IgG4\textsuperscript{−} and 3343 CD3\textsuperscript{−}IgG4\textsuperscript{+} cells in ADR001 were retained after quality control filtering. Datasets were integrated using SCTransform integration (Hafemeister and Satija 2019)\textsuperscript{137} using the recommended parameters and regressing the % mitochondrial read content. PCA and UMAP dimensional reduction (dims = 1:30) and clustering (res = 0.3) was then performed using RunPCA, RunUMAP, FindNeighbours, and FindClusters. All differential gene expression analysis were carried out on log normalised gene expression values (using NormalizeData, default parameters) using the MAST algorithm\textsuperscript{138} within FindMarkers. GOBP analysis was carried out using the XGR package (Fang et al. 2016) using the “lea” algorithm. scTCR data was analysed using scRepertoire (Borcherding and Bormann et al 2020).\textsuperscript{139} Cells were considered of the same clone if they contained a matching TRB sequence and CDR3 gene.
3 CHARACTERISING THE IMMUNE LANDSCAPE IN TRACERx RENAL COHORT

3.1 CHAPTER INTRODUCTION

T cell differentiation is important in determining how the T cells respond to tumour cells. CD8 T cell differentiation involves several steps. The initial step involves activation of the naïve T cells in the secondary lymphoid structures when the dendritic cells present antigenic peptides on MHC class I. Antigen presentation leads to TCR engagement which then leads to expression of co-stimulatory molecules including but not limited to CD80/86 and 4-1BBL. As a result, inflammatory cytokines such as the IFN-α and β, interleukin-2 (IL-2) and TNF-α are produced. Consequently, CD8 cells gain effector function, clonally expand and become CD8 effectors. After the effector T cells perform their function at the periphery, a proportion of them (5-10%) become memory T cells. Transcriptional regulation and cytokine production determine the effector-to-memory T cell differentiation. The terminal effector cells express high levels of KLRG1 but are low in IL-7Rα, CD27 and BCL-2. On the other hand, memory T cells are low in KRLG1 but express high levels of IL-7Rα, CD27 and BCL-2. The memory T cells consist of subsets of cells including stem central memory T cells (Tscm), central memory T cells (TCM) and effector memory T cells (TEM). Tscm cells retain their progenitor function and have increased self-renewal ability. It is unclear whether the Tscm cells have a naïve T cell origin. However, their transcriptional profile shows that they are the least differentiated subset of memory cells and they express EOMES, TBX21 and KLRG1. They proliferate in response to IL-17 and phenotypically they are CCR7-CD62L-CD45RA-CD45RO-. They were also shown to be more tumour reactive compared to the TEM and TCM cells. TEM and TCM cells have different phenotypes. Tcm cells are CD45RA-CCR7+ and they also express high levels of CD62L which allows circulation of the Tcm cells in the blood stream. On the other hand, Tem cells are are CD45RA CCR7- and have lower levels of CD62L. CCR7 and CD62L play an important role in homing of T cells to the lymph nodes. Both TEM and TCM can express CD27 and CD28 which are co-stimulatory molecules. TEM and TCM cells also differ in their function and expression of transcription factors. TEM cells express intermediate levels of Tbet and Eomes and lack expression of Blimp-1 and Bcl6. In addition, they retain their cytotoxic function and can perform differentiate into effector cells following antigen engagement. On the other hand, TCM cells have express low levels of Tbet but high levels of Eomes as well as expressing BCL6, TCF7 and STAT-3. They are less differentiated cells which do not retain any effector function.
Another subset is the effector memory cells that re-express CD45RA (Temra). Temra cells are terminally differentiated, and they also have properties of senescence. However, they can retain their cytotoxic function and can produce cytokines such as IFN-γ and TNF-α. They do not have a high proliferative capacity and cell death occurs soon after activation. They also do not express the surface markers necessary for cell migration such as CCR7 and CD62L. In addition, it has been shown that CD57 expression on Temra cells is associated with terminal differentiation and increased cytotoxic activity whereas CDR57+ Temra cells have higher proliferation and differentiation capacity.

Circulating TEM and TCM cells can become resident in the tissue and become tissue resident memory (TRM) cells. TRM cells express CD69 and CD103 which is usually induced by secretion of cytokines such as IL-33 and TNF-α. CD69 is a C-Lectin and CD103 is an integrin. They bind to the E-cadherin on the epithelial cells which allows for the TRM cells to become resident in the tissue they migrate to. In addition, CD103 is also involved in TCR signalling and therefore plays a role in the cytokine production pathway. Transcription factors Blimp-1 and Blimp-1 homolog Hobit play an important role in differentiation of cells to TRM cells. TRM cells have been observed both in CD8 and CD4 effector cells. Presence of TRM cells have been associated with better prognosis in different cancer types.

TRACERx Renal (TRAcking Cancer Evolution through therapy[Rx]; NCT03226886) is a translational study that allows for multiregional evaluation of genomics and immune landscape in renal cell carcinoma. The study is open across different sites in the UK and is run by a multidisciplinary team of surgeons, histopathologists, oncologists, radiologists, nurses and researchers. As part of the study, longitudinal samples of primary and metastatic RCC tumours, peripheral blood and urine are collected from patients. For the tumours, multiregional sampling is performed. The study aims to investigate the correlation between ITH and clinical outcomes in RCC as well as identifying the genomic drivers of primary and metastatic disease and understanding the immune landscape in RCC. It has previously been shown that T cells constitute an important part of the immune landscape in RCC. In this project, the immune landscape of a cohort of TRACERx Renal patients were analysed to evaluate the T cell subset with a focus on T cell differentiation and their phenotypes.

We hypothesized that tumour, peripheral blood and normal tissue have different T cell differentiation subsets with a high exhausted T cell population in the tumour and T cell infiltration in the tumour can have a prognostic value.
Aims in this chapter are:

- To identify the abundance of CD8, CD4 effector and Treg cells in peripheral blood, normal tissue and TILs
- To identify the T cell differentiation subsets
- To understand the phenotype of the different T cell subsets in PBMC, normal tissue and TILs
3.2 RESULTS

3.2.1 Main characteristics of the lymphocytes in TRACERx Renal Cohort

Multiregional samples were collected from 27 primary nephrectomies (57 regions) from patients with clear cell renal cell carcinoma prior to any systemic anti-cancer treatment. Blood was also collected from 21 patients at the time of the nephrectomy. Tumour adjacent normal renal tissue (referred to as ‘normal tissue’ in this thesis) was collected from 11 cases. T cell profiles including differentiation and checkpoint expression patterns were evaluated in the tumour, normal renal tissue and PBMC. As discussed in the main and chapter introductions, understanding the T cell differentiation in the peripheral blood and tissue is important in gaining a better understanding the role of T cells in antitumor immunity. In addition, checkpoint inhibition has revolutionized cancer treatment after discovery of PD-1 and CTLA-4. However, not all patients or cancer types respond to currently available checkpoint inhibitors. Therefore, mapping the checkpoint expression on T cells is important in identifying new checkpoints contributing to T cell response and immune escape in cancer as well as identifying new targets for immunotherapy. The median values were taken for the samples with multiregional tissue. Multiple t tests using the Sidak-Bonferroni method was used to compare the T cell subsets between TILs, PBMC and normal tissue. The demographics and clinical data is not available for the patients. Therefore, the data was not correlated with any clinical outcome, demographics or detailed histological subtype data. CD8 T cells is the most abundant T cell subset in all three tissue types followed by CD4 effector T cells and Tregs (Figure 3.1A and B). Abundance of CD8 and CD4 effector cells were similar in PBMC, normal tissue and TILs with no statistical significance. However, the level of Tregs was higher in the TILs compares to PBMC and normal tissue (p= 0.029 and p= 0.001 respectively) (Figure 3.1B). It has previously been shown that the CD8 to Treg and CD8 to CD4 effector ratios have a prognostic value in certain cancers including melanoma and breast cancer. In the ccRCC patients, we observed no difference in the CD8 to CD4 effector ratio in PBMC, normal tissue and TILs. However, the CD8 to Treg ratio was higher in the normal tissue compared to PBMC and TILs (p=0.003 and p=0.002, respectively). Similarly, the CD4 effector to Treg ratio was also higher in the normal tissue compared to PBMC and TILs (p=0.002 and p=0.002, respectively) (Figure 3.1C).
Figure 3. 1 Comparison of T cell subsets in tumour, normal tissue and PBMC in ccRCC.

(A). Heatmaps and UMAPs show the CD3 pre-gated T cell phenotypes in tumour (n=27), normal tissue(n=11) and PBMC (n=21). (B). Comparison of CD8, CD4 effector and Treg frequencies out of the CD3 cells in different tissue types is shown. Median value for multiregional tumour areas was used per patient. (C). Comparison of T cell ratios in between PBMCs, normal tissue and TILs is shown. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
3.2.2. CD8 cells have a different phenotype in different tissue types

The expression profile on CD8 cells was evaluated and compared between PBMC, normal tissue and TILs. It was seen that the CD8 cells in the TILs had a higher level CD103 cells compared to the PBMCs (p= <0.001) which suggests presence of TRM cells in the tumour microenvironment compared to peripheral blood. Similarly, normal tissue also had more TRM cells compared to the PBMC (p=<0.001). In addition, HLA-DR was higher in the TILs compared to normal tissue and PBMC (p=<0.001 for both). HLA-DR is transmembrane glycoprotein which are expressed in activated lymphocytes, B cells and antigen presenting cells. HLA-DR is encoded by the telomeric region of the MHCII gene cluster on chromosome 6. HLA-DR expression is upregulated in antigen experienced cytotoxic T cells. Increased expression of HLA-DR both on T cells and on tumour cells has been associated with better clinical outcomes in several cancers including breast cancer, squamous cell carcinoma of the larynx and colorectal cancer. Markers associated with T cell dysfunction such as Eomes, PD-1, TIM-3, CD39 and CTLA-4 are expressed at higher levels in the TILs compared to normal tissue (p=<0.001, p=<0.017, p=<0.001, p=<0.001 and p=0.003) and PBMC (p=0.004, p=<0.001, p=<0.001 and p=0.001). CD38 which is another marker of dysfunction/exhaustion was expressed at higher levels in the TILs and normal tissue compared to PBMC (p=<0.001 and p=0.002, respectively). ICOS is a co-stimulatory molecule expressed on T cells and causes T cell activation. It has a structure similar to CD28 which is another co-stimulatory molecule. However, different to CD28, upregulation of ICOS leads to IL-10 production rather than IL-2. Both TCR engagement and CD28 are required to induce ICOS upregulation in a T cell. It has been shown that treatment with agonistic ICOS antibody leads to upregulation of cytotoxic CD8 cells and CD4 effectors as well as up to 40% reduction in the tumour size in preclinical mouse models. In the TRACERx Renal cohort, ICOS was found at higher levels in the TILs compared to the PBMC and normal tissue (p=<0.001 and p= 0.006, respectively). The phenotypes observed in the PBMC were different compared to TILs and normal tissue. PBMCs had higher expression of CD45RA compared to normal tissue and TILs (p=<0.003 and p=<0.0001, respectively) suggesting higher abundance of Temra cells in the PBMC. CD27 was also found in higher levels in the PBMC compared to TILs (p=0.04). Expression of KLRG1 and TIGIT was also higher in PBMC compared to TILs (p=0.001 and p=0.013, respectively). KLRG1 stands for killer-cell lectin like receptor G1 and has a co-inhibitory function. Binding to E-cadherin is critical for KLRG1 to perform its inhibitory function. It is expressed in antigen experienced T cells and NK cells. It has been shown that the T cells that express KLRG1 are able to produce cytokines but they have lost their ability to proliferate. KLRG1 expression is higher in CD8 cells compared to CD4.
cells. The expression level increases with age and also in more differentiated memory T cells.\textsuperscript{172,173,174} TIGIT (T cell immunoglobulin and ITIM domain) is a co-inhibitory molecule that is expressed on T cells and NK cells following activation.\textsuperscript{175} TIGIT has three ligands, CD155, CD112 and CD113, which are expressed on macrophages, B cells, dendritic cells and several organs in the body including lungs and kidneys as well as tumour cells.\textsuperscript{176} It has been shown that TIGIT expression on CD8 cells impairs the tumour specific cytotoxic function of CD8s.\textsuperscript{177} TIGIT expression on T cells has been associated with worse prognosis in multiple cancers including colorectal cancer, melanoma and gastric cancer.\textsuperscript{178,179,180} In mouse glioblastoma mouse models, it was seen that combination treatment with anti-TIGIT and anti-PD1 therapy to increased survival, increase number of effector CD8 cells as well as reduction in Tregs.\textsuperscript{181} In addition, Granzyme B was higher in the PBMCs in relation to normal tissue and TILs. (p=0.005 and p=0.006, respectively) (Figure 3.2A).
Figure 3. 2 Comparison of CD8 T cell phenotypes in tumour, normal tissue and PBMC in cCRCC.

(A). Dot plot shows expression of differentiation markers on CD8 cells in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. (B). Dot plot shows the checkpoint expression profile on CD8 cells in tumour (n=25), normal tissue (n=11) and PBMC (n=21) samples. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM). Eomes, Hobit, Ki67, Granzyme B and CTLA-4 are intracellular markers. Other markers shown in the plots are intracellular.
After analysing the phenotype of CD8 cells, we then looked at the different differentiation subsets in the PBMC, normal tissue and TILs. We have observed that the abundance of T cells with different differentiation subsets was different in all three (Figure 3.3A and B). There are more Temra (CD45RA^+CCR7^-) and naïve (CD45RA^+CCR7^+) T cells in PBMC compared to normal tissue and TILs (p=0.004, p=0.015 and p=<0.0001 p=0.006, respectively). On the other hand, TRM (CD45RA^CD103^+) CD8 cells were more abundant in the TILs and normal tissue compared to PBMC (p=<0.0001 and p=<0.0001, respectively) (Figure 3.3A).

Given we observed significant differences in the T cell differentiation subsets across the different tissue types, we have looked at the phenotypic features of these different T cell subsets. One of the main observations was that the Temra cells were high in CD57 across all tissue types. In PBMC, Temra cells had higher Granzyme B expression compared to normal tissue and TILs (p=0.003 and p=0.013, respectively (Figure 3.4A). On the other hand, HLA-DR and CD38 higher in the normal tissue and TILs (p=0.011 and p=<0.0001) and CD38 (p=0.045 and p=<0.001) compared to PBMC (Figure 3.4A and B). PBMCs also had a higher level of CD27 compared to the TILs (p=0.002). TRM phenotype was only compared between normal tissue and TILs as the PBMC has negligible amount TRM. TRM cells had very low levels of Ki67, KRLG1 and CD25 in both tissue types. However, PD-1, Eomes, TIM-3, CTLA-4, CD38 and CD39 which are associated with T cell dysfunction were higher in the TILs compared to normal tissue (p=<0.0001, p=<0.0001, p=<0.0001, p=0.002, p=0.032 and 0=0.029 respectively). HLA-DR and CD57 which are associated with antigen experience and terminal differentiation were also higher in the TILs compared to normal tissue (p=<0.0001 and p=<0.0001, respectively). Hobit was also higher in the TILs (p=0.004). ICOS, TIGIT and GITR which are were also expressed at higher levels in the TILs (p=0.006, p=0.044 and p=0.012 respectively) compared to normal tissue.
Figure 3. 3 CD8 cells have different differentiation subsets in different tissue types in ccRCC.

(A). Dot plot shows CD8 differentiation subsets in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM). (B). Concatinated FACS plots show CD8 differentiation subsets in TILs (n=27), normal tissue (n=11) and PBMC (n=21) samples.
Figure 3.4 Expression profile in CD8 Temra and TRM cells

(A). Dot plots show expression pattern in CD8 Temra cells in tumour (n=27), normal tissue(n=11) and PBMC (n=21) samples. (B). Dot plots show checkpoint expression pattern in CD8 Temra cells in tumour (n=27), normal tissue(n=11) and PBMC (n=21) samples. (C). Dot plots show expression pattern in CD8 TRM cells in tumour (n=25), normal tissue(n=11) and PBMC (n=21) samples. (D). Dot plots show checkpoint expression pattern in CD8 TRM cells in tumour (n=25), normal tissue(n=11) and PBMC (n=21) samples. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
3.2.3. PD-1+ and PD-1- CD8 cell have different expression profiles

PD-1+ CD8 cells have previously been described as dysfunctional cells. We therefore analysed the characteristics of the PD-1+ cells across different tissue types and had compared it with the PD-1- CD8 cells. Both PD-1+ and PD-1- CD8 cells had higher CD103 expression in the normal tissue and TILs suggesting a TRM phenotype which is in alignment with the results explained above (p< 0.001 for all). HLA-DR and CD38 levels were higher in the in normal tissue and TILs compared to PBMC both in PD-1+ CD8 (p=0.009 and p< 0.001 for HLA-DR; p< 0.001 and p< 0.001 for CD38, respectively) and PD-1+ CD8 cells (p=0.015 and p< 0.001; p< 0.001 and p< 0.001 for CD38, respectively). In addition, Eomes expression was higher in the PD-1+ CD8 TILs compared to both normal and PBMC. On the other hand, PBMC had higher levels of CD45RA, CD57 and Granzyme B expression compared to normal tissue (p=0.001, p< 0.001 and p< 0.001, respectively) and TILs (p< 0.001 for all three markers) and Hobit compared to normal tissue (p=0.003) which resembles the Temra phenotype observed earlier in the chapter (Figure 3.5A).

Comparison of expression pattern on PD-1+ and PD-1- CD8 cells in the TILs showed that PD-1+ CD8 cells had higher levels of CD103 (p=0.0003) suggesting a more abundant TRM subset PD-1+ CD8s compared to PD-1- CD8 cells in the TILs. On the other hand, CCR7 (p=0.002) and CD45RA (p< 0.001) levels were higher in the PD-1- CD8 cells suggesting a higher abundance of TCM and Temra populations compared to PD-1+ CD8s. In addition, PD-1- CD8 cells were also higher in Granzyme (p< 0.001) suggesting a more cytotoxic phenotype compared to PD-1+ CD8 cells. Eomes, CD38, CD39, TIM-3 (p< 0.001 for all 4 makers) which are markers associated with a dysfunctional phenotype were found to be higher on PD-1+ CD8 cells compared to the PD-1- CD8s suggesting a more dysfunctional phenotype of the PD-1+ CD8 cells in the ccRCC cohort. In addition, CD27, CTLA-4, ICOS and GITR (p< 0.001, p=0.001, p< 0.001 and p=0.001 respectively) as well as the T cell activation marker 4-1BB (p< 0.001) and Hobit (marker of tissue residency and effector function) (p=0.04) were also expressed at higher levels on the PD-1+ cells compared to the PD-1- ones (Figure 3.6A). In contrast to the TILs, PD-1+ CD8 and PD-1- CD8 cells in normal tissue and PBMC has more similar expression profiles. In the normal tissue, PD-1+ CD8 cells had higher CD103 (p=0.005) suggesting a higher proportion of TRM cells. In addition, the T cell activation and exhaustion marker CD38 (p=0.009) and CD27 (p=0.009) expression was higher in the PD-1+ CD8 while PD-1+ CD8 cells had higher Granzyeme B (p=0.006) and TIGIT (p=0.048). (Figure 3.7A). In PBMC, PD-1+ CD8 had higher levels of CCR7 (p< 0.001) which suggests a more naïve phenotype compared to PD-1+ CD8 cells. On the other hand PD-1+ CD8 cells had higher levels of CD57 (p< 0.001), CD38 (p=0.009) and CD27 (p=0.009) suggesting a more
significant proliferation history, terminal differentiation and T cell activation (Figure 3.8A). Some of the markers were found to be co-expressed in CD8 cells in PBMC, normal tissue and TILs (Figure 3.1A). Temra cells which also co-expressed CD57 and Granzyme B as well as Eomes were higher in the PBMC compared to normal tissue (p=0.02 and 0.02, respectively) and TILs (p=<0.001 and p=<0.001, respectively) (Figure 3.9A). In addition, TRM cells which co-express CD57 and PD-1 are higher in the normal tissue compared to PBMC (p=0.02). This population is also higher in TILs compared to PBMC (p=0.002). TRM cells which co-express CD38 and CD38 and TIM-3 in addition to CD57 and PD-1 are higher in TILs compared to normal tissue (p=0.002 and p=0.005, respectively) and PBMC (p=< 0.001 and p=< 0.001, respectively). Compared to PBMC, normal tissue also had higher levels of CD57^+PD-1^+CD38^+ and CD57^+PD-1^+CD38^+TIM-3^+ TRMs (p=<0.001 and p=0.02, respectively) (Figure 3.9B). These suggest a more dysfunctional phenotype in TILs and normal tissue.
Figure 3. 5 Expression profile in PD1+CD8 and PD-1+CD8 cells
(A). Dot plots show expression pattern in PD1+CD8 cells in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. (B). Dot plots show expression pattern in PD-1+CD8 cells in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. (n=25), normal tissue (n=11) and PBMC (n=21) samples. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
Figure 3. 6 Comparison of expression profile in PD1⁺CD8 and PD-1⁻CD8 cells in TILs

(A). Dot plots show comparison of expression profile in PD1⁺CD8 and PD-1⁻CD8 cells in TILs (n=27). Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
Figure 3. 7 Comparison of expression profile in PD1+CD8 and PD-1+CD8 cells in normal tissue

(A). Dot plots show comparison of expression profile in PD1+CD8 and PD-1+CD8 cells in normal tissue (n=11). Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
Figure 3. Comparison of expression profile in PD1+CD8 and PD1-CD8 cells in PBMC (A). Dot plots show comparison of expression profile in PD1+CD8 and PD1-CD8 cells in PBMC (n=21). Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
Figure 3. Dot plots show co-expression pattern on CD8 Temra cells in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. Dot plots show co-expression pattern on CD8 Temra cells in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
3.2.4 CD4 effector cells have different T cell subsets in different tissue types

Most of the tumour killing activity is believed to be carried out to be by the CD8 cells. One of the main reasons for this that the tumour cells mainly express MHCI which is recognised by CD8 cells. On the other hand CD4 cells recognise MHCII and are believed to have a helper role for the CD8s to perform their cytotoxic function. However, recent studies show that CD4 cells also play an important anti-tumour function, independent of the CD8 cells. Studies in mouse models suggest that naïve CD4 cells that get activated in the lymph nodes secrete cytokines at the tumour site which leads to activation of macrophages and NK cells. In the clinical setting, differentiated CD4 effector cells have been associated with better response to chemotherapy in uveal melanoma and breast cancer patients. We analysed the differentiation states in the CD4 effector cells (CD4⁺FoxP3). Naïve CD4 cells were higher in PBMC compared to normal tissue (p=0.0004) and TILs (p=<0.0001). In addition, TCM CD4 cells were higher in the PBMC compared to normal tissue (p=0.006) but the level was similar in TILs. On the other hand, TRM CD4 effector cells were higher in TILs compared to the PBMC (p=0.003). Normal tissue also had a higher proportion of TRM cells compared to the PBMC (p=0.02) (Figure 3.10A and B). This pattern is similar to the differentiation pattern described in CD8 cells earlier in this chapter.

Although the expression pattern in differentiation states was described for CD8 cells, expression pattern of naïve, TCM, Temra and TRM cells was not analysed due to the smaller number of CD4 cells. Similar to CD8 cells, CD4 effectors also had higher levels of CD103 in the TILs compared to PBMC (p=<0.001) suggesting a significantly higher level of TRMs in the TILs. HLA-DR expression was also higher in the TILs compared to PBMC (p=<0.001) and normal tissue (p=<0.001) suggesting a history of more antigen experience. On the other hand, PBMC had higher levels of CD45RA compared to the TILs suggesting a more abundant Temra population in the PBMC (p=0.006). In addition, CD27 and CD25 levels were also higher in the PBMC compared to normal tissue (p=0.02 and p=<0.001, respectively) and TILs (p=<0.001 and p=<0.001, respectively). Compared to PBMC, TILs were also found to express higher levels of checkpoints which are involved in a more dysfunctional T cell phenotypes and these include PD-1, TIM-3, CD39 and CD38 (p=<0.001, p=<0.001, p=<0.001 and p=0.002, respectively). PD-1 was also higher in normal tissue compared to PBMC (p=0.021). In addition, co-inhibitory marker CTLA-4 and TIGIT were higher in the TILs (p=<0.001 and p=0.005, respectively) and TIGIT was higher the normal tissue (p=0.009, respectively) compared to PBMC. ICOS, which is a co-stimulatory molecule, was higher in the TILs compared to PBMC (p=0.005). Comparison of the normal tissue and TILs also showed higher
CD39, TIM-3 and KLRG1 on the TILs (p=0.006, p=0.02 and p=0.009) respectively (Figure 3.11A).

Figure 3. 10 Comparison of CD4 effector differentiation subsets in different tissue types

(A). Dot plot shows CD4 effector differentiation subsets in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM). (B). Concatenated FACS plots show CD4 effector differentiation subsets in TILs (n=25), normal tissue (n=11) and PBMC (n=21) samples.
Figure 3. 11 Comparison of the expression pattern on CD4 effector T cells in tumour, normal tissue and PBMC in ccRCC.

A. Dot plot shows expression of differentiation markers on CD4 effector T cells in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples.

B. Dot plot shows expression of checkpoints on CD4 effector T cells in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
3.2.5 PD-1⁺ and PD-1⁻ CD4 effectors cells have different phenotypes

Phenotype of PD-1⁻ and PD-1⁺ CD4 effector cells were analysed for PBMC, normal tissue and TILs. CD103 expression was higher in the TILs (p=0.006) and normal tissue (p=0.001) compared to PBMC in the PD-1⁺ CD4 effector cells suggesting a TRM phenotype. On the other hand, CD103 expression was less in the PD-1⁻ CD4 effector cells across all tissue types. Both in PD-1⁻ and PD-1⁺ CD4 effector, CD27 was higher in the PBMC compared to the TILs (p= <0.001 and p= 0.02). In addition, CD27 and TIM-3 levels were also higher in the normal tissue compared to TILs in the PD-1⁺ CD4 effector cells (p=0.003 and p=0.008, respectively). Although there was no significant difference in the CCR7 expression in the PD-1⁺ CD4 effector cells, CCR7 was higher in the PBMC compared to the normal tissue (p=0.005) and TILs (p=0.04) in the PD-1⁻ CD4 cells suggesting the PD-1⁻ cells have a more naïve phenotype compared to PD-1⁺ CD4s. In addition, PD-1⁺ CD4 TILs expressed higher levels of CD38 (p= <0.001), CD39 (p= <0.001), TIM-3 (p= <0.001), CTLa-4(p=0.01), GITR (p=0.01) and TIGIT(p=0.02) compared to PBMC. In the PD-1⁺ cells, CTLa-4, CD39 and TIM-3 expression was higher in the TILs compared to the PBMC (p=0.01, p=0.02 and p=0.02, respectively) (Figure 3.12A and B).

Comparison of the PD-1⁻ and PD-1⁺ CD4 effector cells in the TILs show a higher expression of CCR7 in the PD-1⁻ cells suggesting either a naïve or TCM phenotype (p=0.002). On the other hand, PD-1⁺ CD4 effectors had a higher expression of Eomes (p= <0.001). In addition, CTLa-4, CD38, TIM-3 and GITR were also higher in the PD-1⁺ cells compared to the PD-1⁻ CD4 effectors (p= <0.00, p= <0.001, p=0.008 and p=0.002, respectively) (Figure 3.13A). In the normal tissue, CD103 expression is higher in the PD-1⁺ cells suggesting a higher levels of TRMs (p=0.003). Eomes, CTLa-4 and CD38 expression were also higher in the PD-1⁺ CD4 effector cells in the normal tissue (p=0.003, p=0.01 and p=0.008 respectively). In addition, the co-stimulatory checkpoints CD27 and GITR were also higher in the PD-1⁺ cells compared to PD-1⁻ CD4 effector cells (p=0.03 and p=0.01, respectively) (Figure 3.14A). Proliferation marker Ki67(p=0.001), KLRG1 (p=0.01) and CD101(p=0.02) were also expressed at higher levels in the PD-1⁺ CD4 effectors. Analysis of the PBMC showed a higher Eomes, CD57 levels in the PD-1⁺ cells (p= <0.001 for both) as well as Granzyme B (p=0.001) and CD27 (p=0.001) while the CCR7 expression was higher in the PD-1⁻ cells (p= <0.001) (Figure 3.15A).
Figure 3. 12 Expression profile in PD-1^+ CD4 effector and PD-1^- CD4 effector cells

(A). Dot plots show expression pattern in PD1^+ CD4 effector cells in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. (B). Dot plots show expression pattern in PD-1^- CD4 effector cells in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
Figure 3. 13 Comparison of expression profile in PD1+CD4 effector and PD-1- CD4 effector cells in TILs

(A). Dot plots show comparison of expression profile in PD-1+CD4 effector and PD-1-CD4 effector cells in TILs (n=27). Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
Figure 3.14 Comparison of expression profile in PD-1⁺CD4 effector and PD-1⁻ CD4 effector cells in normal tissue

(A). Dot plots show comparison of expression profile in PD-1⁺CD4 effector and PD-1⁻ CD4 effector cells in normal tissue (n=11). Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
Figure 3. 15 Comparison of expression profile in PD1⁺CD4 effector and PD-1⁻
CD4 effector cells in PBMC

(A). Dot plots show comparison of expression profile in PD1⁺CD4 effector and PD-1⁻
CD4 effector cells in PBMC (n=21). Sidak-Bonferroni multiple t-test was used for statistical
analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples
where difference between samples is not statistically significant (p>0.05). Error bars show
mean +/- standard error of the mean (SEM).
3.2.5. Phenotype of Tregs in TILs, normal tissue and PBMC

In addition to analysing the CD8 and CD4 effector cell phenotypes, Tregs were also analysed. The Tregs were defined as CD4+FoxP3+ cells. Due to the small number of Tregs, differentiation states and expression pattern on PD-1+ and PD-1- Tregs were not analysed. PBMCs were found to have higher CCR7 and Granzyme expression compared to normal tissue (p= <0.001 and p=0.003, respectively) and TILs (p=<0.001 and p=0.01, respectively). On the other hand, HLA-DR expression was higher on the TILs compared to PBMC (p=<0.001). Co-stimulatory marker GITR and ICOS as well as the activation marker 4-1BB were found at higher levels in the TILs compared to the PBMCs (p=<0.001 for all three markers). PD-1, CTLA-4, CD39 and TIM-3, which are markers associated with T cell dysfunction were expressed at higher levels in the TILs compared to the PBMC (p=<0.001 for all four markers). CTLA-4, GITR and CD39 were higher in the TILs compared to normal tissue (p=0.006, p=.0.002 and p=0.01, respectively). In addition, TIGIT was also higher in the TILs compared to normal tissue and PBMC (p=0.001 and p=<0.001, respectively) (Figure 3.16A).
Figure 3. 16 Comparison of expression profile in Tregs

(A). Dot plots show comparison of expression profile of Tregs in tumour (n=27), normal tissue (n=11) and PBMC (n=21) samples. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM).
3.3 CHAPTER DISCUSSION

In this chapter, T cell phenotypes in the TILs, normal tissue and PBMC in ccRCC patients were analysed. One of the main limitations of the analysis was the lack of clinical outcome and demographic data. Therefore, the analysis was mainly descriptive. Another limitation was that there was a difference in the sample size for TILs, PBMC and normal tissue due to availability of samples. Although the analysis was very descriptive and limited, it still provided insight into understanding the differences in T cells between TILs, normal tissue and PBMC in treatment naïve ccRCC patients. The data showed that the T cell phenotypes in PBMC, normal tissue and TILs were different. However, the checkpoint expression pattern in the normal tissue was similar to the TILs to certain degree.

In more detail, the results showed that CD8 cells were the most abundant T cell subset in TILs, normal tissue and PBMC followed by CD4 effector cells and Tregs. In the CD8 subset, naïve and Temra cells were found in higher levels in the PBMC. On the other hand, TRM cells were more abundant in the TILs and normal tissue compared to PBMC with highest level in the TILs. CD8 TILs had higher expression of PD-1, TIM-3, CTLA-4, CD38 and Eomes which suggests a dysfunctional CD8 T cell phenotype. In addition, TILs also had a higher levels CD39, GITR, ICOS and HLA-DR. CD8 TILs with a TRM differentiation also had higher expression level of PD-1, TIM-3, CTLA-4, Eomes, CD38, CD39, GITR, ICOS and Hobit compared to normal tissue. However, the expression of these markers was much lower in the Temra cells in the TILs except for Hobit and HLA-DR. This shows a difference in the phenotype of Temra and TRM CD8 cells in addition to the differences in different tissue types. This data suggests that there is a dysfunctional phenotype in the CD8 TILs with co-expression of PD-1, TIM-3, CTLA-4, CD38 and Eomes and this dysfunctional phenotype is also observed in the TRM CD8 cells whereas the Temra cell do not have the dysfunctional state. This suggests that the differentiation into the dysfunctional phenotype mainly happens in the TRM CD8 T cells as previously shown before. The literature on the role of CD39 has been controversial. On the one hand, it has been proposed that CD39 is a marker of terminally dysfunctional T cells as the CD39+ CD8 cells in mouse models and breast cancers patients had reduced cytokine production and upregulated markers of T cell dysfunction. On the other hand, CD39+ TRM CD8 cells were associated with better overall survival in patients with head and neck squamous cell carcinoma, high grade ovarian serous cancer and non-small cell lung cancer. In addition, tumour reactive T cells in functional assays in these patients also had a CD39+ TRM phenotype and CD39 was upregulated upon TCR activation. More research is needed to clarify the role of CD39 in the renal cancer. Given the increased CD39
expression in ccRCC specifically in TILs, correlating this expression with clinical outcome can potentially have a predictive role. It has previously shown that the PD-1+ and PD-1- T cells have different characteristics and they have a predictive role in response to anti-PD-1 therapy. Evaluating PD-1+ and PD-1- CD8 cells showed that both cell types had different phenotypes in different tissue types. The most pronounced difference between the PD-1+ and PD-1- cells was seen in the TILs. CD103 and Hobit levels were higher in the PD-1+ CD8 cells in the TILs compared PD-1- CD8 cells which suggests a bigger proportion of TRMs in the PD-1+ CD8 cells. On the other hand, PD-1+ CD8 cells had higher CCR7 and CD45RA expression suggesting a more abundant naïve and Temra CD8 cells. Higher HLA-DR expression on PD-1+ cells also indicated history of more antigen experience compared to PD-1- CD8 cells. In addition, higher Eomes, CTLA-4, TIM-3, CD38 and CD39 expression on the PD-1+ CD8 cells shows a more dysfunctional phenotype in this subset of cells and highlights these checkpoints as potential target for combination immunotherapy. Current standard of care for renal cell carcinoma is combination anti-PD1 and anti-CTLA-4 therapy. Out of these markers, CD39 and TIM-3 were highest in the TILs with negligible levels in the PBMC and normal tissue. Their specificity for the tumour tissue and co-expression on the PD-1+ CD8 cells makes them a potential candidates for combination immunotherapy. CD39 is important in converting ATP to adenosine which has anti-inflammatory properties and therefore blocking CD39 is suggested to have a pro-inflammatory effect on the TME. Consequently there are several phase I and phase II clinical trials with anti-CD39 antibodies in the solid tumours. In addition, in melanoma mouse models, combination therapy with anti-CD39 and anti-TIM-3 blocking antibodies showed increased reduction in tumour size and improved survival. Profile of PD-1+ and PD-1- CD8 cells were similar both in normal tissue and PBMC with exception of higher CD38 and CD27 expression on the PD-1+ CD8s and higher CCR7 and granzyme B on the PD-1- CD8s.

Most of the therapies and work on tumour immunology and immunotherapies focused on the CD8 T cells until now. However, more recent studies highlight the potential role of CD4 effector cells in antitumour immunity. Therefore, we phenotyped the CD4 cells in detail. Similar to CD8 cells, CD4 cells also had higher CD103 expression on the TILs and normal tissue compared to PBMC suggesting a bigger TRM population in the TILs and normal tissue. In addition, naïve CD4 cells were higher in the PBCM compared to TILs and normal tissue which is similar to the CD8 findings. The checkpoint expression pattern on the CD4 cells was also similar to CD8 cells with higher expression of HLA-DR, PD-1, Eomes, CTLA-4, TIM-3, CD38 and CD39 on the TILs compared to PBMC and normal tissue. These makers were also found in higher levels in the TILs in the PD-1+ CD4
effectors cells in addition to higher GITR expression and TIGIT. Expression of these checkpoints suggest a more dysfunctional CD4 phenotype in the CD4 effector TILs. Higher expression of HLA-DR indicated the prolonged exposure to antigens which could be reason to differentiation into a more dysfunctional CD4 effector phenotype as described previously. Compared to PD-1+ CD8 cells, TIGIT expression is more specific to CD4 effector TILs including PD-1+ CD4 effectors. Given the pro-tumourogenic effect of TIGIT as shown previously, targeting TIGIT can be important in enhancing CD4 mediated immunity and combination therapy with anti-PD-1 therapy can potentially be important in reversing the dysfunctional state of CD4 effector cells in the tumour microenvironment.

In addition, increased KRLG-1 expression in the normal tissue was higher compared to PBMC and TILs suggest a subset of CD4 effector memory cells with higher cytokine secretion. This could potentially represent a transitional state of CD4 effectors cells before they migrate to tumour and differentiate into a more dysfunctional state.

The Tregs were analysed in less detail due to the small cell count. However, phenotypic analysis of the total Tregs showed that Tregs in the TILs had higher expression of HLA-DR as well as the CTLA-4, CD39, GITR, PD-1 and TIM-3, TIGIT and ICOS which similar to the profile seen in CD4 effector TILs. The CTLA-4, TIGIT, and GITR expression was higher on the Tregs compared to the expression in CD4 effector TILs. In addition, there was no statistically significant difference in GITR expression between PBMC, normal tissue and TILs on overall CD4 effectors although it was significantly higher on the PD1+ CD4 effectors in TILs and normal tissue. Therefore, it could be a good target to deplete Tregs given the anti-inflammatory effects Tregs have in the tumour microenvironment. It has been shown that both in glioblastoma mouse models and in phase 1 clinical trial in solid tumours that agonistic anti-GITR antibodies help with depleting the immunosuppressive Tregs and also made the Tregs gain CD4 effector function. In addition, combining the anti-GITR antibody with anti-PD-1 antibody had a synergistic effect and improved survival in mice with glioblastoma. However, the data discussed in this chapter needs to be correlated with clinical outcome to assess which of the markers correlate with better or worse clinical outcome. A bigger sample size, especially in the normal tissue and the PBMC cohort is needed in order to be able make conclusions.

In summary, T cell differentiation is different in tumour and peripheral blood where naïve and Temra cells (in both CD8 and CD4) are mainly found in the peripheral blood whereas TRMs are found the tumour and are seen in negligible levels in the peripheral blood. Characterisation of the CD8 T cells showed an exhausted phenotype in the tumour
and these cells had CD39 upregulation. This highlights CD39 as a potential target for therapy in RCC and correlating this data with clinical outcome is important. Analysis of CD4 effector cells showed an upregulation of TIGIT and GITR in TILs. Studying these two checkpoints further can be important in understanding the role of CD4 T cells in antitumour immunity. CD4 cells have a helper role for cytotoxic CD8 T cells and new immunotherapy targets in CD4 T cells can potentially be useful in developing combination therapies to enhance the effect of existing checkpoint inhibitors. Depleting Tregs has been an important concept in the field of cancer immunotherapy due to the immunosuppressive effect of Tregs. Increased expression of TIGIT and GITR on Tregs highlight them as potential targets for immunotherapy. Engineering antibodies to target CD25 as well as TIGIT or GITR can allow for Treg specific targeting. In addition, it is important to correlate the phenotype described in this chapter with clinical outcome data.
Following the discovery of the role of checkpoint blockade in cancer therapy, clinical trials have shown immunotherapy has an important role in RCC. In the phase III setting, nivolumab, an anti-PD-1 checkpoint inhibitor, was observed to improve overall survival (25 months) compared to the mTOR inhibitor everolimus (19.6 months) in metastatic RCC patients who progressed on first-line therapy. More recently, it was shown that combination therapy with ipilimumab and nivolumab in the neoadjuvant setting improved overall survival and objective response rate compared to sunitinib in patients with treatment naïve advanced RCC. Despite the improved clinical outcome with checkpoint blockade in RCC, the mechanisms of response and resistance to immunotherapy is yet to be clarified. There is no established biomarker to determine which cohort of patients will respond to checkpoint blockade. It has recently been shown that the CD8 infiltration does not predict response to nivolumab therapy in ccRCC. However, there is still no established biomarker which can help identify patients who are likely to respond to anti-PD-1 therapy. In addition, mechanism of resistance to treatment is poorly understood too.

TCR is a heterodimer protein expressed in all T cells and mediates antigen recognition by the T cell. TCR has α and β chains which consist of extracellular domains, variable (V) and constant (C) regions. During T cell development, V, D and junctional (J) alleles go through recombination which leads to diversity of TCRs. Each domain has three complementarity-determining regions (CDR) which are hypervariable regions. Out of the three, CDR3 is the most crucial one in antigen recognition and binding due to its very hypervariable structure. CDR3 recognises and binds to the antigen presented by the major histocompatibility complex (MHC). In the recent years, emerging data in the field showed that TCR repertoire plays an important role in determining response to immunotherapy. Cha and colleagues showed that anti-CTLA-4 therapy increases the diversity of the TCR clones in the tumour and maintenance of these expanded TCR clones is associated with response to immunotherapy in melanoma. In addition, TCR clonality in the peripheral blood was also shown to have a predictive role in response to immunotherapy. Similar results were seen with anti-PD1 treatment where PD-1 mediated immune-editing lead to expansion and maintenance of TCR clones which were important in response to immunotherapy. In multiple cancer types, including RCC, it was shown the certain TCRs are shared between patients and different tumour regions and these clones can be important targets for adoptive T cell immunotherapy. Currently, there are several TCR sequencing protocol available. In this project, we
have used a bespoke TCR sequencing method that was developed in Prof. Benny Chain’s laboratory. In this bespoke method, unique UMIs are ligated to the complementary DNA (cDNA) by using T4 RNA ligase. Using UMIs allows to identify errors which can occur due to amplification or sequencing.\textsuperscript{132}

This chapter will focus on ADAPTeR - A Study of anti-PD-1 (nivolumab) in Therapy as Pre- and Post-Operative Therapy in Metastatic Renal Cell Cancer (NCT02446860). ADAPTeR is a phase II, single-arm, open-label study of nivolumab in patients with metastatic ccRCC patients with no prior systemic therapies. An important translational aspect of the trial was obtaining multiregional biopsies to allow multiple fresh tumour sampling from primary and/or metastatic sites at baseline and week-9 of treatment as well as at nephrectomies when possible. One of the key aims of the study was to evaluate immune correlates of response and changes on therapy. We hypothesised that high T cell infiltration and low macrophage infiltration at baseline will be associated better response to anti-PD-1 therapy. In addition, we also hypothesized that TCR clonality at baseline will be prognostic and will change with anti-PD-1 therapy. In order to test our hypothesis, samples were analysed by using multiplex IF, IHC, flow cytometry and TCR sequencing to answer the questions stated below:

- What is the immune landscape in ccRCC at baseline and how does it correlate with clinical outcome?
- How does the immune landscape in ccRCC change with nivolumab treatment and how does it correlate with response to treatment?
- What is the TCR repertoire in the ccRCC in the tumour and peripheral blood at baseline and on treatment?
- Does the TCR repertoire change with nivolumab treatment in the tumour and in peripheral blood? If so, how does it correlate with response to nivolumab?

The results presented in this chapter are part of several collaborations. The ADAPTeR trial was ran at the Royal Marsden Hospital and the clinical data was collected by Dr. Lewis Au who is a clinical fellow in Dr. Samra Turajlic’s lab. However, the clinical analysis and the clinical figures were made by myself. For the immunofluorescence data, the panel optimisation was done by the Prof. Teresa Marafioti and her team. They have also helped with the staining although I have done the bulk of the staining. In addition, I have done all the analysis of the IF data. The IHC staining was done by Teresa Marafioti’s team. I have done the scoring with her and the analysis was done by me. I have done all the TCR...
experiments in Prof. Benny Chain’s laboratory and the bioinformatics analysis/figures was done by Marc Robert de Massy who is a PhD student in Prof. Sergio Quezada’s laboratory. However, I have contributed to the analysis by generating questions to direct the analysis.

Data presented in this chapter is also used in our article titled ‘Determinants of anti-PD-1 response and resistance in clear cell renal cell carcinoma’ which is accepted to Cancer Cell and I am a joint first author on the paper.

4.2 RESULTS
4.2.1 Overview of the ADAPTeR Study

ADAPTeR is a single arm, open label, phase II study of anti-PD1 (nivolumab) therapy as pre- and post-operative therapy in metastatic renal cell cancer. The population in this trial consisted of patients with metastatic clear cell renal cell carcinoma with predominant clear cell histology. 15 patients were enrolled in the ADAPTeR trial from October 2015 to June 2018. Out of the 15 patients, 13 were males and 2 were females. The intervention in the trial was giving patient nivolumab 3 mg/kg intravenously every two week for eight weeks. Patients who were suitable for nephrectomy after 8 weeks of nivolumab had nephrectomies (n=2). Nivolumab treatment continued after nephrectomy and assessment for nephrectomy until patients did not get any more benefit from the treatment. Patients were followed up until death or withdrawal of consent from follow up. There was no control arm in this study. Primary and secondary endpoints of the study relate to safety and clinical efficacy. Six patients died due to disease progression. The outcome was measured in median PFS and OS. Disease progression was seen in 10 progression patients. The median PFS and OS were 4.1 months and 17.5 months, respectively. To correlate clinical outcome with the translational research findings, clinical response was defined as the percentage of patients with partial response (PR) or stable disease (SD) for ≥ 6 months (‘responder’) and was seen in 33% of the patients (n=5). Clinical non-response was defined as disease progression within <6 months of treatment (‘non-responder’) (n=10, 66% of all patients). Comparison of the responders and non-responders showed that there is no significant difference in age (p=0.835), ECOG performance status (p=0.864) and IMDC risk score (p=0.83) between responders and non-responder. On the other hand, the PFS was significantly higher in the responders (p=0.002) but there was no difference in the OS between the two cohorts (p=0.757). 2 patients (ADR001, a non-responder patient who had <6 months clinical benefit and SD; and ADR013, a responder patient who had ≥6 months clinical benefit and PR) underwent a cytoreductive nephrectomy during the trial (Table 4.1).
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Clinical benefit</th>
<th>Sex</th>
<th>Age (at start of treatment)</th>
<th>ECOG</th>
<th>IMDC Risk Score</th>
<th>Best response</th>
<th>PFS (days)</th>
<th>OS (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR001</td>
<td>&lt;6 months</td>
<td>M</td>
<td>52</td>
<td>0</td>
<td>2</td>
<td>SD</td>
<td>180</td>
<td>832</td>
</tr>
<tr>
<td>ADR002</td>
<td>&lt;6 months</td>
<td>F</td>
<td>56</td>
<td>2</td>
<td>3</td>
<td>PD</td>
<td>44</td>
<td>381</td>
</tr>
<tr>
<td>ADR003</td>
<td>&lt;6 months</td>
<td>M</td>
<td>51</td>
<td>1</td>
<td>3</td>
<td>PD</td>
<td>47</td>
<td>219</td>
</tr>
<tr>
<td>ADR004</td>
<td>&lt;6 months</td>
<td>M</td>
<td>62</td>
<td>0</td>
<td>0</td>
<td>SD</td>
<td>105</td>
<td>740</td>
</tr>
<tr>
<td>ADR005</td>
<td>&lt;6 months</td>
<td>M</td>
<td>52</td>
<td>0</td>
<td>1</td>
<td>PR</td>
<td>124</td>
<td>674</td>
</tr>
<tr>
<td>ADR006</td>
<td>&lt;6 months</td>
<td>M</td>
<td>50</td>
<td>1</td>
<td>3</td>
<td>PD</td>
<td>51</td>
<td>560</td>
</tr>
<tr>
<td>ADR007</td>
<td>&lt;6 months</td>
<td>M</td>
<td>50</td>
<td>1</td>
<td>2</td>
<td>SD</td>
<td>105</td>
<td>532</td>
</tr>
<tr>
<td>ADR008</td>
<td>&gt;6 months</td>
<td>F</td>
<td>59</td>
<td>0</td>
<td>0</td>
<td>PR</td>
<td>608</td>
<td>794</td>
</tr>
<tr>
<td>ADR009</td>
<td>&lt;6 months</td>
<td>M</td>
<td>68</td>
<td>0</td>
<td>1</td>
<td>PD</td>
<td>49</td>
<td>270</td>
</tr>
<tr>
<td>ADR011</td>
<td>&gt;6 months</td>
<td>M</td>
<td>49</td>
<td>0</td>
<td>2</td>
<td>PR</td>
<td>377</td>
<td>377</td>
</tr>
<tr>
<td>ADR012</td>
<td>&gt;6 months</td>
<td>M</td>
<td>80</td>
<td>1</td>
<td>2</td>
<td>SD</td>
<td>158</td>
<td>221</td>
</tr>
<tr>
<td>ADR013</td>
<td>&gt;6 months</td>
<td>M</td>
<td>63</td>
<td>1</td>
<td>2</td>
<td>PR</td>
<td>315</td>
<td>315</td>
</tr>
<tr>
<td>ADR014</td>
<td>&lt;6 months</td>
<td>M</td>
<td>71</td>
<td>0</td>
<td>1</td>
<td>SD</td>
<td>95</td>
<td>188</td>
</tr>
<tr>
<td>ADR015</td>
<td>&gt;6 months</td>
<td>M</td>
<td>74</td>
<td>1</td>
<td>2</td>
<td>SD</td>
<td>256</td>
<td>428</td>
</tr>
<tr>
<td>ADR016</td>
<td>&gt;6 months</td>
<td>M</td>
<td>55</td>
<td>0</td>
<td>1</td>
<td>PR</td>
<td>263</td>
<td>263</td>
</tr>
</tbody>
</table>

Table 4. 1 Clinical characteristics of the patients involved in the ADAPTeR study

Table shows the clinical benefit, gender, age, EOG performance status, progression free survival and overall survival of the patients in the ADAPTeR cohort. (SD=stable disease, PD=progressive disease, PR=partial response, M=Male, F=Female)
4.2.2. T cell infiltration at baseline does not predict response to anti-PD-1 treatment

To investigate immune cells in the tumour regions, we performed multiplex immunohistochemistry (mIHC) and immunofluorescence (mIF) on 61 formalin-fixed paraffin-embedded tumour samples (41 pre-treatment; 20 post-treatment) from 14 patients (Figure 4.1A, B and C). We applied bespoke antibody panels mIHC and mIF to quantify and characterise infiltrating immune cells (Figure 4.2). The median score for each patient was used for two sided Mann-Whitney U statistical analysis.
Figure 4.1 Workflow for mIF and IHC analysis and details of the samples used.

(A). Pathway for mIF analysis is shown. (B). Pathway for mIHC analysis is shown. (C). Details of the patient samples used for mIF and mIHC staining is shown.
Figure 4.2 Immunohistochemistry views of markers used for IF staining

Immunohistochemistry views for the markers used in the mIF staining (CD8, CD4, FoxP3, Pax8, MHCII, NFATc1, CD3, GrzmB and CD163) is shown. The staining is done on ccRCC tissue from the ADAPTeR patients and the images are taken at x40 magnification.

T cell infiltration was evaluated in treatment naïve tumour samples by using mIF. As also demonstrated in other studies previously\textsuperscript{125}, CD8 infiltration was not predictive of response to anti-PD-1 therapy. Evaluation of the T cells subsets out total T cells showed no difference in CD8 infiltration between responders (n=5) and non-responders (n=9) (p=0.6993, Figure 4.3A). In the treatment naïve samples, the level of CD4 effector T cell and Treg (scored as CD4 and FoxP3 double positive cells) infiltration was similar in responders (patients with $\geq$6 months clinical benefit) and non-responders (patients with $\leq$ 6 months clinical benefit from nivolumab treatment) (p=0.6064 and p=0.89811, respectively, Figure 4.3A). Similarly, evaluation of CD8 to Treg and CD4 effector to Treg ratio at baseline did not demonstrate any difference between responders and non-responders (p=0.3434 and p=>0.9999 respectively, Figure 4.3B). In view of the recent literature on CD8 and CD4 double positive cells\textsuperscript{208} abundance of double positive population in treatment naïve samples was analyzed which
showed that CD8 and CD4 double positive cells did not predict response to anti-PD-1 treatment \( (p=0.6064, \text{Figure 4.3A}) \).

T cell infiltration on treatment was also analysed. CD8, CD4 effector and Treg infiltration was similar in responders and non-responders on treatment \( (p=0.905, p=0.904 \text{ and } p=0.606, \text{respectively}) \). Similarly, there was no difference in the abundance of CD8 and CD4 double positive cells on treatment \( (p=0.714, \text{Figure 4.4A}) \). CD8 to Treg and CD4 effector to Treg ratios also did not differentiate between responders and non-responders on treatment \( (p=0.547 \text{ and } p=>0.0999, \text{respectively}) \) \( (\text{Figure 4.4B}) \). We also evaluated the abundance of T cell subsets in relation to total cells counted (density) in a slide and the results remained the same. There was no significant difference in the level of CD8, CD4 effector, Treg and CD8 CD4+ cells in responders and non-responders at baseline \( (p=0.189, p=0.238, p=0.437, \text{and } p=0.190, \text{respectively}) \) and on treatment \( (p=0.318, p=0.318, p=0.261, \text{and } p=0.898, \text{respectively}) \) \( (\text{Figure 4.5}) \).

B cells constitute an important part of humoral immunity and they carry out their cytotoxic function via antibody production by the plasma cells which are more differentiated forms of activated B cells.\(^{209}\) Tumour infiltration with B cells and plasma cells have previously been shown to correlate with better clinical outcome in different cancers\(^{210,211,212}\). It has also previously been reported that B cells and tertiary lymphoid structures (TLSs) are associated with response to immunotherapy in advanced melanoma and non-small cell lung cancer.\(^{213}\) Evaluation of abundance of B cells showed that responders had higher levels of B cells at baseline but there was no difference on treatment \( (p=0.02 \text{ and } p=0.96, \text{respectively}) \) \( (\text{Figure 4.3A, Figure 4.4A and Figure 4.6}) \). On the other hand, there was no difference in the plasma cell infiltration between responders and non-responders at baseline \( (p=0.225) \) and on treatment \( (p=0.535) \) \( (\text{Figure 4.3A, Figure 4.4A}) \).
Figure 4. 3 Immune infiltration RCC samples at baseline

A. Immunofluorescence scoring of T cell, myeloid, B cell and plasma cell frequencies in non-responders and responder at baseline (n=9 and n=5, respectively) in RCC sample in the ADAPTeR cohort is shown. B, CD8 to Treg, CD4 effector to Treg and C, CD8 to myeloid cell ratios on treatment is shown for the same cohort of patients. Two-sided Mann Whitney test is used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). (Blue= Non-responders at baseline, Red=Responders at baseline)
Figure 4. Immune infiltration on treatment

A. Immunofluorescence scoring of T cell, myeloid, B cell and plasma cell frequencies in non-responders and responder on treatment (n=6 and n=3, respectively) in ADAPTeR cohort is shown. B, CD8 to Treg, CD4 effector to Treg and C, CD8 to myeloid cell ratios on treatment is shown for the same cohort of patients. Two-sided Mann Whitney test is used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). (Grey=Non-responders on treatment, Purple=Responders on treatment)
Figure 4.5 Frequency of T cell subsets out of total cells in ccRCC tissue

(A). Frequency of T cell subsets out of the total cells in ccRCC tissue at baseline is shown. (B). Density of T cell subsets out of the total cells in ccRCC tissue on treatment is shown. Two-sided Mann Whitney test is used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). (Blue= Non-responders at baseline, Red=Responders at baseline, Grey=Non-responders on treatment, Purple=Responders on treatment)
**Figure 4.6 Immunohistochemistry images for B cells, plasma cells and PD-1+ cells**

Immunohistochemistry images for B cells (blue), plasma cell (magenta) and PD-1+ (brown) is shown at baseline for a representative non-responder patient (on the left) and a representative responder patient (on the right) is shown. The staining was done on ccRCC tissue taken from the ADAPTeR cohort. The magnification is shown on each image.

### 4.2.3. Level of Granzyme B expression predicts response to anti-PD-1 treatment

Although the T cell infiltration prior to anti-PD1 treatment does not predict response to nivolumab, Granzyme B producing cytotoxic T cells and myeloid cell levels were evaluated. CD163 was used as a myeloid marker but we were not able to identify specific myeloid subpopulations due to absence of other myeloid markers on the immunofluorescence staining panel. There was no significant difference in overall Granzyme B expression in treatment naïve samples ($p=0.0829$, **Figure 4.7A**). Looking closer at those two different immune populations showed that although the CD8 cells had similar Granzyme B expression levels at baseline in responders and non-responders ($p=0.1329$), the overall Granzyme B and Granzyme B+ CD8 cell levels were higher in the responders during anti-PD-1 treatment ($p=0.0238$, $p=0.0476$, **Figure 4.7B**). Despite the non-significant $p$ values in responders, a trend of increase was seen in both markers from baseline to on treatment (**Figure 4.7D**). When the T cell population was compared with the myeloid population, no significant difference was seen in the CD3 to CD163 ratio and CD8 to CD163 ratios between responders and non-responders at baseline ($p=0.1898$ and $p=0.1119$ respectively, **Figure 4.3C**). It has previously been shown that PD-1 expression predicts response to anti-PD-1 therapy in RCC.\textsuperscript{214} However, in our cohort, overall PD-1
expression was scored by mIHC and did not differentiate between responders and non-responders at baseline (p=0.137) or on treatment (p=0.369) (Figure 4.7A and B).

Antigen presentation is important in recognition of the tumour cells by the immune system. MHC I and II are critical in antigen presentation. MHCI consists of an alpha chain and beta-2 microglobulin. MHCI can be expressed by any nucleated cell including the tumour cells. It was previously shown that MHCI expression on melanoma cancer cells is predictive of response to anti-CTLA4 therapy. On the other hand, MHCII expression relies on activation of inflammatory pathways and recent studies show that it can be expressed on immune cells as well as tumour cells. It traditionally presents antigens to CD4 cells. MHCII expression on tumour cells has been associated with response to anti-PD-1 therapy. We therefore, looked at MHCI and MHCII expression in the ADAPTeR cohort. Unfortunately, the MHCI staining was not scorable due to the significant amount of background staining. Therefore, only MHCII expression in relation to CD4, CD8 and Pax8 (tumour marker) cells was analysed. There was no difference in the overall expression of MHCII between responders and non-responders at baseline (p=0.593) and on treatment (p=0.378) (Figure 4.8A). At baseline, responders had more MHCII+CD4 cells compared to non-responders (p=0.027). However, there was no difference in the MHCII+CD8 or MCHII+Pax8 levels between responders and non-responder at baseline (p=0.783 and p=0.804, respectively) (Figure 4.8B). On treatment, there was no difference in the MHCII+CD8, MHCII+CD4 and MCHII+Pax8 levels between responders and non-responders (p=0.788, p=0.787 and p=0.812) (Figure 4.8C). NFATc1 was used to assess the activation status of the TILs. No difference was observed in the NFATc1+CD8 and NFATc1+CD4 level between responders and non-responders at baseline (p=0.639 and p=0.140) or on treatment (p=0.285 and 0.709) (Figure 4.8D-E).
Granzyme B expression in CD3 and CD8 T cells in ccRCC tissue at baseline and on treatment

(A). Granzyme B, Granzyme B+ CD8 and overall PD-1 expression in non-responders and responder in ccRCC tissue at baseline (n=9, n=5, respectively) and (B) on treatment (n=6 and n=3, respectively). (C) Composite image of mIF staining for CD8 and Granzyme B expression in ccRCC tissue at baseline and on treatment is shown. (D) Change in total granzyme B expression, granzyme B expression on CD8 cells from baseline to on treatment is shown (n=3). Two-sided Mann Whitney test is used for statistical analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Statistical significance is not shown for samples where difference between samples is not statistically significant ($p>0.05$). (Blue= Non-responders at baseline, Red=Responders at baseline, Grey=Non-responders on treatment, Purple=Responders on treatment).
Figure 4. 8 MHCII and NFATc1 expression on T cells in ccRCC is shown. (A). Overall MHCII expression in ccRCC tissue in non-responders and responders at baseline is shown. (B). MHCII expression on CD8, CD4 and Pax8 (tumour) cells in ccRCC tissue in non-responders and responders at baseline is shown. (C). MHCII expression on CD8, CD4 and Pax8 (tumour) cells in ccRCC tissue in non-responders and responders on treatment is shown. (D). NFATc1 expression on CD8 andCD4 cells in non-responders and responders in ccRCC tissue in at baseline is shown. (E). NFATc1 expression on CD8 andCD4 cells in non-responders and responders on treatment is shown. Two-sided Mann Whitney test is used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). (Blue= Non-responders at baseline, Red=Responders at baseline, Grey=Non-responders on treatment, Purple=Responders on treatment)
4.2.4 TCR sequencing demonstrates maintained clonal expansion in responders

β-chain of TCRs from 63 tumour samples and 29 PBMC samples from 14 patients, matched at baseline (pre-treatment) and week-9 biopsy (post-treatment) timepoints were sequenced using previously established methods. In order to avoid underestimation of TCR clonal expansion and TCR repertoire diversity, TCR sequences for each patient were pooled at each sampling timepoint. The median number of unique β-chain transcripts were 3,644 for tumor samples and 21,370 for blood samples.

When the similarity between the multiregional samples for each patient was analysed, it was seen that there was a degree of heterogeneity in the TCR repertoire in all the patients. However, patients who had higher levels of similarity between the multiregional samples were the responders (ADR008, ADR016 and ADR013) (Figure 4.9A). Comparison of the TCR repertoire similarity between the regions in the non-responder patient and the regions in the responder patient showed that the on treatment multiregional samples in ADR013 (responder) showed a higher degree of similarity compared to the on treatment multiregional samples in ADR001 (non-responder) (Figure 4.9B).

Effect of anti-PD1 therapy on TCR clonality (low TCR clonality score indicates a more diverse TCR repertoire) in the tumour and peripheral blood was analysed. In the tumour, there was no significant difference in the TCR clonality between the samples at baseline and on nivolumab treatment (p=0.50). Analysis of TCR clonality in the peripheral blood showed that the nivolumab treatment did not have any significant impact on the clonality and it was similar between baseline and in treatment samples (p=0.90) (Figure 14.10A). The intratumoural clonality score was higher in the responders pre-treatment compared to the non-responders (p=0.042) (Figure 14.10B). In addition, the TCR repertoire was more similar in the pre-treatment and on treatment tumour samples in the responders compared to the non-responders (p=0.024) (Figure 14.10C).
Figure 4. 9 TCR repertoire similarity in the tumour samples

(A) The intratumoural similarity in the TCR repertoire from different biopsies taken at the same time point for each patient is shown. Each circle represents a comparison between two samples from the same patient (n = 87 total comparisons from 12 patients). Red circles indicate a pair of biopsies originating from the same site and blue circles indicate biopsies from metastatic sites (B) The similarity in the TCR repertoire in the biopsies taken from post-treatment nephrectomy is shown for ADR001 (left) and ADR013 (right).
Figure 4. 10 TCR repertoire clonality in the tumour and peripheral blood

(A) TCR clonality score for intratumoural and peripheral bloods samples for each patient at pre- and post-treatment time points is shown. (B) Pre-treatment intratumoural TCR repertoire clonality score is shown for responders and non-responders. Each dot represents a patient. Mixed-effect model is used for the p-value. (C) The intratumoural TCR repertoire similarity (using cosine score) between pre-treatment and on-treatment samples is for each patient (n=12) in responders and non-responders is shown. A higher cosine score is seen in the responders (two-sided Mann–Whitney test P value is shown). P values are shown on the figures.
After analysing the clonality of the TCR repertoire, expansion of the TCR clones was analysed. It was seen that the frequency of the expanded TCR clones seen in pre-treatment samples was maintained at a higher degree in the post treatment samples in the responders compared to the non-responders (p=0.024) (Figure 11A and B). This suggests that with nivolumab treatment, TCR clones expanded pre-treatment are maintained during treatment in the responders. In contrast to the findings on the tumour, the maintenance of highly expanded TCRs in responders was not observed in PBMCs (Figure 4.14B).

Figure 4.11 Frequency of expanded TCRs pre and post treatment

(A) The frequency distribution of expanded TCRs in the pre-treatment (red circles; n = 469 individual TCRs combined from 12 patients) and post-treatment (blue circles) intratumoural samples is shown. TCRs that were present both in pre- and post-treatment samples were included. (B) The ratio of expanded pre-treatment clones found post-treatment per patient is shown. Two-sided Mann–Whitney test P value shown.
Building up on the above observations, it can be hypothesized that the maintenance of highly expanded T cell clonotypes among responders can be driven by an antigenic signal that remains stable in the tumour during anti-PD1 therapy. To investigate this, clonotype clustering analysis based on the similarity in amino-acid sequences in the TCRs was performed. Analysis showed that expanded TCRs in tumour samples from responders had more clustering of similar CDR3 sequences both at baseline and on treatment compared to non-responders (Figure 4.12A and B). In addition, the expanded clones pre-treatment which were maintained during nivolumab treatment showed more clustering compared to the clones that were replaced on treatment (Figure 4.12C). We have then looked at clustering at specific patients. In ADR008, which is a responder patient, TCRs clones expanded pre-treatment showed a high degree of clustering (Figure 4.13A). In addition, the expanded clones found at baseline which were maintained during nivolumab treatment showed higher degree of clustering compared to clones which got replaced during treatment (Figure 4.13B). Comparison of ADR001 (non-responder) and ADR013 (responder) showed that ADR013 had a higher level of pre-treatment expanded clones that were maintained on treatment and these clones showed a significantly higher degree of clustering compared to ADR001 (Figure 4.13C). Analysis of the PBMC samples showed that there was no significant difference in the expansion and contraction of TCR clones in responder and non-responders (Figure 4.14B). In addition, there was no significant similarity in the TCR repertoire between pre-treatment and on treatment samples in responders and non-responders (Figure 4.14C). These findings suggest maintenance of clonal expansion of TCR clones by a stable tumour-specific antigenic signal in responders during anti-PD1 therapy.
Figure 4. 12 Clustering of TCR clones in responders and non-responders

(A) Normalised number of clusters that contain expanded TCRs in the pre-treatment intratumoural samples in responder and non-responders is shown. (B) Normalised number of clusters that contain expanded TCRs in the post-treatment intratumoural samples in responder and non-responders is shown. (C) The proportion of expanded TCRs that are part of a cluster is shown. Red circles show the pre-treatment expanded TCRs that are also found post-treatment and the grey circles show the ones which are not found post-treatment. Two-sided Mann–Whitney test P value shown. Two-sided Mann–Whitney test is used for the P value.
Figure 4. 13 Clustering examples in specific patients

(A) Intratumoral CDR3 β-chain sequences found in the pre-treatment intratumoural samples for patient ADR008 are shown. The network shows sequences that are connected to at least one other TCR within the tumor. Clustering was performed around expanded intratumoral TCRs (red circles). (B) Clustering for maintained and replaced expanded clones for ADR008 is shown. (C) Representative network diagrams of post-treatment intratumoral CDR3 β-chain sequences for patient ADR001 (left) and for patient ADR013 (right). Clusters containing expanded sequences are shown.
Figure 4. 14 TCR repertoire clonality in peripheral blood

(A) TCR repertoire clonality score pre-treatment and on-treatment peripheral blood samples is shown for responders and non-responders. Each dot represents a patient. Mixed-effect model is used for the P value. (B) The TCR clones expanded or contracted between pre-treatment and post-treatment in the peripheral blood for each per patient is shown. The data is normalized for the total number of clones tested. Two-sided Mann–Whitney test is used for P value. (C) The TCR repertoire similarity in PBMC (using cosine score) between pre-treatment and on-treatment timepoints for each patient in the responders and non-responders is shown. Two-sided Mann–Whitney test is used for P value; n=12 patients.
4.3 CHAPTER DISCUSSION

In this chapter, clinical and translational data for the ADAPTeR trial was discussed. The median progression free survival and overall survival were 4.1 months and 17.5 months, respectively. Out of the 15 patients involved in the clinical trial, 66% of the patients had <6 months clinical benefit from nivolumab and only 33% of the patients had >6 months clinical benefit. There was no association between age, IMDC risk score and ECOG performance status and response to nivolumab. However, PFS was significantly better in the responders compared to non-responders although there was no statistically significant difference in the OS between the two response groups.

In order to understand the possible mechanisms of response and resistance to nivolumab in the ADAPTeR cohort, we analysed the immune landscape using various methods as discussed in this chapter. In some tumor types, it has been shown that T cell infiltration as well as PD-1 and PD-L1 expression can be predictive of response to anti-PD-1 therapy. However, in ADAPTeR there was no association between CD8, CD4 effector, Treg and CD8⁺CD4⁺ T cell infiltration at baseline or on treatment and response to nivolumab. Similarly, the overall PD-1 expression in the tumour regions was not predictive of response to nivolumab. One of the limitations with the PD-1 scoring in our study was that the PD-1 staining in the mIF had a lot of background which we could not fix despite several attempts. Therefore, PD-1 scoring was done as IHC and as a result we could not assess the PD-1 expression on the specific immune cells. One way of potentially solving this problem can be moving PD-1 from Opal 690 to a less bright fluorophore such as Opal 540. Another potential reason for the high background is likely to be the variable fixation conditions and the tissue quality. We also attempted to stain for IgG4 in the T cell panel in order to image the nivolumab bound cells. However, the optimization was not successful and a potential explanation for this could be that the nivolumab gets stripped off the T cells during the multiple antigen retrieval steps of mIF staining. In addition to T cell infiltration, we have also analysed the macrophage infiltration in tumour regions. However, abundance of macrophages did not correlate with response to anti-PD-1 therapy. One of the limitations in our study was using only one macrophage marker (CD163). CD163 is mainly used as a M2 macrophage marker although it is also expressed on other myeloid cells including monocytes. Therefore, adding another macrophage marker such as CD68 which is expressed on M1 macrophages can help with having more comprehensive analysis of the macrophages rather than looking at only one subtype. On the other hand, there was a higher level of B cell infiltration in the responders at baseline. Although this is an important finding of the study which is in line with the literature, taking
this finding a step forward and looking at the TLS in this cohort can be more informative given the recently published predictive and prognostic role of TLS in various solid tumours.\textsuperscript{223}

Although T cell infiltration was not predictive of response to nivolumab, responders had higher Granzyme B expression on CD8 cells on treatment. Although the change in Granzyme B expression on CD8 cells from baseline to on treatment was not statistically significant, there was trend of increase in Granzyme B expression with nivolumab treatment. Increased Granzyme B\textsuperscript{+} CD8 cells with nivolumab treatment suggest that CD8 cells gain a cytotoxic phenotype with anti-PD-1 therapy. In addition, this cytotoxic phenotype does not directly translate into a cytotoxic function and it needs to be validated by \textit{in vitro} functional assays. Although this is an important finding, the responder cohort on treatment only had 3 patients. Therefore, this finding should be considered as a hypothesis generating finding and needs to validated in a bigger validation cohort. One of the advantages of using mIF is understanding the spatial distribution of the cells which can provide important clues about the communication between different cells in the tumour microenvironment. In ADAPTeR, there was no difference in the proximity or distance between different T cells subsets with each other and the tumour cells between the responders and non-responder. However, it was not possible to look at spatial distance between all cell types due to the limited number of markers we can analyse in each panel. Therefore, using other platform such as CODEX which allows imaging an area with up to 60 markers assessing the communication between these cells will allow a more in depth analysis.\textsuperscript{224} However, with CODEX technology only a limited area in the tumour can be analysed. Therefore, more recent technologies such as slide-seq can be a better choice which will allow analysing multiple regions with over 50 markers at protein and transcriptional level.\textsuperscript{225}

Another important aspect of the translational work in the ADAPTeR study was TCR sequencing. Analysis of the TCR repertoire in the tumour regions and in the PBMC showed that the PBMC was not representative of the tumour. This is alignment with the TCR data published in other solid tumours such as ovarian cancer and oesophageal cancer. In addition, the TCR clonality score was higher in the in the pre-treatment tumour samples in the responders. However, there was no difference in the clonality in the PBMC between responders and the non-responders at baseline and on treatment. One of the most important findings of the TCR analysis was that maintenance of the expanded pre-existing TCR clones at baseline through nivolumab treatment is associated with response to therapy. Whether checkpoint inhibition leads to expansion of pre-existing clones or causes
replacement of pre-existing clones with new clones is a hot topic in the immunotherapy field which is yet to be understood in more detail and the contradictory results have been published so far. Although expansion of TCR clones on treatment was recently shown to be associated with response to checkpoint blockade in RCC, maintenance of pre-existing expanded clones through treatment and its role in response to treatment has not been shown before and it is a novel finding in our study. In contrast to our findings in ADAPTeR, Yost and colleagues suggested that the expanded clones were new clones that replaced pre-existing TCR clones in the tumour. Within the context of non-small cell lung cancer, it has been shown that following anti-PD-1 therapy, TCR clones which have expanded in the peripheral blood migrate to the tumour and replace the existing T cells in the tumour. These studies support a clonal replacement mechanism following checkpoint inhibition rather than expansion of pre-existing T cell clones which suggests immunotherapy can play an important role in causing migration of new clones to the tumour site and is contradictory to the findings of our study. On the other hand, our findings suggest that the existing T cell clones prior to therapy are important in the determining whether the patient will respond to immunotherapy as they would expand in response to checkpoint inhibition. Another important result in ADAPTeR was that responders had more clustering which suggests they shared more similar TCR clonotypes. In addition, more clustering was seen in the expanded TCR clones compared to the non-expanded clones. This clustering pattern has been described before and suggests that the clones that cluster together recognize a common antigen. Although our findings suggest that TCR clonal expansion and maintenance of these clones can be predictive of response to anti-PD-1 therapy in RCC, these findings need to validated in a bigger cohort given the small sample size in this study. Validating these findings in other tumour types is also important in order to see if this concept can provide clinically relevant prognostic insights in other cancer types. In addition, this technique does not allow us to differentiate if the TCR clones are coming from CD8, CD4 effector or Treg cells. Therefore, doing bulk TCR sequencing from FACS sorted CD8, CD4 effector or Treg cells or doing single cell RNA sequencing can be used to overcome this problem.
5 UNDERSTANDING THE EFFECT OF ANTI-PD-1 THERAPY ON PBMC IN RENAL CELL CARCINOMA

5.1 CHAPTER INTRODUCTION

It has previously been shown that anti-PD1 therapy changes the immune landscape in the peripheral blood can be used as a predictive biomarker for response to anti-PD-1 therapy in melanoma patients. In specific, they observed an increase in the central memory T cells and expression of activation markers on the T cells following anti-PD1 treatment. Analysis of the myeloid compartment showed that a higher level of monocytes in the PBMC post-treatment is associated with response to checkpoint inhibition. In cohort of 17 NSCLC patients and 7 RCC patients, Julia et al. have shown that a decrease in T cell abundance and increased TIM-3 expression in CD8 and CD4 T cells in PBMC following anti-PD1 therapy was associated with progressive disease. PBMC samples from the ADAPTeR cohort which were taken at baseline and on treatment were analysed to evaluate the effects of nivolumab on the immune landscape in peripheral blood.

Although the immunotherapies have focused on modulating T cells so far, many studies suggest the crucial role of myeloid cells in cancer. Myeloid cells consist of a heterogeneous group of cells including monocytes, macrophages and dendritic cells. Monocytes differentiate into macrophages and dendritic cells in the tissue. Dendritic cells are key in antigen presentation while macrophages are important in phagocytosis of antigens and pathogens. Presence of macrophages in the tumour microenvironment (TME) was shown to impact clinical outcome in different cancer. Macrophages polarisation determines the type of impact macrophages will have in the clinical outcome. M1 macrophages have pro-inflammatory function and while M2 macrophages are have an anti-inflammatory role. Therefore, M1 macrophages have been associated with better clinical outcome. On the other hand, M2 macrophages are associated with poor clinical outcome. Indoleamine 2,3-dioxygenase (IDO) and Arginase-1 are suggested to be important for M2 macrophages to mediate their anti-inflammatory effects. In addition, myeloid-derived suppressor cells (MDSC) are myeloid cells that follow an incomplete differentiation pattern from the progenitor state and the have been associated with poor outcome in cancer. DCs have different subsets with different functions in the TME. Conventional DCs (cDCs) in the TME is correlated with better clinical outcome and they are found to express CLEC9a. Plasmacytoid DCs (pDCs) were also shown to have an antitumour affect by pro-inflammatory effects, especially by promoting secretion of interferon and Fcγ receptor II (CD32) is suggested to be important for this function. B cells are an important part of humoral immunity and their function includes antigen recognition and antibody production. It has been shown that they play an
essential role in determining response to immunotherapy.\textsuperscript{213,243} Although most of the research on the role of myeloid cells in cancer has been on myeloid cells in the tumour microenvironment, in the recent years studies have shown that myeloid cells in the peripheral blood are also informative and important, especially in the context of response to immunotherapy. For instance, in patients with metastatic melanoma a higher level of monocytes in the peripheral blood was associated with better prognosis and response to anti-PD-1 therapy.\textsuperscript{230} In pancreatic adenocarcinoma patients, MDSC which were seen in the tumour microenvironment were also detected in the peripheral blood and they had the same phenotype as the MDSCs in the tumour microenvironment.\textsuperscript{244} A similar picture was seen in RCC patients in a study by Rodriguez and colleagues and they have shown that argine-1 producing MDSC frequency increases in patients with RCC.\textsuperscript{245} However, the data on the frequency and role of myeloid cells in the peripheral blood in RCC and the effect of immunotherapy in this population of cells is very limited. Therefore, we looked at the myeloid subsets in the peripheral blood and the effect of anti-PD-1 therapy on these subsets in ccRCC patients.

Fc$\gamma$ receptor (Fc$\gamma$R) expression plays an important role in the function of immune cells subsets. Fc$\gamma$Rs are proteins that belong to the family of immunoglobulins. Fc$\gamma$Rs are expressed on different immune cells including the monocytes, macrophages, dendritic cells and natural killer cells which allows them to bind to different antibodies. This consequently leads to activation or inhibition of the immune responses depending on the subtype of the Fc$\gamma$R. There are four main types of Fc$\gamma$Rs: Fc$\gamma$RI(CD64), Fc$\gamma$RII (CD32), and Fc$\gamma$RIII (CD16). Fc$\gamma$RI is a high affinity receptor for IgG and the rest have low to medium affinity. Fc$\gamma$RI and Fc$\gamma$RIII have activating function whereas Fc$\gamma$RII has an inhibitory function. The activating Fc$\gamma$Rs carry out their activating function via the immunoreceptor tyrosine-based activation motif (ITAM) located in their cytoplasmic domain. Similarly, Fc$\gamma$RII performs its inhibitor function via the immunoreceptor tyrosine-based inhibitory motif (ITIM).\textsuperscript{246–248} In addition to the role of Fc$\gamma$Rs in immune cell function, they also affect the efficacy of the therapeutic antibodies as they affect antibody binding to the immune cells. More recently, it has been shown that high affinity polymorphisms affect binding and impact of therapeutic antibodies. For instance, in non-Hodgkin lymphoma patients, it was seen that $FCGR3A$-158V polymorphism in Fc$\gamma$RIII affects response to the anti-CD20 antibody Rituximab.\textsuperscript{249} In addition, it was seen that the polymorphism in of Fc$\gamma$RIII VV head and neck squamous cell carcinoma cells affect the efficacy and ADCC of cetuximab antibody.\textsuperscript{250} Within the context of checkpoint inhibitors, it has been shown that CD16a-V158F polymorphism has been associated with better clinical response to ipilimumab in melanoma patients.\textsuperscript{247}
We hypothesized that higher frequency of myeloid cells such as monocytes or NK cells as well as FcγRs in the peripheral blood can have a predictive role in response to anti-PD-1 therapy.

In this chapter, the questions we want to answer are as following:

- What are the T cell subsets in the peripheral blood in the ADAPTeR cohort and how do they change with nivolumab?
- Which myeloid cells do we see in the peripheral blood in the ADAPTeR cohorts?
- Does nivolumab affect the abundance of myeloid subsets in the peripheral blood?
- What is the FcγR expression profile in the different immune subsets in the peripheral blood and how does it change with nivolumab?
- Do the questions above correlate with response to nivolumab treatment in the ADAPTeR cohort?
5.2 RESULTS

5.2.1 CD8 T cell phenotypes pre- and post-anti-PD-1 therapy in the Peripheral blood

In addition to analysing the tumour immune microenvironment in the ADAPTeR cohort, immune phenotype of peripheral blood was also analysed by using high dimensional flow cytometry. At baseline, 6 non-responder and 3 responder samples were analysed. On treatment, there were 6 non-responder and 4 responder samples. Due to limited sample availability and small sample size, the analysis is mainly descriptive.

T cell subsets were analysed and characterised for all patient groups (Figure 5.1) and the gating strategy for all immune cell subsets is shown in Figure 5.2. CD8 and CD4 effector cells constituted majority of the T cells both in responders and non-responders at baseline and on-treatment. At baseline, non-responders had a median level for CD8 and CD4 were 22.5 % and 56.4 % compared to 2.1% of Tregs. On the other hand, the responders had a median of 43.0 % CD8s which is higher compared to non-responder while the median CD4 effector and Treg levels were lower compared non-responders (39.0% and 0.73%, respectively). Despite the difference in the median percentages, the difference in the abundance of CD8, CD4 effector and Treg cells between responders and non-responders at baseline (p=0.787, p=0.785, p=0.732 respectively) or on treatment (p=0.857, p=>0.999, p=0.857 respectively) was not statically significant (Figure 5.3 A and B). T cell subsets in the PBMC were also analysed. As discussed in TRACERx renal chapter, we have shown that TRM cells are present in PBMC in negligible amounts. Therefore, in the ADAPTeR cohort we focused more on naïve (CD45RA⁺CCR7⁺), Temra (CD45RA⁺CCR7⁻) and TCM (CD45RA⁻CCR7⁺) cells. At baseline, CD8s in non-responders had high levels of Temra cells (median=33.8%) followed by naïve T cells (median= 24.5%) and TCM (median=8.33%). Responders at baseline also followed a similar pattern (median=58% for Temra cells, median=19.2% for naïve cells and median=2.7% for TCM) and the Temra cells were higher in the responder compared to the non-responder in percentage. However, the difference in Temra, naïve and TCM cells was not statistically significant between non-responder and responders (p=0.862, p=0.535 and p=0.341, respectively) at baseline or on treatment (p=0.347, p=0.637 and p=0.572, respectively) (Figure 5.3 C and D). On treatment, the most abundant subset both in the non-responders and responders was Temra cells and was higher in the responders compared to non-responders (median values 58.5% and 30.4%, respectively). Naïve and TCM cell levels were higher in the non-responders (median values 24.5% and 10.1%, respectively) compared to responders (median values 19.2% and 2.57%, respectively) at baseline. The pattern was similar on treatment too. Although there was no difference in the level of CD4 effector naïve, Temra and TCM subsets between non-
responders and responders at baseline (p=0.954, p=0.750 and p=0.209, respectively) and on treatment (p=0.822, 0.776 and p=0.734, respectively), the profile was different compared to CD8 cells. In the CD4 effectors, naïve cells were more abundant compared to Temra and TCM whereas Temra cells made up a higher percentage compared to naïve and TCM cells in the CD8 cells. At baseline, non-responders had a median of 40.9% naïve CD4 effectors, 1% Temra and 35.1% TCM cells versus a median of 41.7% naïve, 1.08% Temra and 42.2% TCM cells in the responder. The profile on treatment was similar both in responders and non-responders with no statistical significance (p=0.822, p=0.776 and p=0.734 for naïve, Temra and TCM CD4 effectors, respectively) (Figure 5.3 E and F).

Figure 5.1 T cell phenotypes in the peripheral blood
The heatmaps and UMAPs show the expression of T cell differentiation markers and checkpoints in the peripheral blood in responders (R) and non-responders (NR) at baseline and on treatment. Heatmaps and UMAPs allow dimension reduction analysis and each cluster represents a different phenotype with co-expression of different markers shown in the heatmap. There are 10 clusters in each heatmap and UMAP and each cluster is represented with a different colour. The scale bar on the right hand side of the heatmap shows the frequency of each cluster in the samples.
Figure 5. 2 Gating strategy used for flow cytometry analysis
The main gating strategy for flow cytometry analysis is shown above. As a first step, lymphocytes are identified. This is followed by single cell and live cell gating. Then, CD45 cells are identified out of the live cells. Out of the CD45 gate, CD3+ T cells, CD56+ NK cells, CD19+ B cells and CD15+ neutrophils and myeloid cells are identified.
Figure 5. 3 Frequency of T cells subsets in the peripheral blood

(A). Abundance of CD8, CD4 effector and Tregs in non-responders and responders at baseline and (B) on treatment is shown. (C). Frequency of CD8 naïve, Temra and TCM cells in non-responders and responders at baseline and (D) on treatment is shown. (E). Frequency of CD4 naïve, Temra and TCM cells in non-responders and responders at baseline and (F) on treatment is shown. Two-sided Mann Whitney test is used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). (Blue= Non-responders at baseline, Red=Responders at baseline, Grey=Non-responders on treatment, Purple=Responders on treatment)
After analysing the T cell differentiation subsets, the phenotype of CD8 and CD4 effector cells in the peripheral blood were analysed. There was no statistical difference between responders and non-responders at baseline and on treatment in any of the markers analysed on CD8 or CD4 cells. However, we have observed that certain markers were expressed at higher levels compared to others. On CD8s cells, high levels of CD45RA and CCR7 reflect the presence of Temra, naïve and TCM populations as described previously. CD57 expression which is associated with history of extensive cell division and terminal differentiation was also expressed in all cohorts in moderate levels (median expression 14.6% in non-responder and 29.5% in responders at baseline; 29.9% in non-responders and 24.3% in responders on treatment). CCR4 (C-C chemokine receptor type 4) was also expressed at moderately high levels. CCR4 is a chemokine receptor which is usually expressed on Th2 cells and Tregs and binds to CCL17 (also called thymus- and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine). CCR4 plays an important role in haematological malignancies and treatment with humanised anti-CCR4 monoclonal antibody showed a clinical benefit in adult T cell leukaemia-lymphoma, peripheral T cell lymphoma and cutaneous T cell lymphoma. More recently, Krieg and colleagues showed that CCR4 was upregulated on CD8 cells in the peripheral blood in responders in melanoma following anti-PD-1 treatment. In the ADAPTeR cohort, at baseline, CD8 T cells in non-responders had higher median expression of CCR4 (17.7%) compared to responders (7.38%) although it was not a statistically significant difference (Figure 5.4A). On treatment, the median expression was similar between responders (14.35%) and non-responders (15.5%) (Figure 5.34B). CD27 was another marker that was expressed in moderately high levels on CD8 cells in responders and non-responders are baseline and on treatment. CD27 is glycoprotein that belongs to the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor family. It is a costimulatory molecule expressed on the surface of T cells (predominantly in the PBMC) and is upregulated following TCR activation. When the CD27 ligand CD70 binds CD27, it leads to activation and proliferation of the T cell and B cells. In addition, CD27 is also important in effector and memory T cell differentiation. Due its role in T cell activation, CD27 is considered as a promising target for immunotherapy. Varililumab which is a humanised IgG1 anti-CD27 monoclonal antibody with an agonistic function. It has been tested on RCC and melanoma patients in a phase 1 clinical trial which showed clinical benefit as well as causing an increase in T cell activation markers and increase in terminally differentiated effector memory cells. In the ADAPTeR cohort, non-responders had median CD27 expression level of 27.4% compared to a median expression of 14.8% in responders at baseline (Figure 5.4C). On treatment, CD8 cells in the non-responder group had a
comparable CD27 expression level of 18.3%. This level was marginally higher in the responder cohort with a median of 23.2% (Figure 5.4D). Although this is not a statistically significant difference, it does suggest a degree of activation in CD8 cells in the peripheral blood following nivolumab treatment. CD8 cells that are CD45RA-CD27+CCR4+ are classified immature memory T cells which secrete type 1 and type2 cytokines. It was also shown that the CCR4 on these cell binds get activated by chemokines produced by macrophages and therefore play a role in T cell-macrophage interaction. Eomes was also another maker that was expressed at moderate levels in the CD8 cells. It is a T-box transcription factor and plays an important role in memory T cell differentiation and effector function in CD8 and CD4 cells. In addition, high levels of Eomes expression and co-expression with PD-1, TIM-3 and CTLA-4 is associated with an exhausted T cell state both in chronic infection and cancer. It was recently shown that increased Eomes expression on CD8 cells is associated with loss of CD226 which is important in TCR mediated T cell activation and response to immunotherapy. There was no statistical difference in the Eomes expression level between different groups and the median Eomes level in the non-responder group at baseline was 7.5% compared to 9.0% in the responders (Figure 5.4C). Eomes expression was also comparable in the non-responders (median expression of 11.7%) and responder cohort (median expression of 12.1%) on treatment (Figure 5.4D). KLRG-1 was also expressed at moderate levels with similar expression in non-responders (median value of 22.9%) and responders (median value of 22.3%) at baseline. On treatment, responders (median of 28.7%) had similar KLRG-1 levels compared to non-responders (median 30.7%). Similar to the PBMC profile seen in the TRACERx Renal cohort, peripheral blood in ADAPTeR also had high levels of granzyme B expression suggesting a cytotoxic phenotype. The median expression levels were 30% and 42.9% in the non-responder and responder groups at baseline respectively (Figure 5.4C). On treatment, the median expression levels were 43.8% in the non-responder cohort and 58.8% in the responder cohort which suggests a trend of increased granzyme B expression in the peripheral blood with nivolumab treatment (Figure 5.4D).
Figure 5. 4 Phenotype of CD8 cells in the peripheral blood

(A) Expression of differentiation markers is shown on CD8 cells at baseline and (B) on treatment. Expression of checkpoints on CD8 cells is shown at baseline (C) and on treatment (D). Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM). (Blue= Non-responders at baseline, Red=Responders at baseline, Grey=Non-responders on treatment, Purple=Responders on treatment.)
5.2.2 CD4 T cell phenotypes pre- and post-anti-PD-1 therapy in the peripheral blood

The phenotype of the CD4 effector cells in the peripheral blood was also analysed. The profile of CD4 effector cells was similar to what we observed in CD8 cells. Similar to CD8 cells, CD4 effectors had high levels of CD45RA and CCR7 which reflects the previously described Temra, naïve and TCM populations. In contrast to CD8 cells, CD4 effectors had a lower level of CD57 expression in the in non-responder (median of 0.1% on the CD4 effectors versus 14.6% on CD8s) and responder (median of 1.8% on the CD4 effectors versus 29.5% on CD8s) at baseline. The pattern was similar on treatment too where the non-responder (median 5.7%) and responder (median of 1.3%) cohorts has lower levels of CD57 expression compared to CD8s (median of 29.9% and 24.3%, respectively). This suggests that the CD8 cells were more terminally differentiated and had gone through more cellular division. As also observed in CD8 cells, CCR4 was also observed at moderate levels on CD4 effector cells in non-responders (median of 24.1%) and responders (median of 16.8%) at baseline and on treatment (median of 26.3% and 29.5%, respectively) (Figure 5.5A and B). High levels of CD27 express was observed on CD4 effector cells although there was no statistical difference in different subgroups (median for non-responder and responder groups at baseline: 40.8% and 37.8%, respectively; median for non-responder and responder groups on treatment: 35.9% and 35.2%, respectively) which suggests a moderately activated CD4 effector phenotypes in all PBMC cohorts. Granzyme B expression on CD4 effector cells was lower compared to the expression level on CD8 cells. At baseline CD4 effectors had a median expression of 1.45% in non-responder and 2.44% in responders (compared to 30% and 42.9% on CD8 cells). On treatment, this level was slightly higher with a median value of 8.02% in the non-responders and 14.65% in the responders (compared to 43.8% and 58.8% on CD8 cells). There was no statistical difference in the Granzyme B expression on CD4 cells in the different cohorts (Figure 5C and D).
Figure 5.5 Phenotype of CD4 effectors in the peripheral blood

(A) Expression of differentiation markers is shown on CD4 effectors at baseline and (B) on treatment. Expression of checkpoints on CD4 effectors is shown at baseline (C) and on treatment (D). Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). Error bars show mean +/- standard error of the mean (SEM). (Blue= Non-responders at baseline, Red= Responders at baseline, Grey= Non-responders on treatment, Purple= Responders on treatment).
Figure 5.6 Phenotype of Tregs in the peripheral blood

(A) Expression of differentiation markers is shown on Tregs at baseline and (B) on treatment. Expression of checkpoints on Tregs is shown at baseline (C) and on treatment (D). Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p > 0.05). Error bars show mean +/- standard error of the mean (SEM). (Blue = Non-responders at baseline, Red = Responders at baseline, Grey = Non-responders on treatment, Purple = Responders on treatment).
Tregs made up a very small proportion of the T cells in peripheral blood (up to 2% of the T cells). Analysis of the Treg phenotype showed moderate expression of CCR7 and CD45RA suggesting presence of Temra and naïve Tregs in all 4 cohorts. CD69 which is associated with maintenance of immunosuppressive function of Tregs was expressed at moderate level in the non-responders and responders both at baseline (median levels of 22.5% and 29.5%, respectively) and on treatment (median level of 21.6 and 20.1%, respectively) but with no statistically significant difference. CD38 and CD39 are other two markers which were expressed at relatively higher levels compared to other makers. The CD38 expression was similar in the non-responders (median expression of 28.9%) and responders (median expression of 30.8%) at baseline with no statistical difference. On the other hand, responders (16.2%) had lower CD38 expression on treatment compared to non-responders (median expression of 34.1%) but this difference was not statistically significant. On the other hand, CD39 expression was had a higher median expression level in the responders both at baseline (34.6% versus 11.5%) and on treatment (36.1% and 22.2%). However, this difference was not statically significant. CCR4 was another marker that was expressed at relatively higher levels on the Tregs and comparison of the 4 different cohorts showed that CCR4 was expressed at marginally higher levels in the non-responders compares to responders both at baseline (20.5% versus 16%) and on treatment (32.8 versus 16.2%) although this difference was not statistically significant (Figure 5.6A and B). In addition, Fas and CD27 were two markers with considerably high expression on Tregs. Although there was no difference in the Fas expression level between the responders and non-responders at baseline or on treatment, on treatment samples (63.9% and 70.6% for non-responders and responders, respectively) had higher Fas expression compared to baseline (49.6% and 40% for the non-responders and responders, respectively). CTLA-4 expression was also found to higher in 4 cohorts of Tregs compares to CD4 effectors and CD8 cells (Figure 5.6C and D).

After analysing the phenotypes of the CD8, CD4 effector and Treg cells in the peripheral blood in the ADAPTeR cohort, presence and abundance of nivolumab bound cells in the T cells subsets was assessed by using IgG4 as a surrogate marker as previously described. As expected, IgG4+ cells were found at negligible levels in the baseline samples both in CD8, CD4 effectors and Tregs. IgG4 expression was significantly higher on the nivolumab treated samples in CD8 (p=0.0007) and CD4 effectors (p=0.001) compared to baseline samples. However, there was no difference in the Tregs (p=0.809). Although the nivolumab bound to a small proportion of cells in the PBMC, the highest binding was in the CD4 effectors followed by CD8 cells (Figure 5.7).
Figure 5. 7 Percentage of nivolumab bound T cells in the peripheral blood

(A). Percentage of IgG4+ CD8 cells is shown at baseline and on treatment peripheral blood samples. (B). Percentage of IgG4+ CD4 effector cells is shown at baseline and on treatment peripheral blood samples. (C). Percentage of IgG4+ Tregs is shown at baseline and on treatment peripheral blood samples. *P < 0.05, **P < 0.01, ***P < 0.001.
5.2.3 Myeloid subsets pre- and post-anti-PD-1 therapy in the peripheral blood

After analysing the T cells in the peripheral blood, other immune cell types including B cell, natural killer (NK) cells, neutrophils and myeloid cells were also analysed. Different immune subsets were observed in the peripheral blood in both the responder and non-responder cohort at baseline and on treatment (Figure 5.8A and B). It was previously reported in anti-PD1 antibody treated melanoma patients, responders had lower levels of CD3 in the PBMC compared to non-responders at baseline.\(^{270}\) We asked to the same question in our cohort. It was seen that lymphocytes (CD45\(^+\)CD3\(^+\) cells) made up majority of the immune cells in all four cohorts with a median expression of 53.6% and 62.5% in the non-responder and responders at baseline, respectively. Although slightly higher median percentage of CD3 cells were observed in the responder cohort at baseline, the difference between responder and non-responders was not statistically significant (\(p=0.703\)) (Figure 5.9A). A similar result was observed on treatment. Non-responder and responder cohorts a similar percentage of lymphocytes (median expression of 57.8% and 60.4%, respectively) with no statistical significance (\(p>0.999\)) (Figure 5.9B). NK cells were another subset of immune cells analysed in the ADAPTeR cohort. It has previously been shown that in a cohort of non-small cell lung cancer patients treated with nivolumab, responders had higher levels of natural killer cells in PBMC at baseline compared to non-responders.\(^{271}\) NK cells are an important part of the innate immune system with a cytotoxic function and they have previously been shown to play a role in response to checkpoint blockade in cancer. In our analysis we also evaluated the NK cells in the peripheral blood. NK cells were defined as CD45\(^+\)CD3\(^-\)C19\(^-\)CD15\(^-\)CD11b\(^-\)CD56\(^+\) cells. In ADAPTeR, there was no statistically significant difference in the abundance on natural killer cells in the 4 cohorts analysed. At baseline, the median expression was 8.2% in the non-responders and 7.6% in the responders. Similarly, on treatment non-responders had a median of 6.5% compared to 7.4% in the responders. As previously shown, B cells and tertiary lymphoid structures are associated with improved clinical and response in immunotherapy in renal cell carcinoma\(^{213}\) and other cancers including melanoma\(^{274}\) and sarcoma\(^{275}\). As explained earlier in the thesis, we found that higher B cell infiltration in the tumour at baseline was associated with response to nivolumab. We therefore assessed whether the B cell level in the peripheral blood had any predictive value in response to nivolumab. B cells were defined as as CD45\(^+\)CD3\(^-\)CD15\(^-\)CD56\(^+\)C19\(^+\) cells. In contrast to the tumour, B cell level in the peripheral blood did not have any correlation with response to nivolumab treatment in the ADAPTeR cohort. All four groups had similar levels of B cell. Although the responders at baseline had approximately half the level of B cells observed in non-responders at baseline (median of 9.3% in non-responders and 4.2% in responders), this difference was not statistically significant (\(p=0.393\)). This difference in the sample size is also likely to contribute the this difference as
well as the lack of statistical significance. On treatment, both non-responder and responder had similar levels of B cell (median of 10.6% and 9.3%, respectively) with no statistically significant difference (p=0.981). Neutrophils make up the biggest subset of granulocytes and they are important in innate immunity. Their main roles an in a healthy individual include anti-microbial activity, phagocytosis and degranulation. Neutrophils have a half-life of 7 to 10 days but this period can be extended with the presence of cytokines in the circulation or microenvironment. Increased neutrophil infiltration of tumors has been associated with poor prognosis in several cancers including melanoma, glioma and gastric cancer. Similarly, intratumoural neutrophils are makers of poor prognosis in metastatic RCC. In addition to the intratumoral neutrophil infiltration, raised neutrophil count in the peripheral blood has been also been associated with worse overall survival in metastatic RCC patients. In light of this, we have analyzed the neutrophils in the peripheral blood. There was no statistically significant difference in the level of neutrophils in the peripheral blood between responders and non-responder at baseline and on treatment (p=0.907 and p=0.280, respectively). Neutrophils were less abundant compared to the other immune subsets analysed. At baseline, the median level was 1.5% and 1.9% in the non-responders and responders, respectively. On treatment, the median level was 0.86% and 0.17% in the non-responders and responders, respectively (Figure 5.9A and B).

Myeloid cells which consist of different subsets have also been analysed. CD45+CD3-CD15-CD56-C19- were identified as a general myeloid population. Similar to the pattern of macrophage infiltration seen in the tumors as described earlier in this thesis, no statistical significance was seen in the amount of myeloid cells in the peripheral blood in the 4 subgroups analysed. At baseline, the myeloid population made up 15.4% (median value) of the non-responder cohort and 13.6% (median value) of the responder cohort which was not a statistically significant difference (p=0.990). Similarly, on treatment, the myeloid cells in non-responders and responders were 13.6% and 11.8% (both median values) respectively with no statistically significant difference between the two groups (p=0.979). Myeloid cells were then classified into classical monocytes (CD45+CD3-CD16+HLA-DR+CD14+CD16-CD11b-CD11c+CD33+CD68- cells), dendritic cells (CD45+CD3-CD15-CD56-C19-CD14-CD16-CD11b-CD11c-CLECA9a+) and monocyte derived macrophages (MDM) (CD45+CD3-CD15-CD56-C19-CD14-CD16-CD11b-CD11c-CD33-CLECA9a+CD68+) and monocyte derived macrophages (MDM) (CD45+CD3-CD15-CD56-C19-CD14-CD16-CD11b-CD11c-CD33-CLECA9a+CD68+). More detailed analysis of the myeloid cell subsets showed that there was no significant difference in the abundance of monocytes, CLECA9a+ dendritic cells (DCs) and MDMs between non-responder and responders at baseline (p=0.999, p=0.999 and p=0.999, respectively) and on treatment (p=0.998, p=0.998 and p=0.980, respectively). Compared to the lymphocytes, these myeloid subsets constitute a relatively smaller proportion of the CD45+ immune cells in the peripheral
blood. The median range for classical monocytes was 4.53% and 6.93% across all 4 cohorts. For CLEC9a+ DCs and MDMs the median range was 2.47%-3.25% and 1.78%-4.47%, respectively (Figure 5.9A and B).

Figure 5.8 Phenotype of myeloid cells in the peripheral blood

(A). Heatmap shows the expression myeloid markers on CD45 pre-gated immune cells in the PBMC. (B). UMAPs show the expression profile of myeloid markers on CD45 pre-gated immune cells in non-responders at baseline (Baseline NR), responders at baseline (Baseline R), non-responders on treatment (Treated NR), and responders on treatment (Treated R). Heatmaps and UMAPs allow dimension reduction analysis and each cluster represents a different phenotype with co-expression of different markers shown in the heatmap. There are 10 clusters in each heatmap and UMAP and each cluster is represented with a different colour. The scale bar on the right hand side of the heatmap shows the frequency of each cluster in the samples.
Figure 5. 9 Immune cell subsets in the peripheral blood

(A). Frequency of different immune cell subsets in the peripheral blood for non-responder and responders at baseline and (B) for non-responders and responders on treatment is shown. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05).
After identifying the immune subsets in peripheral blood, we have characterized each subset in more detail. One of the questions we wanted to answer was to see if there is any difference in the FcγR expression in these immune subsets in the peripheral blood of four different clinical cohorts. CD16 expression on NK cells is important for antibody dependent cellular cytotoxicity (ADCC) function. Binding of ligands to CD16 on the NK cells leads to production of IFN-γ and TNF-α. In the ADAPTeR cohort, NK cells expressed moderate to high levels of CD16. At baseline, the median CD16 expression on NK cells was 84.7% in the non-responders and 76.7% in the responders, with no statistically significant difference between the two groups (p=0.998). No significant difference was seen between the two groups on treatment either (p=0.949) with median CD16 level of 85.4% in non-responders and 83.3% in the non-responders. On the other hand, the CD16+ NK cells were less abundant both at baseline (median of 15.3% and 23.3% in non-responders and responders, respectively) (p=0.998) and on treatment (median of 14.6% and 16.7% in non-responders and responders, respectively) (p=>0.999) compared to CD16+ NK cells. This suggests that the NK cells in all cohorts have a higher subset that has greater cytotoxic potential. CD32a is a subtype of CD32 FcγR and has an activatory function on immune complexes. It is found on activated NK cells. CD32b is another subtype of the CD32 FcγR with an inhibitory function on the immune complexes. It is expressed on different immune cells but most expressed on B cells. Both CD32a and CD32b were found to be expressed in very low levels in the NK cells. At baseline, CD32a expression on NK cells in non-responders was 2.25% (median value). Although this level was lower in the responders and was in negligible levels with a median value of 0.35 %, this difference was not statistically significant (p=0.999). On treatment, the non-responders had a median value of 2.05 % and responders had a median of 0.34%. Similar to the baseline cohorts, there was no statistically significant difference between these two cohorts (p=0.979). The small sample sizes in the responder groups both at baseline and on treatment could be a factor contributing to the lack of statistical significance. CD32b expression on NK cells was similar to the CD32a levels. The median expression of CD32b on NK cells at baseline was 2.61% in non-responders and 0.35% in the responders with no statistically significant difference (p=0.973). Results were similar in the treated cohorts where the median expression was 1.39% in the non-responders and 1.85% in the responders (p=0.998). Similar to the CD32a and CD32b, CD64 expression was also low in NK cells in both at baseline (median expression level of 2.09% in non-responders and 0.31% in responders; p=0.973) and on treatment (median expression level of 1.14% in non-responders and 0.62% in responders; p=0.940). It is known that CD38 is expressed on activated NK cells and it has previously been shown that the CD38+ NK cells are also mostly
Activated NK cell phenotype in the tumour has been associated with response to anti-PD1 therapy in metastatic melanoma patients. We therefore wanted to see if CD38 expression on NK cells in the periphery has a similar effect in response to nivolumab in RCC patients. At baseline, both the non-responders and the responders had relatively high levels of CD38 expression with a median expression level of 66.7% and 28.3%, respectively. Although there is a difference in the expression level of non-responders and responders, it was not statistically significant (p=0.996). One of the main reasons for lack of statistical significance is likely to be due to the difference in the sample size in non-responders and responders. A similar pattern was seen in on treatment too where the median expression of CD38 on NK cells in the non-responders was 77.5% and 48.2% in the responders (p=0.870). In addition, the CD38 expression was mainly on the CD16+ NK cells (median expression of 59.5% in non-responders and 25.8% in responders at baseline and p=0.906; median expression of 60.8% in non-responders and 46.5% in responders on treatment and p=0.989) compared to the CD16- NK cells (median expression of 10.9% in non-responders and 8.1% in responders at baseline and p=0.998; median expression of 13% in non-responders and 10.2% in responders on treatment and p=0.973). This suggests that NK cells in the peripheral blood have a more activated phenotype in all four cohorts and a large proportion of these cells also express CD16. However, this does not associate with response to nivolumab in this cohort. We have also looked at the HLA-DR expression on NK cells. The role of HLA-DR expression on NK cells has been a controversial topic. Some studies suggest that it is a marker of NK cell activation. On the other hand, in HIV patients it was shown that the HLA-DR expressing NK cells in the peripheral blood do not have an activated phenotype. However, in the ADAPTeR cohort HLA-DR expression was lower both at baseline (median expression of 9.67% and 5.3% in non-responder and responders, respectively; p=0.991) and on treatment (median expression of 6.98% and 6.02% in non-responder and responders, respectively; p=0.979). CD73 is also known as ecto-5'-endonucleotidase and is important in production of adenosine from AMP after ATP is converted to AMP by CD39. CD73 is expressed on various cells including tumour cells and different types of immune cells and inhibits immune activation. It was shown that the hypoxic microenvironment of the tumour leads to upregulation of CD73 which lead to vascular leakage. High CD73 expression has been associated with immune evasion and poor prognosis in multiple cancers including triple negative breast cancer and glioblastoma. It has been shown that the CD73 expression is mainly seen on the tumour infiltrating NK cells compared to the circulating NK cells and the increased levels of CD73+ NK cells in the tumour has been associated with worse clinical outcomes in sarcoma and breast cancer. They were founds to produce TGF-β as well as inhibiting CD4 T cell function. The median CD73 expression was in negligible amounts (<2%) in all cohorts with statistically significant
difference between the non-responders and responders at baseline (p=0.998) and on treatment (p>0.999) (Figure 5.10A and B).

Figure 5. 10 Phenotype of NK cells in the peripheral blood

(A) Phenotype of NK cells at baseline and (B) on treatment is shown. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). (Blue= Non-responders at baseline, Red=Responders at baseline, Grey=Non-responders on treatment, Purple=Responders on treatment)
Although there was no difference in the abundance of B cells in the different responders and non-responders in the ADAPTeR cohort as described above, we also investigated the expression of HLA-DR, CD73 and CD40 as the unsupervised analysis showed that expression of those three markers were elevated on the B cell as demonstrated in the heatmap in Figure 5.7A. CD73+ B cells were shown to have an immunosuppressive effect on effector T cells and an improved outcome was observed by inhibiting CD73 on B cells in melanoma mouse models. In addition, CD40 expression on B cells is important in B cell maturation and is suggested to play a role in anti-tumour immunity by increasing the antigen presentation and T cell activation ability of B cells. Similar to T cells, HLA-DR expression is marker of activation in B cells and suggests increased interaction with the T cells. It was observed that the HLA-DR expression was above 98% on B cells in groups and there was no statistically significant difference in the response groups at baseline (p=0.998) and on treatment (p=0.831). On the other hand, CD40 expression on B cells was lower compared to HLA-DR with a median expression of 43.9% in the non-responders and 45.8% in the responders at baseline with no statistical difference between the two groups (p=0.998). On nivolumab treatment, the median CD40 expression was 48.9% in the non-responders. Although the CD40 expression was higher in the on treatment in the responders with a median value of 61.6%, the difference between non-responders and responders was not significant (p=0.516). In addition, there was no significant difference observed in CD73 expression on B cells in the two response groups at baseline (p>0.999) or on treatment (p=0.271) (Figure 5.11 A and B).
Figure 5. 11 B cell characteristics in the peripheral blood

(A) Phenotype of B cells at baseline and (B) on treatment is shown. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). (Blue= Non-responders at baseline, Red=Responders at baseline, Grey=Non-responders on treatment, Purple=Responders on treatment.)

DCs are important antigen presenting cells and have different subtypes. CLEC9a is a C-type lectin receptor which is expressed on conventional/myeloid DCs which is a subtype of DCs that is mainly found in the peripheral blood. CLEC9a+ DCs have the ability to cross present both by MHCI and MCHII.305 We have analysed the expression pattern on DCs including the FcγR. In contrast to the NK cells, CD64 was the highest expressed FcγR on the DCs. Both non-responders and responders had high levels of CD64 expression at baseline (median expression of 76.85% and 71.1% respectively; p=0.999) and on treatment (median expression of 86.2% and 70% respectively; p=0.961). In contrast to CD64, other FcγRs were expressed in lower levels in the DCs. The median expression of CD16, CD32a and CD32b at baseline was 4.59%, 7.21% and 1.05% in the non-responders and 4.41%, 16.9% and 1.3% in responders, respectively. The CD16, CD32a and CD32b expression on DCs on treatment was similar to the baseline both in the non-responder cohort (median expression of 2.87%, 13.8% and 0.19%, respectively) and the responder cohort (4.25%, 15.4% and 0% respectively). The difference in expression of these three markers on the DCs between the non-responder and responders was not significant at baseline (p=0.998, p=0.999 and p=0.999, respectively) or
on treatment (p=0.924, p=0.979, p=0.999) (Figure 5.12 A and B). In addition to its activatory function, CD64 expression on the circulating DCs was shown to mediate increased phagocytosis of the antigens suggesting a possible increased phagocytic activity of the CD64+ DCs in the peripheral blood in the ADAPTeR cohort. CD38 is another marker which was expressed at high levels in the DCs. It was previously shown that CD38 plays an important role in DC maturation as well as chemotaxis and migration of DCs in the periphery. The median expression of CD38 was between 82%-93% in the non-responders and responders both at baseline and on treatment with no statistical difference (p=0.999 and p=0.822, respectively). CD206 is a mannose receptor which is expressed on immature DCs and is involved in endocytosis. CD206 expression usually has a negative correlation with CD64 expression in the DCs. To determine the immature DCs, we evaluated the CD206 expression on DCs and our analysis showed that CD206 was expressed in low levels on DCs in the peripheral blood. At baseline, the median expression was 3.48% in the non-responder cohort and 4.41% in the responder cohorts with the significant difference between the two groups (p>0.999). On treatment, the median expression of CD206 was slightly higher in the non-responders (5.27%) compared to responders (1.79%) but this difference was not statistically significant (p=0.822). In addition to CD64 and CD38, we also evaluated the expression level of CD86 on the DCs. CD86 is marker of DC activation and it binds to the CD28 which is an activating receptor expressed on T cells. The median expression of CD86 on the DCs in the peripheral blood was high both in non-responders and responders at baseline (median value of 88.% and 95.5%, respectively) and on treatment (median value of 87.8% and 94.5%, respectively). Moreover, the difference in the expression level did not differentiate between the response groups (p=0.999 and p=0.979 at baseline and on treatment, respectively). CD40 expression is an important marker for DC maturation and it has been shown that engagement of the CD40 expression leads to upregulation of CD86 and cytokine production by the DCs. Compared to CD86 an intermediate level of CD40 expression was observed on the circulating DCs in the ADAPTeR cohort. Non-responder cohort had a median CD40 expression of 50.7% compared to 38.6% in the responder cohort at baseline. Although the expression level in the responder cohort was lower, the difference was not statistically significant (p=0.865). A similar pattern was observed on treatment where there median CD40 expression in non-responders was 56.5% and 49.1% in the responders, with no significant difference between the two groups (p=0.999). In addition, CD73 expression on the DC was negligible with a median expression of <1% in all cohorts. We also looked at the expression of more functional markers such as Indoleamine-2,3-dioxygenase (IDO) and arginase-1 (Arg-1). IDO is an important enzyme in the tryptophan pathway and it’s main function is converting tryptophan to kynurenine. Kynurenine and its metabolites have important immune regulatory and immunosuppressive functions. Arg-1 mediates L-arginine
metabolism and plays an important role in wound healing, fibroblast and collagen production.\textsuperscript{315} It has been shown that Arg-1 expression in DCs down regulate MHCII expression and downregulates T cell based immunity.\textsuperscript{316,317} Both IDO and Arg-1 expression was at negligible levels with median IDO level of <2% in all cohorts and Arg-1 expression of <1% in cohorts at baseline and on treatment (Figure 5.12 A and B).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure5.png}
\caption{Phenotype of dendritic cells in the peripheral blood}
\end{figure}
As described earlier in the thesis, the classical monocytes makes up to 6% of the immune cells in the peripheral blood in the ADAPTeR cohort. Analysis of the FcγRs showed that the classical monocytes had a moderate level of CD32a whereas the CD32b expression was very low. At baseline, non-responders had a median CD32a level of 49.7%. Similarly, the median expression level was 44.2% in the responders. On treatment, the CD32a expression was slightly higher with 66.8% in the non-responders and 54.9% in the responders. However, there was no significant difference in the two cohorts at baseline (p=0.880) and on treatment (p=0.862). In contrast to CD32a, CD32b expression on the classical monocytes was low a median expression of 3.29% in the non-responders and 6.09% in the responders at baseline; 2.12% and 0.65% in the non-responders and responders on treatment, respectively. There was no statistical difference in the two response groups at baseline (p=0.981) and on treatment (p=0.862). Similar to what we observed in the DCs, monocytes both at baseline (median expression of 89.2% in non-responders and 81.1% in the responders) and on treatment (median expression of 95.8% in the non-responders and 77.2% in the responders) had high levels of CD64 expression. Although the responders had slightly lower levels of CD64 expression in the responders both at baseline and on treatment, the differences were not statistically significant (p=0.993 and p=0.538 for baseline and on treatment, respectively). High CD64 expression on monocytes suggest an activated state with more phagocytic activity. We also evaluated CD38 and CD86 expression which are both markers of activation in monocytes. Similar to DCs, the classical monocytes also had high levels of CD38 expression with median expression of CD38 higher than 85% in the all 4 cohorts. However, there was no significant difference in the CD38 level between non-responders and responders at baseline (p=0.997) and on treatment (p=0.138). CD86 was also expressed at high levels on the monocytes at baseline (median expression level of 85.9% and 74.7% in non-responders and responders, respectively) and on treatment (median expression level of 79.3% and 88.1% in non-responders and responders, respectively) with no statistical difference between non-responders and responders (p=0.911 and p=0.999 at baseline and on treatment, respectively). Engagement of the CD40 expressed on the monocytes with its ligand leads activation and cytokine production by the monocytes. CD40 expression was lower compared to CD38 and CD86 expression with median expression of 70.25% in non-responders and 57.3% in responders at baseline and 70.9% and 62.8% in non-responders and responders on treatment, respectively. CD73, Arg-1 and IDO expression on monocytes was less than 1% in all cohorts (Figure 5.13A and B).
Figure 5. 13 Phenotype of monocytes in the peripheral blood

(A) Phenotype of monocytes at baseline and (B) on treatment is shown. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). (Blue= Non-responders at baseline, Red=Responders at baseline, Grey=Non-responders on treatment, Purple=Responders on treatment)
MDM makes up the smallest subsets of immune cells in the peripheral blood in the ADAPTeR cohort as discussed earlier (Figure 5.9A and B). The phenotype of the MDMs was analysed in more detail. It was observed that the MDMs and the classical monocytes had a similar phenotype with higher expression of activatory markers such as CD32a, CD64, CD38 and CD86. The median expression of CD32a was 80.3% in the non-responders and 65.5% in the responders at baseline. On treatment, the non-responder cohort had a median CD32a expression level of 86.4% and the responders had median expression of 65.5%. Although the CD32a expression was lower in the responder groups both at baseline and on treatment, this difference was not statistically significant (p=0.815 and p=0.806, respectively). In contrast to CD32a, CD32b expression was almost at negligible levels both at baseline (median expression of 4.5% in the non-responders and 8.82% in the responders) and on treatment (median expression of 1.35% in the non-responders and 0.45% in the responders). CD64 expression was also high, similar to CD32a with a median expression of 54.6% in the non-responder and 54.9% in the responders at baseline (p=0.999). The CD64 level was higher in the non-responders (74.8%) compared to responders (46.2%) on treatment but this difference was not statically significant (p=0.569). CD38 which is another activation marker was also expressed at high levels in both at baseline and on treatment. However, the non-responder had higher expression of CD38 compared to responders both at baseline (median expression of 71.7% and 68.9%, respectively) and on treatment (median expression of 85.7% and 60.3%, respectively). However, this difference was not statistically significant (p=0.999 at baseline and p=0.309 on treatment). The CD86 expression was also high in all four cohorts with a median expression range between 82.5%-94.1% and with no significant difference in between the non-responders and responders at baseline (p=0.880) and on treatment (p>0.999). Similar to the monocytes the CD40 expression was at intermediate level compared both at baseline and on treatment CD38 and CD86 expression. On the other hand, CD206, CD73, Arg-1 and IDO expression were at negligible levels in all four cohorts. High CD64, CD38 and CD86 expression with low/negligible expression of CD206 suggest that the MDMs observed in the peripheral blood in the ADAPTeR cohort have a pro-inflammatory phenotype as previously also described in other studies (Figure 5.14A and B).
Figure 5. Phenotype of MDMs in the peripheral blood

(A) Phenotype of MDM cells at baseline and (B) on treatment is shown. Sidak-Bonferroni multiple t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance is not shown for samples where difference between samples is not statistically significant (p>0.05). (Blue= Non-responders at baseline, Red=Responders at baseline, Grey=Non-responders on treatment, Purple=Responders on treatment)
5.3. CHAPTER DISCUSSION

Analysis of the peripheral blood in the ADAPTeR cohort showed that CD8 and CD4 effector cells made up a much bigger compartment of the T cells compared to Tregs. Analysis of T cell differentiation patterns in the T cell subsets showed that Temra cells were the most abundant cell population in the CD8 followed by naïve CD8s and TCMs. On the other hand, naïve cells made up the biggest population in CD4 effectors followed by TCM and Temra cells. Detailed phenotypic analysis of the CD8, CD4 effector and Treg cells did not show any statistically significant difference. The lack of statistically significance is likely to be due to the very small sample size. Therefore, the data is mainly descriptive and cannot be used to any conclusions. A bigger Temra population in the CD8 subset with higher expression of CD57 and granzyme B suggest that these cells have gone through significant level of cell proliferation and have a cytotoxic phenotypes after antigen encounter. High levels of CD27 and KLRG-1 also suggest that these cells retain their effector function.143 In the CD4 cells, higher CCR4 expression suggests Th2 differentiation of CD4 cells which secrete IL-4 and IL-5 and lead to upregulation of IgE. Th2 CD4 cells originate from naive cells and then circulate to specific sites based on chemokines secreted.323 In RCC patients, it has been shown that higher CCR4+ CD4 cells was associated with more advanced disease.324 In addition, high levels of CD27 expression on the CD4 effectors suggests a maintained effector function.325 Analysis of Tregs showed that they had a different phenotype compared to CD4 effector and CD8 cells. High level of CD69 expression in the Tregs in the peripheral blood suggests a maintained immunosuppressive function. In addition, higher CCR4 expression (especially the trend in the non-responders although there was no statistically significant difference between the responders and non-responders) suggests a memory Treg differentiation. In addition, CCR4 expression on Tregs plays an important role in activation of Fas/Fas-L pathway and inhibits CD8 proliferation.326 Increased Fas expression in the Tregs in addition to increased CCR4 expression can potentially suggest their increased inhibitory effect on the CD8 cells. Finally, analysis of the nivolumab bound cells in the peripheral blood showed that the nivolumab bound cells made up a small proportion of the T cells subsets peripheral blood (up to 6 %) but the level was significantly higher in compared to treatment naïve peripheral blood samples. This low level could be due to fact that T cells in the blood circulate and we have only captured a proportion during the circulation. In addition, this could also be due to the fact that PD-1 expression in the peripheral blood is less than PD-1 expression in the TILs ad hence the lower drug binding. Another possible explanation can be due to time. These samples were collected several years ago and stored in liquid nitrogen. We do not have any evidence on how long the nivolumab stays bound to T cells and how the storing conditions affect this.
In addition to analysing the T cells, other immune cell subsets including myeloid cells, B cells and NK cell were also analysed in the peripheral blood in the ADAPTeR cohort. No statistical significance was seen in any of the comparisons. The most abundant immune cell subset in the peripheral blood was lymphocytes (>50%) and the level of B cells, NK cells, neutrophils and myeloid subsets were much less (median level of up to 15%). Amongst those, neutrophils made up the smallest subset with less than 2% expression. In the myeloid compartment, monocytes made up the biggest group followed by DCs and MDMs. These immune cell subsets were also analysed in more detail to understand their potential functional roles and to see if there was any correlation with response to nivolumab. In the NK cells, more than 70% of the cells expressed CD16 in all four cohorts which suggests these cell have a higher cytotoxic potential. In addition, CD38 which a marker of NK cell activation was high in all cohorts (with a higher trend in the non-responders although the difference was not statistically significant). More specifically, CD38 expression was higher in the CD16+ NK cells which supports the possibility that majority of the NK cells in ADAPTeR peripheral blood have an activated and cytotoxic phenotype. However, this did not correlate with response or resistance to nivolumab. In contrast to the IF data which showed increased intratumoural B cell infiltration at baseline in the responders, there was no difference in the level of B cells in the peripheral blood in different cohorts. In all cohorts, B cells were found to express high levels of HLA-DR and CD40 which suggest a mature and antigen experienced B cell phenotype. On the other, a moderate level of CD73 was also observed on the B cells suggesting a group of B cell with potentially immunosuppressive characteristics.

Analysis of the CLEC9a+ DCs in the peripheral blood showed high expression of CD38 suggesting a more mature DC phenotype in all groups. CD64 and CD86 were also expressed at high levels in the DCs which indicate that these DCs are activated. However, this activated state did not differentiate with response to nivolumab. Evaluation of both the classical monocytes and the MDMs showed high level of CD64, CD86 and CD38 expression in all four cohorts which indicate an activated state with increased phagocytic activity.

Although the data discussed in this chapter provides information about the common phenotypes of the immune cell subsets in the peripheral blood in the ADAPTeR cohort, there was no significant correlation with response or resistance to nivolumab treatment. The samples small size is a significant limitation in this analysis. Therefore, the data discussed is very descriptive and cannot be used to make any conclusions.
DECIPHERING THE EFFECT OF ANTI-PD1 THERAPY IN RENAL CELL CARCINOMA: IN DEPTH ANALYSIS OF A RESPONDER AND NON-RESPONDER

6.1 CHAPTER INTRODUCTION

Despite the revolutionary effect of immunotherapy in cancer treatment, response is still limited to a cohort of patients in different cancer types and the mechanism of resistance to immunotherapy is yet to be understood. Functional state of the T cells is critical in determining the ability of the T cells to respond to immunotherapy and dysfunctional (or exhausted- used interchangeably) T cells were shown to have impaired response to a immunotherapy. Increased tumour mutation burden has been shown to play an important role in T cell dysfunction and is vital in predicting response to immunotherapy. It is suggested that prolonged antigen stimulation alters the effector function of T cells and causes a shift towards a more dysfunctional state. During this shift, IL-2 production, proliferation and chemokine production of the T cells gets impaired which leads to defects in their cytotoxic function. Increased expression of PD-1, CTLA-4, TIM-3, LAG-3 and BTLA-4 on CD8 T cells is associated with an a dysfunctional/exhausted T cell phenotype and poor prognosis in cancer. Co-expression of PD-1 and TIM-3 on CD8 cells has been associated with more severe dysfunctional state. Recently it has been shown that TOX, which is a transcription factor, plays an important role in commitment of effector T cells to an exhausted state. In addition, CD39 expression on tissue resident memory (TRM) T cells was shown to increase in an exhausted phenotype. Miller and colleagues showed that exhausted T cells exist in a biphasic state and a subpopulation of exhausted cell maintain their functional state which is a progenitor-like exhausted state. However, with continued antigen exposure, the progenitor exhausted T cells commit to a terminally exhausted state where they lose their function. Transcription factor TCF1/7 was shown be critical in maintaining the progenitor-like state of exhausted T cells and therefore allow this population of T cells to maintain their tumour reactivity and therefore enhance response to immunotherapy.

Although the information from bulk analysis on the possible mechanism of response and resistance to immunotherapy including anti-PD-1 therapy in different cancers have been very informative, the advances in the single cell technologies advanced the depth and resolution of understanding of mechanism of immune response and resistance to immunotherapies. Single cell RNA sequencing (SC RNA Seq) data form a pan-cancer meta data showed that the CXCL13 and CCR5 expression on T cells correlates with response to anti-PD-1 therapy. A recent study using SC RNA Seq showed a higher levels of TRM CD8 cells as well as increased TCR expansion in RCC patients who
respond to immunotherapy. However, this study included only 6 patients and there was a heterogeneity in the treatment as some patients were treated with anti-PD-1 therapy and some were treated with combination anti-PD-1 and anti-CTLA-4 therapy.

We hypothesized that the non-responder and responder patients will represent the corresponding cohorts in the ADAPTeR trial and the responder patient will have higher granzyme B expression, higher proportion of dysfunctional CD8 T cells as well as higher TCR expansion and clustering compared to the non-responder patient. In order to have a more in depth understanding of the possible mechanisms involved in the response and resistance to anti-PD-1 therapy in the ADAPTeR cohort, we have focused on one responder and one non-responder patient. We have performed high dimensional flow cytometry analysis and SC RNA Seq with TCRSeq in order to answer the following questions:

- What are the characteristics of T cell subsets at a protein expression and transcriptomic level in the responder and non-responder?
- Is there any difference in the protein, transcription and TCR profile of the cells that bind nivolumab and cells that do not bind nivolumab?
- Is there relationship in the characteristics observed following the questions stated above and response to treatment?

The results presented in this chapter were produced as part of several collaborations. I have designed and optimised the 24 colour high dimensional flow cytometry panels used in this chapter under the supervision of Dr. Pablo Becker who is a group leader at Achilles Therapeutics. I have performed all the experiments and the data analysis in addition to the panel design and optimisation. I have also planned and performed the wet lab experiments for the SC RNA/TCR Sequencing. The bioinformatics analysis was performed by Dr. Gordon Beaty who is post-doctoral scientist at the single cell core facility at the UCL Cancer Institute.

Data presented in this chapter is also used in our article titled 'Determinants of anti-PD-1 response and resistance in clear cell renal cell carcinoma' which is accepted to Cancer Cell and I am a joint first author on the paper.
6.2 RESULTS

6.2.1 CD8 T cells have a different immune checkpoint landscape in responder and non-responder patients

Although fresh tumour samples were not available for high dimensional flow cytometry analysis for all the patients, single cell suspension of TILs for on treatment nephrectomy samples were available for ADR001 (a non-responder patient) and ADR013 (a responder patient). Tumour adjacent normal renal tissue was also available for ADR013. Therefore, those two patients were used as representative patients for responder and non-responder cohorts. Bulk tissue excluding the necrotic areas was used for TIL isolation and flow cytometry analysis (Figure 6.1).

Figure 6. 1 Details of the fresh samples used from ADR001 and ADR013

(A) Regions used from the nephrectomy sample to make up the bulk TILs suspension for ADR001 is shown. The numbers indicate the regions collected. Blue circles indicate adequate tumour content and red circles indicate necrotic areas. (B) Regions used from the nephrectomy sample to make up the bulk TILs suspension for ADR013 is shown. Red circles indicate adequate tumour content, pink circles indicate low tumour content and stripy dot indicate necrotic areas.
Abundance of T cell subsets and their phenotypes were analysed in detail for ADR001 TILs, ADR013 normal tissue and ADR013 TILs (Figure 6.2A and B). It was not possible to perform any statistical analysis due to the sample size (n=3) and the analysis is mostly descriptive. The most abundant T cell subtypes was CD8 followed by CD4 effectors in both patients. In ADR001, CD8 cells made up 53.3% of the T cells. ADR013 TILs has a slightly higher CD8 level (68.4%). In addition, the CD8 infiltration in ADR013 normal tissue was lower (49.2%). CD4 effector infiltration was lower compared to CD8 cells and was highest in the ADR013 normal tissue (31.7%). ADR013 TILs has lower levels of CD4 effectors (8.29%) compared to ADR001 (16.0%). Tregs made up the smallest T cell subset with abundance of 2.86%, 3.88% and 1.64% in ADR001 TILs, ADR013 normal tissue and ADR013 TILs respectively (Figure 6.2A).
Figure 6.2 Phenotype of T cell subsets in ADR001 and ADR013

(A) Percentage of C8, CD4 effector and Tregs from the on treatment nephrectomies. ADR001 (non-responder) and ADR013(responder) is shown. Heatmaps and bar graphs in (B) and (C) show the phenotype of T cell subsets. ADR001_T= ADR001 TILs, ADR013_N=ADR013 normal tissue, ADR013_T=ADR013 TILs. Heatmaps and bar graphs allow dimension reduction analysis and each cluster represents a different phenotype with co-expression of different markers shown in the heatmap. There are 10 clusters in each heatmap and bar graph and each cluster is represented with a different colour. The scale bar on the right hand side of the heatmap shows the frequency of each cluster in the samples.
We then evaluated the phenotype of the T cell subsets in more detail. Role of T cell differentiation has been shown to have a prognostic value in response to checkpoint blockade treatment in cancer.\textsuperscript{153,338} Evaluating the T cell differentiation states in ADR001 and ADR013 showed that they had negligible level of naïve CD8 cells (less than 2%). CD8 T cells were then analysed separately (Figure 6.3). The CD45RA\textsuperscript{+}CD103\textsuperscript{+} CD8 population in both patients suggest presence of TRMs which are effector memory cells that become resident in a tissue type after antigen exposure. CD8 cells in ADR001 had a bigger subset of CD57\textsuperscript{+} CD8 cells (23.2%) compared to ADR013 (10%) which suggests a more terminally differentiated phenotype.\textsuperscript{193} CD8 cells were found to express higher levels of PD-1 (29.2% vs 2.25%), Eomes (61.6% vs 40.5%), TIM-3 (13.1% vs 1.35%), CD38 (33.9% vs 19.7%), CD39 (35.7% vs 2.29%) and TOX (11.6% vs 3.03%) in the ADR013 tumour sample compared to ADR001 which are associated with an exhausted T cell phenotype (Figure 6.4A and B).\textsuperscript{339,340} In addition to contributing to an exhausted state, CD39 expression was also suggested to have tumour-specific T cell reactivity.\textsuperscript{341} GITR level was lower in ADR01 than in ADR013 (26.9% vs 37.9% for GITR). CD8 cells in the ADR013 tumour also expressed higher levels of TCF7 (27.5%) compare to ADR001(4.64). A small proportion of the TCF7\textsuperscript{+} CD8 cells co-expressed TOX (4.56%) which was higher in ADR013 in comparison to ADR001 (less than 1%). TCF7 expression is suggested to be important in maintaining progenitor like effector function during prolonged antigen stimulation in T cells.\textsuperscript{112,253,254} Although TCF7 and TOX are expressed in two opposing T cell differentiation function states, recent data suggests that TOX expression is not only associated with T cell exhaustion and is important for antigen driven effector function and its co-expression with TCF7 has been described as a precursor exhausted T cell state which is suggested to be important for a maintained T response to antigen stimulation. This suggests a presence of precursor exhausted and exhausted CD8 cells in the responder patient which is a concept that has been previously been described to a have predictive role in response to anti-PD1 therapy in cancer.\textsuperscript{182} CD8 cells with a cytotoxic phenotype were also higher in the responder compared the non-responder as reflected by the higher Granzyme B expression in ADR013 30.3% (versus 20% in ADR001). HLA-DR which is a marker of antigen experience and activation was also expressed in higher levels on the CD8 cells in the ADR013 (47.5% vs 23.8%) as well as the chemokine receptor CCR4 (28.9% in ADR013 vs 10.1% in ADR001). In addition, the CD8 T cells in the normal region in ADR013 showed a similar phenotype to ADR013 TILs, especially in TCF7, CD39, CD57 and Eomes expression. On the other hand, the CD27 and KLRG1 expression were higher in the normal tissue (25.9% and 21.1%) compared to TILs (10.4% and 8.05%) ADR013.
Figure 6. 3 Phenotype of CD8 cells in ADR001 and ADR013

Heatmaps and bar graphs in (A) and (B) show the phenotype of CD8 cells ADR001_T=ADR001 TILs, ADR013_N=ADR013 normal tissue, ADR013_T=ADR013_TILs. Heatmaps and bar graphs allow dimension reduction analysis and each cluster represents a different phenotype with co-expression of different markers shown in the heatmap. There are 10 clusters in each heatmap and bar graph and each cluster is represented with a different colour. The scale bar on the right hand side of the heatmap shows the frequency of each cluster in the samples.
Figure 6. 4 Comparison of CD8 cells in ADR001 and ADR013

Bar graphs in (A) and (B) show the comparison of CD8 phenotypes in ADR001 TILs, ADR013 normal tissue and ADR013 TILs.
In addition to analyzing the phenotype of CD8 cells, phenotype of CD8 TRM cells were also analyzed in more detail. The expression profile of CD8 TRM cells mirrored the overall CD8 phenotype. TRMs in the ADR001 had higher CD57 expression (26.9%) compared to ADR013(8.2%) suggesting a history of increased proliferation and terminal differentiation. CD69, which is another tissue residency marker and allows for TRMs to remain in the TME, was shown to be a HIF-1 target which gets upregulated in hypoxic conditions. TRMs in the ADR001 had higher CD69 expression (20.7%) than ADR013 (8.79%) suggesting a potential higher hypoxic microenvironment in ADR001. On the other hand, PD-1 (19.4% vs 0.66%), TIM-3 (10.3% vs 1.05%), Eomes (74.2% vs 49.3%), CD39 (15.9% vs 2.13 vs) and TOX (9.24% vs 1.87%) were higher in ADR013 compared to ADR001 suggesting a more exhausted phenotype. Similar to the overall CD8 population, CD8 TRMs in ADR013 has higher TCF7 expression (34.6%) compared to ADR001 (1.96%) which suggest that the CD8 TRMs in the responder patient (ADR013) have a progenitor-like phenotype which is not observed in the non-responder (ADR001). CCR4 expression was also higher on the TRMs in ADR013 (28.5%) than in ADR001 (16.6%). CD38 and GITR levels were high in both patients (CD38: 44.5% and 34.8%, GITR: 27.1% and 36.5% in ADR001 and ADR013 respectively). TRMs in the normal region in ADR013 similar to ADR013 TILs in terms of their phenotype, especially in terms of TOX, CD57, Eomes, CD38, CD39 and Granzyme B expression. However, they had higher CD27 expression and lacked the KLRG1 expression in the overall CD8 cells in the normal region (Figure 6.5 A and B).
6.2.2 Nivolumab bound IgG4+CD8 cells have progenitor exhausted and cytotoxic phenotype in the responder compared to the non-responder

Nivolumab is an IgG4 isotype anti-PD1 antibody. It has previously been shown that anti-human IgG4 can be used to assess PD-1 receptor occupancy by the anti-PD-1 monoclonal antibody. We used anti-human IgG4 antibody as a surrogate for binding of nivolumab to T cells. Therefore, IgG4+ cells were accepted as nivolumab bound cells. Comparison of peripheral blood and TILS in ADR001 and ADR013 showed that the percentage of IgG4+ CD8 cells is much higher in the tumour (73.1% in ADR001 and 46.8% in ADR013) compared to CD8 cells in the peripheral blood (15.5% in ADR001 and 8.74% in ADR013) which suggests a higher affinity of nivolumab for immune cells resident in the tumour microenvironment rather than in the periphery (Figure 6.7B). This difference can also be due to the lower PD-1 expression in the peripheral blood compared to the TILs. In
addition, the IgG4+ CD8 cells and IgG4+ CD8 TRM cells were higher in ADR001 compared to ADR013 suggesting more CD8s including TRMs have bound the drug in the non-responder. In addition, a significant proportion of CD8 cells (26.6%) and CD8 TRM cells (31%) have also bound the nivolumab in the normal tissue in ADR013 (Figure 6.4B and 6.5B). On the other hand, a bigger proportion of the CD8 TRM cells were nivolumab bound in the non-responder (61.3%) compared to the responder (21.9%). Almost none of the CD45RA+ CD8 cells were IgG4+ in both patients suggesting that Temra and naïve CD8 cells did not find nivolumab. However, CD57 expression was higher on the IgG4+ CD8 cells (24.3% in ADR001 TILs, 16.30% in ADR013 Normal and 16.70 in the ADR013 TILs) in both patients compared to the IgG4+ CD8s (12.4% in ADR001 TILs, 7.4% in ADR013 Normal and 1.41% in the ADR013 TILs) and was higher in the non-responder compared to the responder. In addition, PD-1 expression was higher in the IgG4+ CD8 cells in the responder compared to the non-responder patient but the PD-1 expression in the IgG4+ and IgG4- CD8 cells were comparable. The higher level of PD-1 expression could potentially be due to a better ability of the T cells to respond to activation and therefore upregulate PD-1 in the responder compared to the non-responder. IgG4+ CD8 cells also had higher TIM-3 in the responder patient (35.4% in TILs and 24.4% in the normal region) compared to the non-responder patient (3.52%). However, TIM-3 expression on IgG4- CD8 cells was at negligible levels for both patients. A similar pattern was seen for CD39 expression. The IgG4+ CD8 cells had higher CD39 in the responder (54.6% in the TILs and 31.5% in the normal tissue) compared to the non-responder (3.25%). On the other hand, IgG4- CD8 cells in the non-responder and normal tissue in the responder had <1% CD39 expression and the TILs in the responder had a low level of CD39 expression at 6.58%. CD38, which is another marker of T cell dysfunction, was also higher on the nivolumab bound CD8 cells and was found at a higher level in the responder patient (57.4%) compared to the non-responder (22.6%). Eomes was lower on the IgG4+ CD8 cells compared to IgG4- CD8s in both patients but the responder higher expression (54.9%) in relation to the non-responder (36.2%). Further analysis of the nivolumab bound CD8 cells showed that the responder patient had a higher level of Granzyme B expression (38.9% vs 8.75%). However, Granzyme B expression on the non-drug bound cells was lower in the responder (17%) but higher in the non-responder (26.9%) which suggests that the nivolumab is able to induce a cytotoxic function in a higher proportion of CD8 cells in the responder. Granzyme B expression has been shown to be a prognostic factor in other cancer types and our results also suggested that increased level of drug bound cells with higher cytotoxic function correlate with better response to anti-PD1 treatment. Similar to the expression profile of CD8 cells described above, TOX and TCF7 expression were also higher on the nivolumab bound CD8s and the responder had higher TOX
(14.5%) and TCF7 (19.5%) compared to the non-responder (4.1% and 2.7% for TOX and TCF7 respectively). HLA-DR level was also observed at higher levels on the nivolumab bound CD8 cells with more expression in the responder (41.9%) compared to the non-responder (20.7%) suggesting a more activated state and more antigen experience in the responder and on the nivolumab bound cells. (Figure 6.6, Figure 6.7A and B). Co-expression of TCF7 with TOX in a small proportion of IgG4+ CD8 cells indicates the presence of precursor dysfunctional cells which has been associated with maintenance of effector function (Figure 6.7A).\textsuperscript{349} The IgG4+ CD8 cells in the normal tissue in ADR013 had a high level of CD27 expression (67.0%). However, the CD27 level was only 11.3% in the IgG4- CD8 cells. This increased CD27 expression was specific to the normal tissue and was not observed in ADR013 TILs or ADR001 TILs. In contrast to CD27, KLRG1 expression was higher in the IgG4- CD8 cells in the ADR013 normal tissue (25.30%) compared to the IgG4+ CD8 cells (4.17%). In addition, KLRG1 expression was highest in the ADR013 normal tissue compared to the ADR013 and ADR001 TILs.
**Figure 6. 6 Comparison of IgG\(^4\) and IgG\(^4\)+ CD8 cells in ADR001 and ADR013**

Comparison of IgG\(^4\) and IgG\(^4\)+ CD8 phenotypes in ADR001 TILs, ADR013 normal tissue and ADR013 TILs is shown.

<table>
<thead>
<tr>
<th></th>
<th>ADR001 TILs</th>
<th>ADR001 Normal</th>
<th>ADR013 TILs</th>
<th>ADR013 Normal</th>
<th>ADR013 TILs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD103</td>
<td>36.60</td>
<td>28.20</td>
<td>45.90</td>
<td>61.30</td>
<td>37.30</td>
</tr>
<tr>
<td>TOX</td>
<td>1.47</td>
<td>0.77</td>
<td>9.28</td>
<td>4.10</td>
<td>12.70</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>9.24</td>
<td>7.55</td>
<td>30.00</td>
<td>20.70</td>
<td>19.90</td>
</tr>
<tr>
<td>CD45RA</td>
<td>14.80</td>
<td>18.20</td>
<td>2.23</td>
<td>0.46</td>
<td>1.45</td>
</tr>
<tr>
<td>CCR4</td>
<td>6.43</td>
<td>5.08</td>
<td>19.50</td>
<td>7.20</td>
<td>7.97</td>
</tr>
<tr>
<td>CD87</td>
<td>12.40</td>
<td>7.40</td>
<td>1.41</td>
<td>24.30</td>
<td>16.30</td>
</tr>
<tr>
<td>KI67</td>
<td>2.25</td>
<td>1.39</td>
<td>3.17</td>
<td>3.10</td>
<td>2.54</td>
</tr>
<tr>
<td>CD39</td>
<td>0.34</td>
<td>0.46</td>
<td>6.58</td>
<td>3.25</td>
<td>31.50</td>
</tr>
<tr>
<td>CCR7</td>
<td>6.09</td>
<td>0.15</td>
<td>0.00</td>
<td>0.46</td>
<td>0.72</td>
</tr>
<tr>
<td>CD69</td>
<td>11.80</td>
<td>4.47</td>
<td>4.70</td>
<td>0.23</td>
<td>0.00</td>
</tr>
<tr>
<td>TCF7</td>
<td>2.03</td>
<td>5.86</td>
<td>5.29</td>
<td>2.17</td>
<td>13.00</td>
</tr>
<tr>
<td>CXCR5</td>
<td>2.48</td>
<td>4.01</td>
<td>17.20</td>
<td>0.39</td>
<td>0.72</td>
</tr>
<tr>
<td>Eomes</td>
<td>37.60</td>
<td>72.90</td>
<td>75.20</td>
<td>36.20</td>
<td>56.10</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>0.48</td>
<td>0.14</td>
<td>2.02</td>
<td>1.44</td>
<td>4.92</td>
</tr>
<tr>
<td>GITR</td>
<td>24.30</td>
<td>10.30</td>
<td>33.00</td>
<td>29.10</td>
<td>14.40</td>
</tr>
<tr>
<td>4-1BB</td>
<td>1.90</td>
<td>0.28</td>
<td>2.83</td>
<td>4.96</td>
<td>10.20</td>
</tr>
<tr>
<td>CD38</td>
<td>12.50</td>
<td>7.86</td>
<td>11.20</td>
<td>22.60</td>
<td>35.10</td>
</tr>
<tr>
<td>CD228</td>
<td>4.05</td>
<td>3.03</td>
<td>0.54</td>
<td>3.13</td>
<td>2.27</td>
</tr>
<tr>
<td>TIM-3</td>
<td>0.00</td>
<td>0.55</td>
<td>2.56</td>
<td>3.52</td>
<td>24.20</td>
</tr>
<tr>
<td>KLRG-1</td>
<td>12.40</td>
<td>25.30</td>
<td>8.22</td>
<td>0.39</td>
<td>4.17</td>
</tr>
<tr>
<td>CD27</td>
<td>0.48</td>
<td>11.30</td>
<td>3.10</td>
<td>0.65</td>
<td>67.00</td>
</tr>
<tr>
<td>ICOS</td>
<td>0.24</td>
<td>0.14</td>
<td>0.40</td>
<td>1.17</td>
<td>9.47</td>
</tr>
<tr>
<td>Fas</td>
<td>13.60</td>
<td>11.40</td>
<td>7.68</td>
<td>17.20</td>
<td>9.09</td>
</tr>
<tr>
<td>GrzmB</td>
<td>26.90</td>
<td>28.20</td>
<td>17.00</td>
<td>8.75</td>
<td>20.10</td>
</tr>
<tr>
<td>CD25</td>
<td>0.48</td>
<td>1.24</td>
<td>0.40</td>
<td>0.00</td>
<td>1.14</td>
</tr>
<tr>
<td>PD-1</td>
<td>0.48</td>
<td>5.09</td>
<td>17.90</td>
<td>0.78</td>
<td>3.41</td>
</tr>
</tbody>
</table>
Figure 6. 7 FACS plots for CD8 and IgG4+CD8 cells in the ADR001 and ADR013

(A) Co-expression of markers on CD8+ and IgG4+CD8 cells is shown in ADR001 TILs, ADR013 normal tissue and ADR013 TILs. (B) Comparison of IgG4+ CD8 cells in the peripheral blood and in the TILs is shown for ADR001 and ADR013.
6.2.3 CD4 cells have a different phenotype compared to CD8 cells with more naïve-like characteristics in CD4 effectors high expression of Fas and CTLA-4 in the Tregs

We have also analysed the CD4 effector cells in more detail in the responder and non-responder patients. The CD103 expression on the CD4 effectors was less compared to the CD8 cells but similar both patients (5.79% in ADR001, 6.60% in ADR013 TILs and 9.9% in normal tissue in ADR013). On the other hand, CD4 effector has higher CCR7 in ADR001 (18.9%) compared to the negligible (<2%) CCR7 expression on the CD8 cells which suggests that a higher proportion of CD4 naïve and TCM cells. However, CD4 effector CCR7 expression was marginally lower in the responder (6.09% in ADR013 TILs and 2.35% in ADR013 normal). Expression of most markers such as TOX, HLA-DR, CCR4, CD39, CD38, CD69, Eomes and TIM-3 were significantly lower on the CD4 effector cells in both patients compared to CD8 cells. Although TCF7 and PD-1 were also expressed at lower levels compared to CD8 cells, they were found at higher levels on the CD4 effectors in the responder (18.8% and 24.9% for TCF7 and PD-1 respectively) than the non-responder (5.45% and 1.83%). In addition, the HLA-DR and CCR4 levels were much lower on the CD4 effectors in relation to CD8 cells and the comparison between the two patients shows that the non-responder has higher HLA-DR and CCR4 (18.8% and 16.2%) expression than the responder (10.7% and 5.8% for HLA-DR and CCR4 respectively). On the other hand, CTLA-4 expression was higher on the CD4s and was highest in the non-responder (35.8%) followed by the responder TILs (13.8%) and responder normal region (6.64%). A striking difference between the CD8s and CD4 effectors was that the CD4 effector cells had a significantly higher expression of Fas which is a protein that belongs to the TNF receptor family and is involved in cell death.\textsuperscript{350} The non-responder patient had the highest Fas expression (68%). Although the Fas expression was less in the ADR013 TILs (52.9%) and normal tissue (59.6%), the level of expression of still high. Fas\textsuperscript{+} CD4 effector cells in the non-responder co-expressed CTLA-4, ICOS and CD25 which was not observed in the responder patient. In addition, a small population of the CD4 effector cells bound nivolumab compared to CD8 cells reflect by the IgG4+ CD4 effector cells: 15.30%, 18.8% and 28.7% in ADR01 TILs, ADR013 TILs and ADR013 normal tissue respectively (Figure 6.8A, B and C).
Figure 6. 8 Comparison of CD4 effector cells in ADR001 and ADR013

Bar graphs in (A) and (B) show the comparison of expression pattern on CD4 effectors in ADR001 TILs, ADR013 normal tissue and ADR013 TILs. (C). FACS plots show co-expression of Fas with CD25, CTLA-4 and ICOS in ADR001 and ADR013 TILs, ADR013 normal tissue.
IgG4+ CD4 effector cells were also analysed in order to understand the phenotype of the nivolumab bound CD4 effector cells in more detail. PD-1 expression in the responder (11.3%) was higher compared to the non-responder (2.13%). However, PD-1 expression was higher on the IgG4+ CD4 effector cells (27.4%) in the responder compared to the IgG4+ CD4 effectors. In contrast to the CD8 cells, the non-responder patient had higher levels of HLA-DR and CCR4 both in the IgG4+ CD4 effector (38.2% and 26.6% in non-responder versus 17.7% and 6.45% in the responder) and IgG4+ CD4 effector populations (15.9% and 23% in non-responder versus 6.67% and 13.3% in the responder). Similar to the observations in the CD8 cells, TCF7 was found at a higher level in the responder patient compared to the non-responder both in IgG4+ (21.0% versus 2.13%) and IgG4+ CD4 effectors (40.7% versus 10.5%). It was also seen that the nivolumab bound CD4 cells had less TCF7 expression in both patients. This suggests that the CD4 effectors in the responder have a higher progenitor like function and this is more prominent in the cell which have not bound nivolumab. Similar to the overall CD4 population, a high level of Fas expression was seen in both in the IgG4+ and IgG4- CD4 effector cells. The highest expression was in the non-responder patient both on the IgG4+ (57.90%) and IgG4- CD4 effector cells (62.50%). In the responder patient the Fas expression level was lower (28% on the IgG4+ CD4 effectors and 41.9% on the IgG4- CD4 effectors). CTLA-4, ICOS, Eomes and GITR were also expressed at moderate levels in the IgG4+ and IgG4- CD4 effector both in the responder and non-responder. CTLA-4 expression was higher on the IgG4- cells in the non-responder (40% versus 11.3% in the responder) compared to the IgG4- cells. ICOS expression was higher on non-responder in both IgG4+ (28.1% versus 14%) and IgG4- (16.5% versus 4.03) CD4 effector cells and the IgG4+ cells had higher ICOS levels in both patients compared to IgG4- cells. On the other hand, Eomes expression was similar between the two patient in nivolumab bound cells (38.6% in the non-responder and 38% in the responder) and was higher compared to the non-drug bound CD4 effectors (4.76 in the non-responder and 14.5% in the responder). In contrast, the responder had higher GITR expression in both subsets (34% in the IgG4+ cells and 27.4% in the IgG4- cells) compared to the non-responder (22.8% in the IgG4+ cells and 17.8% in the IgG4- cells). In addition, CD4 effectors in the normal region in the responder patient also had higher levels of Fas (42.4%), ICOS (27.3%), CTLA-4(19.2%), Eomes (25.3%) and CD27(35.4%) on the nivolumab bound cells (Figure 6.9).
**Figure 6.** Comparison of IgG4\(^-\) and IgG4\(^+\) CD4 effector cells in ADR001 and ADR013

Comparison of IgG4\(^-\) and IgG4\(^+\) CD4 effector phenotypes in ADR001 TILs, ADR013 normal tissue and ADR013 TILs is shown.
In addition to the CD8 cells and CD4 effector cells, we have also characterized Tregs. Both patients had a high level of CCR4 expression with similar expression levels in the responder (48.7%) and the non-responder (47.9%). Another chemokine receptor, CXCR5, was also found at a higher level in the responder (20.5%) compared to the non-responder (0%). Similar to CD4 effector cells, the responder patient (15.4%) had higher level of TCF7 compared to the non-responder (1.71%). Similarly, CXCR5 expression was also higher in the responder (20.5%) and no expression was seen in the non-responder. On the other hand, HLA-DR expression was higher in the non-responder (23.1%) compared to the responder (5.13%). Analysis of the checkpoint expression showed that PD-1 expression on the Tregs in the responder patient was higher in the responder (20%) compared to the non-responder (5.1%). CTLA-4, GITR, ICOS and Fas were expressed at higher levels in both patients compared to the checkpoints. Both the non-responder and the responder patient had similar levels of CTLA-4 (54.2% versus 52.2%), GITR (41.7% versus 39.1%) and Fas (27.1% versus 34.8%) expression. However, ICOS expression on Tregs was higher responder (39.1%) compared to the non-responder (20.8%). Analysis of the IgG4+ cells showed that the 17.9% of the Tregs bound nivolumab in the responder while the only 5% of the Tregs in the non-responder bound the drug (Figure 6.10). Fas+ Treg co-expressed CTLA-4, ICOS, GITR and CD25. CTLA-4 and CD25 co-expression level was higher in non-responder. In addition, CTLA-4+ Tregs also co-expressed CD25, ICOS and GITR which higher in the non-responder (Figure 6.11). Due to the small number of Tregs, IgG4+ and IgG4- Tregs were not characterized in more detail.
Figure 6. 10 Comparison of Tregs in ADR001 and ADR013

Bar graphs in (A) and (B) show the comparison of Treg phenotypes in ADR001 TILs, ADR013 normal tissue and ADR013 TILs.
Figure 6. 11 FACS plots showing co-expression pattern in Tregs in ADR001 and ADR013

FACS plots show co-expression of Fas ,CD25, CTLA-4, ICOS and GITR in ADR001 TILs, ADR013 normal tissue.
6.2.4 Nivolumab binding correlates with upregulation of T cell activation genes and TCR clonal expansion

In order to characterise the nivolumab bound and non-nivolumab bound T cells in more detail, we have performed SC RNaseq with gene expression profile and TCR sequencing on the FACS sorted IgG4+CD3+ and IgG4 CD3− TILs from the ADR001 (non-responder) and ADR013 (responder). The number of IgG4 CD3+ cells used for the SC RNaseq analysis for ADR001 was 6799 and the number of IgG4+CD3+ cells were 4394. In ADR013, we used 9377 IgG4+CD3+ and 10,614 IgG4+CD3+ cells. The T cell subset phenotype classification was done as following: CD8 (CD8+CD4−FoxP3−), CD4 effector (CD8−CD4+FoxP3−) and Treg (CD8−FoxP3+). The most abundant T cell subtype was CD8 followed by CD4 effectors and Tregs in both patients (Figure 6.12A). We then assessed if there was a difference in the transcription profile of nivolumab bound and non-bound T cells subsets between the responder and the non-responder. The results shows that the nivolumab bound cells had similar transcriptional profiles in the responder and non-responder patients in CD8, CD4 effector and Treg cells (Figure 6.12B). Then we focused on the CD8 cells for rest of the analysis as CD8 was the most predominant T cells subset in both patients. Differential gene expression and GOBP gene set enrichment analyses of IgG4+ CD8 cells showed upregulation of T cell activation, inflammatory response and cytotoxic activity pathways in both patients which suggests that the CD8s that bind to nivolumab have similar transcriptional profiles overall (Figure 6.12C). TCR analysis showed that CD8 cells had the most clonal expansion in with higher abundance of expanded and hyperexpanded clones. The responder had more clonal expansion compared to the non-responder (Figure 6.13A and B). In addition, the clonal expansion was higher on the IgG4+ CD8 cells compared to the IgG4− CD8s (Figure 14A). When the transcriptional profile of IgG4+ CD8 with different degrees of TCR clonal expansion was analysed, we saw that the expanded IgG4+ CD8 cells in the responder upregulated genes involved in cytotoxicity including Granzyme K which was not the case in the non-responder (Figure 6.13C). On the other hand, expanded IgG4− CD8 cells in the non-responder upregulated genes involved in T cell migration and metabolism (Figure 6.13D). Using the bulk and SC TCR sequencing data, we did clustering analysis on CD8 cells which allowed to cluster the TCRs with similar sequences (therefore, similar structures) together. The TCRs which cluster together are likely to recognise and bind the same antigens due to sharing a similar TCR structure. The bulk TCR data was used to identify which expanded TCRs were existing pre-treatment and which ones are new on treatment. In the responder patient, there was significant clustering of the clones and most of the were nivolumab bound cells. There both new and pre-exiting expanded clones in the clusters. On the other hand, there was almost no clustering in the non-responder patient (Figure 6.14B). scRNAseq data also confirmed flow
cytometry findings, with higher expression of GZMB, TCF7, TIM3, and CD39 expression on IgG4+CD8+ T-cells in ADR013 compared to ADR001 (Figure 6.15A and B).
**Figure 6. 12 Characteristics of Nivolumab bound and non-bound T cells**

(A) The merged overall abundance of CD8, CD4 effector and Treg cells is shown by the UMAP and the proportions of each T cell subtype in ADR001 and ADR013 is shown in the bar graph. (B) Shows the differential gene expression between IgG4+ and IgG4− T cell subsets in both patients. Average logFC is plotted for responder versus non-responder. Regression line is plotted using a linear model, colours indicate whether a logFC change was found significant in either or both patients. (C) GOBP pathway analysis of genes preferentially upregulated in nivolumab bound CD8 cells in ADR001 and ADR013 is shown. Circle size indicates the number of genes overlapping with GOBP term.
Figure 6. 13 Expanded TCR clones in ADR001 and AD013

(A) UMAP of scRNA Seq data from non-responder and responder coloured by frequency of TCR clones. (B) Clonal proportion plot of CD8, CD4 effector and Treg compartments in non-responder and responder. (C) Heatmaps showing top genes which positively correlated (Pearson’s correlation, IgG4+CD8+ cells only) with TCR expansion in the responder. (D) Heatmaps showing top genes which positively correlated (Pearson’s correlation, IgG4+CD8+ cells only) with TCR expansion in the non-responder patient.
Figure 6. 14 TCR clonal expansion and clustering in nivolumab bound CD8 cells

(A) Degree of TCR clonal expansion in nivolumab bound or unbound cells is shown. (B) The clustering network of post-treatment intratumoural CDR3 β-chain sequences for ADR001 and ADR013 is shown. Expanded intratumoural TCRs found in the bulk TCR-seq data were used as a basis for the analysis. The expanded clones only found in the post-treatment repertoire (blue circles) and clones that were found both pre-and post-treatment (orange circles) are shown. TCR clones with an IgG4+ phenotype that were detected in the scTCR repertoire but not found expanded in the bulk TCR repertoire are also shown (yellow circle). The clustering for clones seen in IgG4- cells (top panel) or in IgG4+ cells (bottom panel) in the single-cell data. Clustering network derived from bulk post-treatment tissue (grey circles).
Figure 6. 15 Single-cell gene expression analysis of CD8+ and IgG4+CD8+ T-cells.

Single-cell RNAseq expression of Granzyme B, TCF7, TOX, HAVCR2 (TIM-3), CD38, ENTPD1(CD39) and PDCD1(PD-1) on (A) CD8+ and (B) IgG4+CD8+ T-cells in ADR013 (responder) and ADR001 (non-responder) are shown.
6.3 CHAPTER DISCUSSION

This chapter focuses on the two patients from the ADAPTeR trial: ADR001 (non-responder) and ADR013 (responder). T cells subsets were analysed in depth by using high dimensional flow cytometry and SC RNA Seq/TCR Seq. Although the data in this chapter is descriptive due to the small sample size, we provide novel and in depth findings about the phenotype of T cells in the responder and non-responder patient as well as the characteristics of the T cells that find Nivolumab. The data is mainly used for hypothesis generating and should be validated in a bigger cohort of patients.

Phenotypic analysis of the CD8 cells showed that the responder patient has higher expression of PD-1, Eomes, TIM-3, CD38, CD39 and TOX compared to the non-responder patient. This indicates the a more exhausted phenotype in the responder patient compared to the non-responder. In addition, the responder patient was also found to have higher expression of TCF7 which suggests a maintained progenitor-like phenotype. In addition, similar to the findings in the mIF data at a cohort level, the responder patient had higher Granzyme B expression compared to the non-responder suggesting a more cytotoxic phenotype of the CD8 T cells. TRM CD8 cells showed a similar phenotype to the phenotype described above in the responder and non-responder patients. One the novel aspects of our data was that we also analysed the nivolumab bound (IgG4+) TILs which has not been done before apart from peripheral blood in a melanoma cohort. Characterisation of the IgG4+ CD8 cells showed that the nivolumab bound CD8 cells had a more dysfunctional phenotype in the responder patient marked by higher expression of PD-1, Eomes, TIM-3, CD38, CD39 and TOX. They also had higher TCF7 and Granzyme B expression in the responder suggesting that these cells have better ability to get activated and gain a cytotoxic phenotype following anti-PD1 therapy and they also retain their progenitor function. In alignment with our findings, it has previously been shown that the dysfunctional CD8 T cells and ones with TCF7 expression expand more with anti-PD1 treatment and this is associated with response to anti-PD-1 therapy. In the responder patient, CD8 cells had a similar phenotype to TILs except for higher CD27 and KLRG-1 expression which suggests a highly proliferative effector cells with cytotoxic ability in the normal tissue. These cells are likely to be in a transitional state to becoming more dysfunctional as they migrate into the tumour area and get more antigen exposure.

Analysis of the CD4 cells showed that the CD4 effectors had a different phenotype compared to the CD8 cells. A more naïve phenotype and lower expression of markers
associated with a dysfunctional phenotype compared to CD8 cells. CD4 effector cells had high levels of Fas expression in both patients. However, Fas+ CD4 effector cells in the non-responder co-expressed CTLA-4, ICOS and CD25 which was not observed in the responder patient. Analysis of the IgG4+ CD4 effector cells showed higher expression of TCF7 in the responder suggesting a progenitor-like phenotype in the nivolumab CD4 effectors too. In addition, Fas expression was mainly on the IgG4+ CD4 cells. Tregs were found to expressed high levels of CTLA-4 which co-expressed high levels of CD25 in the non-responder patient. If this co-expression is validated in non-responders in a bigger cohort, combination therapy with anti-CTLA-4 antibody with anti-CD25 antibody which is currently in clinical trial can have potential therapeutic effects by depleting Tregs.354

T cells including the nivolumab bound IgG4+ T cells were analysed in more detail via SC RNA Seq and TCR Seq. One of the key findings was that nivolumab bound CD8, CD4 effector cells and Tregs had similar gene expression profile in the responder and non-responder patients. T cell activation, inflammatory response and cytotoxic activity pathways were upregulated in the nivolumab bound cells in both patients which indicate nivolumab causes similar effects on the cells it binds regardless of the intrinsic characteristics of the cells. Flow cytometry findings of higher TIM-3, CD38, CD39, TOX, TCF7 and Granzyme B expression on the CD8 cells in the responder patient was also confirmed in the SC RNA Seq analysis. Similar findings were also shown in a recent SC RNA Seq analysis in a cohort of RCC patients.126,355 However, we saw a discrepancy in the PD-1 expression pattern between the flow cytometry and SC RNA Seq data. This could be due to a lag in translation of the transcriptional profile to protein expression. TCR Seq analysis showed that the TCR expansion (especially hyper-expansion) mainly took place in the CD8 cells and it was more prominent in the responder patient. Comparison of the expanded CD8 cells between the responder and non-responder showed genes involved in cytotoxic function such as Granzyme K were upregulated in the responder whereas the non-responder had upregulation of integrins and chemokine receptors such CXCR6. In addition, nivolumab bound cells had more TCR expansion irrespective of clinical outcome. However, in the responder patient nivolumab bound cells had significantly more clustering. These finding suggest that CD8 cells are the main players in response to antigenic stimulus and CD8 cells that bind nivolumab that are able go through clonal expansion have a more cytotoxic phenotype and recognise a similar antigen. Our sample size (n=2) is a significant limitation in our data but our findings provide important hypothesis generating clues about mechanisms of actions of anti-PD-1 therapy and as well as response to anti-PD-1 treatment. Therefore, confirming these findings in validation cohort is critical.
7 CONCLUSION

In this project, the immune landscape in RCC was evaluated in treatment naïve and anti-PD1-treated samples. In addition, the phenotypes in tumour, tumour adjacent normal tissue and peripheral blood were compared to each other. Analysis of the TRACERx renal cohort showed that the highest Temra population is seen in the peripheral blood. CD8 Temra cells were positive for CD57 and Granzyme B. On the other hand, TRM population was highest in the TILs and seen in negligible amounts in peripheral blood. The phenotype of CD8 TRM cells were different compared to CD8 Temra cells and they had higher expression of co-inhibitory checkpoints including PD-1, TIM-3, CTLA-4, transcription factor Eomes as well CD38 and CD39 which suggest a dysfunctional phenotype in the tumour. In addition, PD-1\(^+\) and PD-1\(^-\) CD8 cell also had different phenotypes in the TILs with a more higher level of makers of T cell dysfunction in the PD-1\(^+\) CD8 cells. The CD4 effector cells had a more naïve phenotype in the PBMC. However, in the tumour CD4 effector cells had higher expression of PD-1, TIM-3, CD38 and CD39 compared to tumour and peripheral blood suggesting a dysfunctional phenotype in the CD4 effector cells too. Although this data provides useful information about the T cell phenotypes found the blood, normal tissue and tumour areas in ccRCC patients, lack of clinical data is a major limitation in this dataset.

Within the context of anti-PD-1 treatment, the results in the ADAPTeR cohort showed that T cell and myeloid cell infiltration did not predict response to nivolumab treatment. On the other hand, B cell infiltration at baseline was predictive of response to nivolumab treatment. Anti-PD-1 treatment led to an increase in Granzyme B production in the CD8 which was associated with response to nivolumab. In contrast to other studies, in ADAPTeR anti-PD-1 treatment did not cause expansion of new TCR clones with nivolumab but maintenance of TCR clones present at baseline through therapy was associated with response. In addition, maintained expanded clones showed more clustering in the responders which suggest they respond to the same maintained antigen. Comparison of TCR repertoire in the tumour and in peripheral blood showed that peripheral blood was not reflective of the tumour. Analysis of the different immune cells in the peripheral blood showed that there was no correlation with response to anti-PD-1 therapy and any of the phenotypes seen in the PBMC. However, the sample size in the peripheral blood cohort was very small which was a major limitation and therefore, a bigger sample size is needed to be able to draw more accurate conclusions.
Analysing the responder and non-responder patient showed that in the responder overall and nivolumab bound CD8 cells had a more dysfunctional phenotype with TCF7 and Granzyme B expression which indicates a progenitor-like exhausted T cell state with the ability to preserve cytotoxic function. SC RNA Seq and TCR Seq analysis of IgG4+ and IgG4− T cells showed that the nivolumab bound T cells upregulate pathways of T cell activation regardless of response to treatment. However, drug bound cells have more expanded CD8s and these expanded CD8 cells have more cytotoxic phenotype with more clustering in the responder compared to the non-responder patient. This suggests that in the responder, dysfunctional CD8 cells have retained their progenitor-like state and ability to get activated via antigenic stimulus and they maintain this ability through continuous antigen stimulation over time and through treatment which leads to a cytotoxic phenotype. Although the data is based on only two patients, it is based on in depth analysis and it generated important hypothesis which need to be validated in a bigger cohort.

8 FUTURE WORK

There were similar limitations in this study which warrant more work in the future. In order to understand the phenotype described in the TRACERx renal cohort and assess if they have any predictive value, the phenotype data needs to correlated with the clinical data. Given the important TCR Seq findings in the ADAPTeR cohort, doing TCR Seq for the TRACERx renal samples and correlating with the T cell phenotypes can provide useful information. Analysing the B cells, NK cells and myeloid cells in addition to the T cell phenotypes and correlating this data with clinical outcome should be carried out as this information will provide a better and more comprehensive understanding of the immune landscape in ccRCC.

For ADAPTeR cohort, expanding on the imaging with more advanced imaging techniques such as Slide-Seq will allow to stain for significantly more markers which will allow for a more in depth phenotypic and spatial understanding of the immune landscape. In addition, the flow cytometry and SC RNA Seq/TCR findings in the ADAPTeR cohort need to validated in a bigger cohort. In order to understand the functional characteristics of the T cells in the ADAPTeR and TRACERx cohorts, functional assays such MANAFEST assay can be performed.
9 APPENDIX

9.1 Papers during the PhD


9.2 Conference presentations during the PhD

ADAPTeR: A phase II study of anti-PD1 (nivolumab) therapy as pre- and post-operative therapy in metastatic renal cell carcinoma. Lewis Au, Kevin Litchfield, Emine Hatipoglu, Marc Robert de Massy, Andrew Rowan, Stuart Horswell, Fiona Byrne, David Nicol, Nicos Fotiadis, Roberto Salgado, Steve Hazell, Jose Ignacio Lopez, Lyra Del Rosario, Lisa Pickering, Martin Gore, Benny Chain, Sergio Quezada, James Larkin, Charles Swanton, Samra Turajlic. ESMO 2019 (Poster Presentation)
10 REFERENCES


15. Shankaran, V. *et al.* IFNγ and lymphocytes prevent primary tumour development and 


   (2011).

   (2002).


22. Ribas, A. & Wolchok, J. D. Cancer immunotherapy using checkpoint blockade. *Science* 
   (2018).


   prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review 

119. Nakano, O. et al. Proliferative Activity of Intratumoral CD8+ T-Lymphocytes As a 
   Prognostic Factor in Human Renal Cell Carcinoma: Clinicopathologic Demonstration of 


121. Granier, C. et al. Tim-3 Expression on Tumor-Infiltrating PD-1+CD8+ T Cells 

122. Şenbabaoğlu, Y. et al. Tumor immune microenvironment characterization in clear cell 
   renal cell carcinoma identifies prognostic and immunotherapeutically relevant 

123. Gerlinger, M. et al. Ultra-deep T cell receptor sequencing reveals the complexity and 

124. Braun, D. A. et al. Progressive immune dysfunction with advancing disease stage in 


261. Kondo, T. & Takiguchi, M. Human memory CCR4+CD8+ T cell subset has the ability to produce multiple cytokines. Int. Immunol. 21, 523–532 (2009).


345. Labiano, S. et al. CD69 is a direct HIF-1α target gene in hypoxia as a mechanism enhancing expression on tumor-infiltrating T lymphocytes. *OncoImmunology* **6**, e1283468 (2017).


