Identification of urine derived lymphocytes as a non-invasive measure of the tumour immune microenvironment in bladder cancer

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Thesis submitted for the degree of Doctor of Philosophy (Ph.D.)

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**Declaration**

‘I, Yien Ning Sophia Wong 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.’

10\(^{th}\) Aug 2020

..........................  .................................................................
Date  Signature

Yien Ning Sophia Wong
Abstract

Despite the advances in systemic immunotherapy with immune checkpoint blockade, only a small fraction of patients with bladder cancer respond to this treatment. This highlights a need to better understand drug resistance and identify rational immunotherapeutic combinations. This thesis explores the immune checkpoint landscape of the bladder tumour microenvironment (TME) in three main groups of patients with bladder cancer: non-muscle invasive bladder cancer (NMIBC), muscle invasive invasive bladder cancer (MIBC), and metastatic urothelial bladder cancer.

The immune checkpoint landscape between NMIBC and MIBC in the bladder TME was very similar, but distinct to peripheral blood. Interestingly, I observed the presence of urine-derived lymphocytes (UDLs) in NMIBC as previously described, but also in MIBC. We identified UDLs as a readily accessible source of T cells in 32 patients with MIBC, and demonstrated that UDLs have a remarkably similar immunological T cell phenotype and TCR repertoire to tumour infiltrating lymphocytes (TILs) in patients with MIBC. Interestingly, patients with MIBC who were found to have high urinary lymphocytes, at the time of cystectomy, in particularly high expression of PD-1 (PD-1hi) on CD8+, had a reduced recurrence free survival.

Furthermore, I developed a lab protocol for the in vitro expansion of cryopreserved UDLs. Expanded UDLs produced IFNg and TNFa when restimulated with autologous tumour samples. In addition, with collaborative efforts, we explored the antigenic specificity of T cells and observed the presence of neoantigen reactive T cells (NARTs) in the peripheral blood of a single patient.

Finally, in nine patients with metastatic bladder cancer, I showed a poor prognostic association in patients with high UDL CD3+ count and an exhaustive phenotype of CD8+ PD-1hi UDLs as detected at the end of treatment, which could also be tracked longitudinally in metastatic patients undergoing systemic therapy. Taken together, the data suggest a role for UDLs as an immune biomarker to track response to systemic therapy. However, this requires a large prospective study to validate these findings. Crucially, the data above highlight the importance of incorporating UDL analysis in all future studies with a view to integrating into routine clinical practice in patients with bladder cancer.
Impact Statement

Checkpoint inhibitors (CPI) targeting the PD-1 and CTLA-4 pathway have revolutionised and led the way in the systemic treatment of several solid cancers, including bladder cancer. There are a number of other checkpoint receptors and ligands that are currently being investigated as immunotherapy targets in early phase trials. However, to understand which checkpoint targets are the most relevant in bladder cancer, longitudinal mapping of the immune checkpoint landscape in non-muscle invasive bladder cancer and muscle invasive bladder cancer (MIBC) is vital to help identify novel immune-oncology (IO) targets and uncover the optimal combinatorial immunotherapies for bladder cancer.

Currently, several non-validated predictive biomarkers of response to systemic immunotherapy rely upon tumour biopsies. However, accessibility to longitudinal tumour sampling prior and during therapy is a major limitation in understanding the immune tumour microenvironment (TME). Accordingly, there is an unmet need to develop non-invasive methods to assess the immune TME. It is essential to use sensitive tools such as multi-parametric flow cytometry and T cell receptor (TCR) repertoire analysis, to study the immune TME, as these tools allows for in-depth characterisation of individual T cells. Furthermore, it is critical to study the functional status of the immune cells found in a dynamic TME using genomics and immunological pipelines to understand the interaction between tumour antigens and T cell activation.

The discovery that urine derived lymphocytes (UDLs) have a remarkably similar immunological T cell phenotype and TCR repertoire to tumour infiltrating lymphocytes (TILs) in bladder cancer highlights the potential and unique opportunity to use UDLs as a window to the bladder immune TME. Interestingly, the worse outcome is in patients with high urinary lymphocytes and CD8⁺ PD-1⁺ indicates that UDLs could also be used as a prognostic marker and perhaps this group of patients may benefit from adjuvant therapy, with close monitoring for recurrence. Taken together, the data support the use of UDLs as a dynamic liquid biopsy that provides an insight into the bladder immune TME. Importantly, UDLs may be used to identify actionable IO targets with potential prognostic value in patients with MIBC.

Crucially, UDLs may be used to track the evolving and dynamic immune landscape of patients with metastatic bladder cancer throughout the course of their disease and treatment. The findings in the metastatic cohort of patients reaffirms the hypothesis that the presence of a high UDL count and effector PD-1⁺ T cells at the end of their treatment
is associated with worse outcome. I also found that the UDL count is dynamic during systemic treatment, highlighting its use as a predictive marker to treatment response. Furthermore, the expansion of UDLs from cryopreservation makes it an extremely attractive non-invasive source of cells that could subsequently be used for adoptive cellular immunotherapy for patients with bladder cancer. However, a large prospective study is needed to validate this. In summary, urine-derived lymphocytes may provide a window to the bladder TME and track the dynamic and evolution of the immune landscape longitudinally in patients with bladder cancer, with a potential to serve as a prognostic and predictive biomarker of systemic therapy.
Acknowledgement

I would like to express my utmost gratitude to my scientific supervisor, Professor Sergio Quezada, for his invaluable advice and infectious passion for science – always encouraging us to look beyond our data. You have also made me appreciate the importance of working hard, but also playing hard at the same time. To my clinical supervisor, Dr Mark Linch, thank you for clinical and translational oversight throughout my PhD. Thank you for the opportunities you have given me from writing papers to setting up clinical trials – I am forever grateful. Thank you both for your support professionally and personally.

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To my husband, Wesley and son, Zac – thank you for your unwavering support, love and understanding throughout this process, and for sacrifices you have made. I am forever grateful. Last but not least, to my late father, Dr Su Yong Wong, who has always encouraged me to pursue a scientific route – this PhD is for you. I hope I have made you very proud.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>4-1BB</td>
<td>CD137</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD4eff</td>
<td>CD4 effector T cells</td>
</tr>
<tr>
<td>cfDNA</td>
<td>Circulating free deoxyribonucleic acid</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>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinases</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>FDA</td>
<td>Food and drug administration</td>
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<tr>
<td>FoxP3</td>
<td>Forkhead box P3</td>
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<tr>
<td>GzmB</td>
<td>Granzyme B</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible T-cell co-stimulator</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IO</td>
<td>Immuno-oncology</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIBC</td>
<td>Muscle invasive bladder cancer</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NART</td>
<td>Neoantigen reactive T cell</td>
</tr>
<tr>
<td>NCT</td>
<td>Clinicaltrials.gov National Clinical Trial number</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NSM</td>
<td>Nonsynonymous mutation</td>
</tr>
<tr>
<td>NT</td>
<td>Non-tumour tissue</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>New York esophageal squamous cell carcinoma 1</td>
</tr>
<tr>
<td>ORR</td>
<td>Objective response rate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
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<tr>
<td>OS</td>
<td>Overall survival</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>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>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>pREP</td>
<td>Pre-Rapid Expansion Protocol</td>
</tr>
<tr>
<td>REP</td>
<td>Repaid Expansion Protocol</td>
</tr>
<tr>
<td>RFS</td>
<td>Recurrence free survival</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour associated antigen</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</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>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>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TSA</td>
<td>Tumour specific antigen</td>
</tr>
<tr>
<td>TURBT</td>
<td>Transurethral resection of the bladder tumour</td>
</tr>
<tr>
<td>UDL</td>
<td>Urine-derived lymphocyte</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Cancer and the Immune System

The immune system defends our bodies against pathogens including infectious agents and cancer cells. It can be categorised into two groups: innate immunity and adaptive immunity, consisting of a variety of immune cells. These distinct groups of immune responses usually exploit a unique repertoire of haematopoietic effector cells, however there are unconventional T cells that bridge the two groups (Figure 1.1) (1). The innate immune system is the first line of defence that responds rapidly after the appearance of an antigen, without needing prior exposure. Whilst the innate immune system is readily available to fight a whole host of pathogens, its limitation is its inability to recall prior exposure to pathogens. This is in contrast to the adaptive immune system which provides an antigen specific response that also produces an immunological memory by increasing protection against reinfection with the same pathogen. Innate immunity is crucial to the initiation and maintenance of adaptive immunity, while mounting their own effector responses against pathogens, for instance natural cytotoxicity for natural killer (NK) cells, and phagocytosis for macrophages and polymorphonuclear cells. The innate immune cells also contribute to effector responses after the induction of antibodies, through the expression of Fc receptors for antibodies, via antibody-dependent cellular phagocytosis or antibody-dependent cell cytotoxicity (2).

Figure 1.1: Adaptive and innate immune cellular responses involved in the anti-tumour immune response.
Adapted from Wong et al. 2017 (1). Figure depicts key cellular components that are involved in the adaptive and innate immune responses.
The adaptive immune response is categorised into two groups: humoral immune response and cell-mediated immune response. In humoral immune response, B cells produce proteins called antibodies to fight pathogens; while in cell-mediated immune responses, the cells themselves fight against the pathogens or cancer cells. These B and T lymphocytes recognise a broad range of antigens in a specific way via the T and B cell receptors (3).

Immune cells have the ability to recognise and eliminate pre-cancerous and/or cancerous cells based upon the expression of tumour specific antigens or molecules induced by cellular stress. This process is known as immune surveillance. However, in spite of immune surveillance, some tumour cells escape the functioning immune system and progress in cancer growth. This observation led to the generation of an updated concept, known as “cancer immunoediting”.

1.1.1 Cancer Immunoediting
In the early 20th century, Paul Ehrlich, a Nobel Prize winner for his work in immunology, first predicted that the immune system could halt tumour growth by proposing that molecules such as antibodies could be used to deliver toxins to cancer cells. But it wasn’t until the mid 20th century that Frank MacFarlane Burnet, recipient of the 1960 Nobel Prize, and Lewis Thomas proposed the concept of “immune surveillance”, where the immune system would recognise and eradicate the cancer cells based upon tumour-specific antigens. The hypothesis of “immune surveillance” remained contentious over the next 50 years as experiments using mice with spontaneous mutations generated immunocompromised but not entirely immunodeficient mice (4). It wasn’t until the late 1990s, that Robert Schreiber’s group found that the adaptive immunity helps control tumour formation by collaborating with interferon gamma (IFNg) pathways to protect against 3-MCA-induced tumour formation and growth (5, 6). As scientists began to understand that the relationship between the immune system and cancer is substantially more complex, a modified hypothesis of “cancer immunoediting” was more widely accepted, encompassing both “immune surveillance” and tumour progression (7). “Cancer immunoediting” is composed of three phases: elimination, equilibrium and escape (Figure 1.2)

The first phase is “elimination”, which encompasses the original theory of “immune surveillance”. This process involves both the innate and adaptive immunity to destroy developing tumour. If this process of elimination is unsuccessful, the tumour microenvironment enters a state of dormancy into the second phase of “equilibrium”. After chronic antigenic stimulation, the tumour cells undergo changes that help their
survival through selection pressure, a process also known as cancer immunoediting. Finally, the tumour cells may escape and evade the immune system through various mechanisms and become clinically detectable, in the last phase of “escape” (7, 8).

![Diagram](https://example.com/diagram.png)

**Figure 1.2:** The three “E”s of Cancer Immunoediting. Permission to reuse figure from Elsevier (8). Normal cells (grey) can transform into tumour cells (red) (top). The process of cancer immunoediting may happen even at early stages of cancerogenesis (bottom).

### 1.1.2 T cell mediated immunity in cancer

The principle of the immune system is to reach an equilibrium of attacking immune cells to defend us against pathogens such as viruses, bacteria and malignant cells, whilst having suppressive immune cells for adequate tolerance towards self-antigens and thereby avoiding autoimmunity or allergy.

Tumours are primarily distinct from their normal tissue counterparts in both the biological behaviour and antigen composition (9). Tumour associated antigens (TAAs) are tumour antigens that are shared between individuals, and have differential expression in tumour compared to normal tissues. For example, melanoma-associated antigen (MAGE) is highly expressed in malignant melanoma but is also expressed in normal testicular cells. This shared expression pattern, gives cancer cells an opportunity to escape from the immune system due to self-tolerance. Tumour-specific antigens (TSAs), for example neoantigens, are exclusively expressed in tumour cells, making them extremely appealing targets for immunotherapy as theoretically they should not lead to autoimmunity compared to TAAs. Hence, there has been an intensification of effort to
test strategies in targeting neoantigens over the last decade. There are now a number of examples where targeting neoantigens have successfully led to anti-tumoural immune responses and improved survival (10). Moreover, there have been several studies demonstrating that these neoantigens are critical for an effective anti-tumour T cell response to checkpoint inhibitors (CPI) (11-14).

The generation of cancer immunity can be described in a cyclic way known as the “Cancer-Immunity Cycle” (15). Tumour elimination by the immune system is principally via cell-mediated immune responses. I will focus on the T-cell mediated immune response in killing of cancer cells. Cancer antigens (TAA and TSA) like neoantigens are released and captured by antigen presenting cells (APCs) such as dendritic cells for processing. However, to mount an anti-cancer T cell response, it must have signals to prevent peripheral tolerance to the tumour antigens induced. The APCs present the captured antigens via the major histocompatibility complex (MHC) molecules – MHC class I and MHC class II. These MHC molecules are membrane proteins on APCs that are responsible for the binding and displaying of exogenous and endogenous peptides from antigens at different intracellular sites. Typically, MHC class I molecules bind and present intracellular peptides derived from housekeeping proteins or viral proteins from a virally infected cell. MHC class II molecules bind exogenous peptides which have been phagocytosed and processed by APCs. However, MHC class I molecules can also access exogenous peptides that have been internalised by endocytosis or phagocytosis, known as cross presentation. This is an important phenomenon for generation of an effective immune response, utilising cytotoxic CD8+ T cells (3).

The antigen presentation on MHC class I and MHC class II molecules prime and activate CD8+ and CD4+ T cells respectively, causing an effector T cell response against cancer antigens that has been viewed as non-self or where central tolerance was not complete. For this process to happen, a two-signal model must happen. Signal 1 is antigen specific and a stimulation signal occurs when the antigen/MHC complex is recognised by the T cell receptor (TCR). A second signal then occurs when co-stimulation of molecules from a B7 superfamily ligand (e.g. CD80/86), which are normally expressed on APCs, engages with its corresponding receptor, CD28 and deliver a positive signal to T cells when bound to its co-stimulatory receptor. If signal 2 does not occur, the T cell will become anergic (3). This two-signal model is required for T cell proliferation, differentiation and survival.

A crucial balance of effector T (Teff) cells and regulatory T (Treg) cells, that develop upon recognition of self-antigens and dampens down immune responses, is vital at this
juncture in determining the nature of the immune response. If there is an activated T cell response, the activated Teff cells will traffic to and infiltrate the tumour. These Teff cells will recognise and bind to the cancer cells through the interaction between its TCR and its cognate antigen bound to MHC class I, and thereby killing the target cancer cell. This effective killing of the cancer cell will release more cancer-specific antigens and the “Cancer-Immunity Cycle” will repeat itself again, allowing for more breadth and depth of response with each subsequent cycle (15). In theory, this should be self-propagating leading to an accumulation of immune-stimulatory factors that should amplify and broaden T cell responses. However, there may be inhibitory factors that lead to an immune regulatory feedback mechanism, which can stop the development or restrict the immunity, such an overbalance of Treg cells. This “Cancer-Immunity Cycle” is depicted in Figure 1.3 (15).

Figure 1.3: The Cancer-Immunity Cycle.
Permission to reuse figure from Elsevier (15). The interaction between the immune system and tumour antigens is a cyclical process involving tumour antigen recognition by the immune system with antigen presentation, priming and activation of the immune system. If no inhibitory factors are activated by the cancer cell and/or immune system in this process, killing of cancer cells will occur. Legend: APCs, antigen presenting cells; CTLs, cytotoxic T lymphocytes.
1.1.3 Immune checkpoints

The birth of cancer immunotherapy began in the late 19th century when William Coley observed a patient with sarcoma achieved a complete remission of his tumour following acute infection with the bacteria *Streptococcus pyogenes*, known as “Coley’s toxins”. Coley went on to inject these toxins into patients with bone and soft tissue sarcoma, and he witnessed some remissions (16). Whilst this practice was not widely accepted at the time, it led to the use of *Bacillus Calmette-Guerin* (BCG) as the first cancer immunotherapy, which remains a standard of care for superficial bladder cancer today (17).

Since then, there has been a paradigm shift in the world of cancer immunotherapy, particularly utilising the adaptive immune system via checkpoint inhibition. The better understanding through basic and translational research of how the immune response to cancer is regulated has drastically changed the landscape of cancer immunotherapy, particularly in solid cancers. A key turning point in the history of cancer immunotherapy was the discovery of an immune checkpoint molecule that was expressed at high levels on activated T cells *in vitro*, known as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (18). It was believed that CTLA-4 acted as a co-inhibitory receptor and was responsible for the down-regulation of T cell activity. Several groups in the mid 1990s, suggested that CTLA-4 would restrict the activity of tumour reactive T cells by acting as an immune checkpoint, with successful tumour rejection in cancer mice models using CTLA-4 blockade (19-21).

Immune self-tolerance is broadly classified into two forms: central tolerance and peripheral tolerance. Central tolerance is the main mechanism of self-tolerance, where immune editing occurs in the thymus and self-reactive T cells are eliminated by negative selection (22). However, not all self-reactive T cells are eliminated by this method and additional tolerance mechanisms are needed. Peripheral tolerance deals with this by suppression of these self-reactive T cells that enter the periphery. This is regulated through the T cell responses intrinsically, and via extrinsic control such as Tregs by suppressing T cell responses (22). T cell response is also regulated by a balance between co-inhibitory and stimulatory signals through immune checkpoints. These immune checkpoints are vital under normal physiological conditions in preventing autoimmunity in the form of “self-tolerance”. Thereby, one of the tumour escape and resistance mechanisms is dysregulation of these immune checkpoint proteins.

Antagonistic antibodies of these co-inhibitory receptors or agonistic antibodies of co-stimulatory signals, result in a magnitude of T cell responses, unleashing an anti-tumour
immune response that has revolutionized the therapeutic landscape of solid cancers, including bladder cancer. The two most studied immune checkpoint molecules are cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4; also known as CD152) and programmed cell death protein 1 (PD-1; also known as CD279), which are both co-inhibitory receptors. Numerous co-inhibitory and stimulatory checkpoint targets have now been identified that have promising potential, based upon pre-clinical data and/or are under development (Figure 1.4) (23, 24).

Figure 1.4: T cell responses are regulated by multiple co-inhibitory and stimulatory checkpoint molecules.
Permission to reuse figure from Springer Nature and adapted from Pardoll (23). The key ligand-receptor interactions between T cells and antigen-presenting cells (APCs) discussed in this thesis are shown. Legends: B7RP1, B7-related protein 1; CTLA4, Cytotoxic T-lymphocyte-associated antigen 4; GAL9, galectin 9; ICOS, inducible T cell co-stimulator; PD-1, programmed cell death protein 1; PDL, PD-1 ligand; TIM3, T cell membrane protein 3.

1.1.3.1 Immunoglobulin superfamily (IgSF)

T lymphocytes do not normally respond to the ligand-receptor interactions, unless the T cells recognise their cognate antigen via the TCR first (Figure 1.4). These responses can happen at the initiation of T cell responses in lymph nodes via dendritic cells as APCs or in the peripheral tissues or tumours, where there is regulation of effector responses. Several of these ligands bind to many receptors, and are capable of delivering both co-inhibitory and stimulatory signals. Generally, pairs of co-inhibitory and stimulatory receptors that bind the same ligand(s), like CD28 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), show unique expression with the co-inhibitory receptor being frequently upregulated after T cell activation, and the stimulatory receptor expressed on resting and naive T cells. The B7 family is a key family of membrane-bound ligands which
binds both co-inhibitory and stimulatory receptors. All of the B7 family members and their known ligands belong to the immunoglobulin superfamily (IgSF) (23).

### 1.1.3.1.1 B7-CD28 Family

#### 1.1.3.1.1.1 Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)

The first immune checkpoint molecule to be discovered was CTLA-4, which is expressed at increased levels on T cells when activated (18). It is an inhibitory receptor member of the IgSF, specifically the B7-CD28 family. CTLA-4 is comprised of an extracellular IgV-like domain made up of the B7 binding motif (MYPPPY), a stalk consisting a cysteine that mediates homodimerization, a transmembrane domain, and a cytoplasmic tail (18).

CTLA-4 is not expressed on the T cell surface, but rather, it is primarily expressed inside T cells within intracellular vesicles. After signal 1 and activation of T cells, CTLA-4 is translocated to the cell surface, where it competes with CD28 for the binding of their ligands expressed on APCs. The greater the stimulation through the TCR (and CD28), the larger the amount of CTLA-4 is deposited on the T cell surface. Hence, CTLA-4 operates as a signal dampener to provide a consistent level of T cell activation despite a hugely varying ligand affinity for the TCR (23). CTLA-4 competes with CD28 for the binding of its ligands, CD80 and CD86, at an affinity that is 10 times higher compared to CD28, delivering an inhibitory signal to T cells (25). “Signalling-independent” T cell inhibition via CTLA-4 also occurs through sequestration of its ligands, CD80/CD86 from CD28 engagement, in addition to the removal of these co-stimulatory ligands from APC which degrade inside CTLA-4 expressing cells (26).

CTLA-4 is primarily expressed by T cells and is constitutively expressed by Tregs in high levels. However, for maximal anti-tumour effect, targeting CTLA-4 on both Teff and Treg cells in pre-clinical mouse models of cancer was needed (27). The role of CTLA-4 for controlling T cell hyperactivation is shown in Ctl4−/− mouse models, which resulted in rapid progression of lymphoproliferative disease with multiorgan lymphocytic infiltration, leading to tissue destruction and death in weeks (28, 29). There were initial doubts in inhibiting CTLA-4 as there was no tumour specificity to the CTLA-4 ligands and due to the rapid progression and lethal hyper- and autoimmunity seen in the Ctl4−/− mouse models. In the mid 1990s, Allison et al. were the first to demonstrate that administration of CTLA-4 antibodies resulted in tumour regression in mouse models (19). This key finding and his subsequent work led to his Nobel Prize win in 2018, as well as the production and testing of a fully humanised monoclonal CTLA-4 antibody, ipilimumab in humans. This led to the seminal randomized Phase III trial which demonstrated durable
responses in patients with metastatic melanoma, resulting in the US Food and Drug Administration (FDA) approval in 2010 and subsequent approval in the UK (30).

1.1.3.1.1.2 Programmed cell death-1 (PD-1)

Programmed cell death-1 (PD-1) is a co-inhibitory cell surface molecule and IgSF member. The PD-1 receptor comprises of an N-terminal IgV-like domain, a transmembrane domain and a cytoplasmic tail domain that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (31). Phosphorylation of ITIM and ITSM motifs occurs when PD-1 engages with one of its ligands programmed death-ligand 1 (PD-L1; also known as B7-H1 and CD274) or programmed death-ligand 2 (PD-L2; also known as B7-DC and CD273) (Figure 1.4). This phosphorylation leads to the recruitment of SH2-domain containing protein tyrosine phosphatases (SHP-1 and/or SHP-2) to the ITSM cytoplasmic region of PD-1, which inhibits downstream signals of TCR, via PI3K/AKT and mitogen-activated protein kinases/extracellular signal-related kinases (MAPK/ERK) pathway, thereby inhibiting cell cycle progression, cytokine expressions and transcription factors related to effector T cell function, like Tbet and EOMES (32, 33). Therefore, signalling of PD-1 can transform T cells jointly in various ways to inhibit immune responses.

In contrast to CTLA-4 where it plays a major role during the initial T cell activation stage, the key function of PD-1 is to dampen T cell activity in peripheral tissues when there is an inflammatory response to an antigen and to reduce autoimmunity. This is an important adaptive resistance mechanism within the tumour microenvironment (TME) (34). PD-1 is expressed more broadly compared to CTLA-4. PD-1 is absent on naïve cells but upregulated following T cell activation (31), and is found on B cells, myeloid-derived cells and natural killer (NK) cells (35). Hence, PD-1 inhibition not only increases the effector T cells activity in the TME, it is likely to increase the activity of NK cells, and enhance B cell antibody production (23). Moreover, chronic antigenic stimulation and exposure due to chronic viral infection and cancer, can lead to persistent high levels of PD-1 expression (36-41). This leads an exhausted and anergic state in the antigen-specific cognate T cells, which is partially reversible by PD-1 inhibition (36). PD-1 is also vital in the maintenance of peripheral self-tolerance. In vivo mouse models using C57BL/6 PD1−/− and BALB/c PD-1−/− transgenic mice have been shown to develop lupus-like autoimmune disease (such as arthritis and glomerulonephritis) and autoimmune dilated cardiomyopathy respectively (42, 43). Moreover, in a non-obese diabetic (NOD) PD-1−/− mouse model, the onset and frequency of type I diabetes is accelerated (44).
Taken together, these findings show that PD-1 has a major role in inhibiting immune responses and maintaining peripheral T cell tolerance. There have since been several monoclonal antibodies targeting the PD-1 (pembrolizumab and nivolumab) and PD-L1 axis (atezolizumab, durvalumab and avelumab) (45-50) that have been approved by the European Medical Agency (EMA) and the US FDA for patients with metastatic bladder cancer in the second-line setting or as first-line therapy in patients who are ineligible for platinum based chemotherapy.

1.1.3.1.1.3 Inducible T-cell co-stimulator (ICOS)

Contrary to CTLA-4 and PD-1, inducible T-cell co-stimulator (ICOS; also known as CD278) is a co-stimulatory receptor expressed on T cells. ICOS also belongs to the IgSF B7/CD28 family and interacts with ICOS ligand (ICOS-L; also known as B7RP1, CD275) (Figure 1.4). The ICOS receptor consists of a YMFM SH2 binding domain within its cytoplasmic tail. Following engagement with ICOS ligand, ICOS activates the downstream signalling pathways of PI3K/AKT, leading to the activation of transcription factors involved in cell growth, effector function and survival. In addition, ICOS induce secretion of IL-4 and IL-21 through engaging the C-MAF pathway (51).

ICOS plays a vital role in T cell activation and proliferation (52), with upregulation of ICOS expression in naïve and resting T cells following T cell activation. Additionally, ICOS is constitutively expressed by Treg cells at high levels compared to naïve subsets. ICOS-mediated co-stimulation is less powerful than CD28-mediated co-stimulation, largely due to CD28 co-inducing IL-2 secretion (53).

Pre-clinical models of melanoma have demonstrated that ICOS or ICOS-L deficient mice had impaired responses to CTLA-4 inhibition compared to wild-type mice, suggesting that the ICOS pathway is critical for optimal therapeutic effect with anti-CTLA-4 inhibition (54). Moreover, combination therapy with anti-CTLA-4 and ICOS engagement via a tumour cell vaccine expressing ICOS-L enhanced better outcome in melanoma and prostate cancer mouse models, reiterating the key role of ICOS in mediating maximal therapeutic responses to anti-CTLA-4 therapy (55).

In a Phase I trial, ICONIC, using an anti-ICOS agonistic monoclonal antibody (mAb), JTX-2011 in combination with or without nivolumab, has shown that it is well tolerated with some antitumour responses in heavily pre-treated gastric cancer and triple negative breast cancer (56). Further trials using JTX-2011 are ongoing, in particular NCT03989362, which is looking at PD-1/PD-L1 pre-treated patients with non-small cell
lung cancer and urothelial cancer, in combination with ipilimumab. There is also another anti-ICOS agonistic mAb, GSK3359609 in early phase clinical trials, looking at combination with or without anti-PD-1/PD-L1 in advanced solid cancers including urothelial cancers (NCT02723955, NCT03693612). There is an antagonistic anti-ICOS mAb, MEDI-570 that is currently being tested in patients with peripheral T-cell lymphoma (NCT02520791), but not yet in solid cancers. Interestingly, there is a new fully human anti-ICOS IgG1 kappa mAb, KY1044 that selectively binds to ICOS with high affinity and has a dual mechanism of action: it preferentially depletes of intratumoural ICOSHigh Tregs as well as stimulates ICOSlow Teff cells. It is currently being tested in combination with or without atezolizumab in advanced solid cancers (NCT03829501).

1.1.3.1.2 TIM subfamily members

1.1.3.1.2.1 T-cell immunoglobulin and mucin-domain containing-3 (TIM-3)

T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) is also a member of the IgSF, but specifically a member of the TIM subfamily. TIM-3 is a type 1 transmembrane protein, comprising of a single IgV domain, a mucin domain, a stalk, and a cytoplasmic tail consisting a tyrosine-based signalling motif (35, 57). There are several candidate ligands for TIM-3 described, some of which have a primary role in the myeloid compartments, like high mobility group box 1 protein (HMGB1) and phosphatidylserine (PtdSer). There are two T cell related ligands that have been described, galectin-9 (Gal-9) (Figure 1.4) and carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1) (58). Gal-9 suppresses T helper (Th1) cell response (59) and anti-TIM-3 antibody enhances anti-tumour immunity and suppresses established tumour (60).

TIM-3 expression has been shown on activated T cells and on myeloid cells including DCs, NK cells, and monocytes. Interestingly, TIM-3 expression can be upregulated by an inflammatory environment rich in cytokines (IL-2, IL-7, IL-15, IL-21) independent of TCR or antigenic stimulation (61). A major role of TIM-3 is its ability to inhibit cytotoxic T cell responses. TIM-3 was upregulated on antigen specific CD8+ T cells in patients with progressive human immunodeficiency virus (HIV) infection. Additionally, the TIM-3 expressing cells did not proliferate with re-antigenic stimulation nor produce cytokines like interferon gamma (IFNg) (62).

TIM-3 has been shown to be co-expressed with PD-1, a phenotype associated with impaired T cell function and chronic antigenic stimulation, particularly on tumour-specific CD8+ T cells (63-66). Pre-clinical in vivo mouse model work demonstrated that PD-1/TIM-3 co-expression on CD8+ T cells had the most exhausted phenotype, and failed
to proliferate and produce IFNg, IL-2 and tumour necrosis factor (TNF) (64). Moreover, blocking both PD-1 and TIM-3 resulted in enhanced anti-tumour immune responses and reversal of T cell exhaustion and dysfunction in both in vivo and in vitro work (64, 66). Moreover, the use of TIM-3 blockade following adaptive resistance to PD-1 blockade showed a survival advantage in pre-clinical models (67). There are several early phase clinical trials with anti-TIM-3 mAb, mostly in combination with or without anti-PD-1/PD-L1 antibodies as backbone that are currently recruiting in solid cancers (NCT02817633, NCT03099109, NCT02608268, NCT03744468) with two bispecific anti-PD-1/TIM-3 antibodies also being investigated in early phase studies (NCT03752177, NCT03708328).

1.1.3.2 Tumour necrosis factor receptor superfamily (TNFRSF) members

A second family of the regulatory ligand-receptor pairs is the tumour necrosis factor receptor superfamily (TNFRSF) members, which engages with cognate TNF receptor family molecules. These receptors primarily deliver co-stimulatory signals when bound to their cognate ligands. TNFRSF members activate intracellular signalling pathways that may promote cell survival or trigger apoptosis either through their death domains or TNF receptor associated factor (TRAF) mediators (68). TRAFs are trimeric proteins that interact with the cytoplasmic tails of trimeric TNFRSF receptors upon engagement of these receptors to their respective ligands. I will focus on 4-1BB (also known as CD137; TNFRS9) for my thesis.

1.1.3.2.1 4-1BB

4-1BB is a co-stimulatory molecule that is expressed following acute T cell activation. 4-1BB is a glycosylated type I membrane protein comprised of four cysteine-rich pseudo repeats (CRDs) forming the extracellular domain, a short helical transmembrane domain, and a cytoplasmic signalling motif. There can be one to four CRDs that form the extracellular domains of TNFRs and usually form elongated structures (69). 4-1BB ligand (4-1BBL) is the main ligand of 4-1BB (Figure 1.4) and is primarily expressed on APCs, including B cells, dendritic cells (DC), and macrophages (70). Intracellular signalling pathway is induced via TRAF1 and TRAF2 following binding of 4-1BB to its ligand. This causes activation of the NF-κB, AKT, p38 MAPK and ERK pathways, resulting to increased expression of survival genes and decreased in the expression of pro-apoptotic Bim (68).

4-1BB is expressed on a subset of immune cells upon activation, including NK cells and T cells (71). Both CD4+ and CD8+ T cells express similar levels of 4-1BB. However, in
pre-clinical models, there seems to be more upregulation of 4-1BB in CD8⁺ T cells following T cell activation both in vivo (72, 73) and in vitro (74). Melero et al. were the first to demonstrate that 4-1BB agonist mAbs eradicated large, established tumours in mice. Moreover, the lasting anti-tumour activity shown in their model was predominantly mediated by CD8⁺ T cells and was associated with memory responses (75). In pre-clinical models, anti-4-1BB was shown to increase IFNg production, T-cell survival, and the cytolytic activity of antigen-specific T cells (76), as well as reduced tumour load (77).

Two mAbs targeting 4-1BB have been developed and tested clinically: Urelumab (BMS-663513, Bristol-Myers Squibb) is an fully humanised IgG4 agonistic, non-ligand–blocking mAb (78), and Utomilumab (PF-05082566; Pfizer) is a fully humanised IgG2 agonistic mAb (79). Despite promising data with anti-4-1BB mAbs, there has been limited clinical benefits in early clinical trials, mainly with severe transaminitis with urelumab doses of ≥1 mg/kg. Trials using lower doses of urelumab at 0.1 mg/kg every 3 weeks were safer (78). The lower dose of urelumab is currently under clinical evaluation as a monotherapy and in combination with other immune-oncology agents. Finally, utomilumab has shown a favourable safety profile with some anti-tumour activity (80) and trials looking at combination therapy with utomilumab are underway.
1.2 Bladder cancer

1.2.1 Epidemiology
Bladder cancer is the 8th most common cancer, and the most common malignancy involving the urinary system. Every year, there are over 400,000 new cases of bladder cancer diagnosed worldwide (81). In the UK, a total of 10,171 new bladder cancer cases were diagnosed in 2015, equating to 15.6 per 100,000 persons (82). Globally, Europe has the highest incidence of bladder cancer of 17.7 per 100,000 persons, particularly in Southern Europe (21.8 per 100,000 persons), followed by Western Europe (19.7 per 100,000 persons) (83). Other geographical areas with a high incidence of bladder cancer include North America (19.5 per 100,000 persons) and North Africa (15.1 per 100,000 persons) (83). The peak age incidence of bladder cancer is between 60 – 70 years old and is rare in patients under the age of 40 years old. The male incidence of bladder cancer is three-fold higher than in females, which is partially attributed to higher tobacco use in males (84). Environmental exposure has been shown to play a major role in the development of bladder cancer, with cigarette smoking, the leading cause of bladder cancer. In a large cohort study of nearly 500,000 participants, current smokers (HR 4.06; 95% CI 3.66-4.50) and former smokers (HR 2.22; 95% CI 2.03-2.44) have an increased risk of developing bladder cancer compared to non-smokers (85). Occupational risk factors such as workers exposed to polycyclic aromatic hydrocarbons (cooks, waiters, aluminium workers, petroleum workers, chimney sweeps, seamen) and aromatic amines (tobacco, dye, hair dressers, printers, rubber workers, leather workers) have a higher risk of developing bladder cancer (86). About 40% of bladder cancer cases can be prevented by minimising on these environmental exposure (82). Moreover, the reduction in smoking and occupational risk factors in the UK led to a nearly 40% fall in bladder cancer compared to the early 1990s (87).

1.2.2 Bladder cancer subtypes, pathological and molecular classification
Although urothelial carcinoma (transitional cell carcinoma; TCC) represents about ~90–95% of all bladder tumours, there is a wide spectrum of bladder cancer histological subtypes, including squamous cell carcinoma (SCC; 2–5%), adenocarcinoma (0.5–2%), small cell carcinoma (<1%), and other less common histologies (88, 89). Owing to the high incidence of TCC, historically it is the most studied histological subtypes, while other histologic subtypes like SCC is understudied and frequently excluded from clinical trials. Bladder cancer is staged according to the depth of invasion using the tumour-node-metastasis (TNM) system. Bladder tumours are classified into non-muscle invasive
bladder cancer (NMIBC) [CIS, pTa, pT1] and muscle invasive bladder cancer (MIBC) [pT2, pT3, pT4] (Figure 1.5) (90) (91). NMIBC accounts for about 75% of all bladder cancer diagnosed and MIBC accounts for the remaining 25% at diagnosis (92).

Bladder cancer is a heterogeneous disease molecularly that is characterized by genomic instability and a high mutation rate (93). There are six biologically relevant consensus molecular classes, starting with the largest subtype – basal/squamous (35%), followed by luminal papillary (24%), luminal unstable (15%), stroma-rich (15%), luminal non-specified (8%), and neuroendocrine-like (3%) (94) as represented in Figure 1.6. This molecular classification is facilitated by transcriptome profiling, which allows for more precise patient stratification according to prognosis and therapeutic options.

Figure 1.5: A schematic diagram of bladder cancer pathological stage and grading. Permission to reuse figure from Springer Nature (90). The Tumour–Node–Metastasis (TNM) system staging of bladder cancer is demonstrated.

Figure 1.6: Summary of the main molecular subtypes in bladder cancer. Figure from Elsevier (94). A schema representation of the tumour microenvironment and the dominant characteristics. Legends: MIBC, muscle-invasive bladder cancer; NK, natural killer.
Pathological and clinical information from mouse models and human samples demonstrate that there are two potential distinct pathways of pathogenesis of NMIBC and MIBC (Figure 1.7). Low-grade papillary tumours from NMIBC can arise from minimal dysplasia and simple hyperplasia. These have been shown at a molecular level by loss of heterozygosity (LOH) of chromosome 9 and activating mutations of genes encoding for fibroblast growth factor receptor 3 (FGFR3), telomerase reverse transcriptase (TERT), phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (PIK3CA), which encodes p110α of PI3K and stromal antigen 2 (STAG2), which encodes cohesion subunit SA-2. These genes have major role in cell division, proliferation and growth. Although, NMIBC tumours recur often, they are genetically stable. MIBC arises from flat dysplasia and carcinoma in situ (CIS), which normally express TP53 mutation in addition to LOH at chromosome 9, but far fewer FGFR3 mutations. Contrary to NMIBC, MIBC is genetically unstable and accumulate several genomic alterations. Moreover, high-grade non-invasive papillary tumours may develop from flat dysplasia. Low-grade papillary NMIBCs might progress to MIBCs and characteristically acquire either loss of cyclin-dependent kinase inhibitor 2A (CDKN2A) loss, which encodes p16 and p14ARF, or an FGFR3 mutation.

Figure 1.7: Urothelial cancer pathogenesis pathways.
Permission to reuse figure from Springer Nature (90). Potential pathogenesis pathways are demonstrated based upon molecular and histopathological observations. The purple and blue pathways show the two potential major pathways with distinct molecular and histopathological features that have been recognised over the last 20 years. Percentages at diagnosis and key molecular features are shown. Solid arrows indicate pathways for which there is histopathological and/or molecular evidence, while uncertainty is indicated by dashed arrows. Legends: ARID1A, AT-rich interactive domain 1A; EMT, epithelial–mesenchymal transition; RHOGDI1, RHO-GDP dissociation inhibitor 2; ZEB1, zinc-finger E-box binding homeobox 1.

1.2.3 Treatment options in bladder cancer
At initial diagnosis, about 75% of patients present with NMIBC, while about 20% present with MIBC, and the final 5% with metastatic disease (95). There has been a paradigm
shift in treatment landscape for bladder cancer at all stages since the arrival of CPI. Success in targeting the immune system is not unexpected considering that bladder cancer is one of the cancer types with the most somatic mutations (93). The CPI targeting the PD-1/PD-L1 pathway has revolutionised the treatment of this tumour type with several drugs having FDA approval including atezolizumab, nivolumab, durvalumab, avelumab, pembrolizumab. In this section, I will discuss the current gold standard treatment in all stages of bladder cancer, and the emerging therapeutic options pertaining to CPI.

1.2.3.1 Non-muscle invasive bladder cancer

NMIBC is primarily surgically treated by endoscopic transurethral resection of the bladder tumour (TURBT). If deemed intermediate or high-risk disease, a risk-based adjuvant intravesical instillation of chemotherapy or Bacillus Calmette-Guerin (BCG) is given to reduce the risk of disease recurrence respectively (96). Mitomycin C (MMC) is the most common intravesical chemotherapy used. The current UK recommendation is 6-weekly instillation of Mitomycin C (MMC) for intermediate-risk disease and further maintenance MMC is not recommended (97, 98), which is similar to the protocol of the MMC arm in the Southwest Oncology Group (SWOG) 8795 protocol (99).

BCG therapy is one of the most successful immunotherapies against cancer and remains the gold standard for patients with high risk NMIBC (100), showing that immunotherapy remains an effective management for bladder cancer. Evidence suggests that live BCG attaches to the urothelial cells and is internalised by bladder cancer cells, leading to antigen presentation and immune cell activation, along with cytokine secretions (101). However, despite BCG being the mainstay of treatment for over 40 years, the exact mechanism of action remains elusive.

Maintenance BCG treated patients had a significantly higher recurrence free survival compared to induction BCG alone (maintenance: 76.8 months vs induction: 35.7 months; P=<0.001), with an absolute 5-year survival advantage of 5% in the maintenance arm, confirming its superiority (102). Subsequently, two further meta-analysis have reported a reduction in risk of disease progression in maintenance BCG treated patients (103, 104). However, BCG therapy is limited by substantial local and/or systemic side effects, with a discontinuation rate in up to 30% of patients owing to poor tolerance. Moreover, there is considerable rates of treatment failure with up to 20% of patients experiencing early recurrence at 1 year and 55% after 5 years (95). Overall, about 40% of patients will develop recurrence and approximately 20% of patients will progress to MIBC within 5
years despite maintenance BCG (105). Currently in this group of BCG failure/unresponsive patients, the recommendation is for radical cystectomy (106) which has significant morbidities and mortality.

The use of CPI in the NMIBC setting is growing with several trials currently assessing drugs targeting the PD-1/PD-L1 pathway, namely nivolumab, pembrolizumab, atezolizumab, durvalumab and avelumab across a variety of NMIBC disease risk status and BCG-exposure settings. One of the very first phase I trial with monotherapy CPI, ipilimumab showed that 67% of patients with T1-T2 localised urothelial carcinoma had downstaging of tumour post therapy (107), giving a rationale behind administration of CPI in this setting. Recently, the FDA approved the use of intravenous pembrolizumab monotherapy in patients with BCG-unresponsive, high risk NMIBC, who are ineligible for or have elected not to have cystectomy. The phase II results demonstrated a 3-month complete response of 40.2%, with a 52.5% durability of response at 12 months. However almost half of patients with complete response have experienced recurrent NMIBC (108).

There are currently several randomised Phase III trials looking at high risk NMIBC in combination with BCG, either in BCG naïve patients or patients with persistent or recurrent disease following adequate BCG induction. This includes the use of pembrolizumab (KEYNOTE-676; NCT03711032); nivolumab (CheckMate 7G8; NCT04149574); durvalumab in BCG-naïve patients (POTOMAC; NCT03528694); and atezolizumab in BCG-naïve patients (ALBAN; NCT03799835). There are also a couple of trials assessing intravesical pembrolizumab (PemBla; NCT03167151) and in intravesical pembrolizumab in combination with BCG (NCT02808143).

1.2.3.2 Muscle invasive bladder cancer

The gold standard treatment for patients with MIBC is cisplatin based neoadjuvant chemotherapy, followed by radical cystectomy (109). The 5-year survival following radical cystectomy for localised MIBC is only about 50%, with common progression to metastases (109). In patients who are not suitable for radical cystectomy, radical radiotherapy is an option for these patients, with comparable outcomes to cystectomy having been reported (110, 111). Survival rates have remained mainly similar in the last 30 years, suggesting a demand for new effective treatments in the right sequential combinations (112).

Meta-analysis of randomised controlled trials of neoadjuvant chemotherapy in localised bladder cancer demonstrated a 5% improvement in absolute 5-year survival (113). There
was about a 30% complete response rate seen at cystectomy samples following neoadjuvant chemotherapy, with a relative risk of overall survival (OS) of 0.45 (95% CI 0.36-0.56, \( p<0.001 \)) (114). In the adjuvant setting, the evidence to support chemotherapy post radical cystectomy is lacking. In a randomised phase III trial with a median follow-up of 7 years, 284 patients were randomly assigned to immediate or deferred adjuvant chemotherapy post radical cystectomy for pT3-T4 or positive lymph node bladder cancer. There was no significant improvement in overall survival between the two arms (adjusted HR 0.78, 95% CI 0.56-1.08; \( P=0.13 \)) (115). Therefore, neoadjuvant chemotherapy remains the mainstay of treatment in patients fit for systemic chemotherapy. Recently a randomised, placebo-controlled phase II study, NEO-BLADE evaluated if the addition of nintedanib, an oral triple kinase inhibitor to neo-adjuvant platinum based chemotherapy improved outcome. Although, the study did not meet its primary endpoint of an improvement in pathological complete response rate, patients treated with nintedanib showed a progression free survival (PFS) and OS benefit at 12 and 24 months (116). However, only 10-20% of eligible patients received neoadjuvant chemotherapy, and of these over a third of patients received non-cisplatin based therapy (117-119). The reason for this is unclear and could be one or a combination of the following reasons: poor renal function, co-morbidities and patients’ preference. Therefore, it is important to identify new therapies in this setting. Neo-adjuvant use of CPI has been associated with pathological complete response in MIBC (120, 121) and therefore would be a good option to explore in this setting.

In the neoadjuvant setting with CPI, ABACUS investigating 2 cycles of atezolizumab (120) and PURE-01 investigating 3 cycles of pembrolizumab (121) have both recently been reported. In ABACUS, Powles and colleagues reported a pathological complete response (pCR) as defined as pathological pT0 disease at cystectomy of 31%, while in PURE-01, Necchi and colleagues reported a pCR of 37%. The ABACUS trial included higher-risk patients than in PURE-01 as patients included were ineligible for cisplatin and a key inclusion criteria required for trial entry was residual disease in the bladder prior to receiving atezolizumab. There are several other trials looking at combination IO or with chemotherapy: pembrolizumab in conjunction with gemcitabine, with or without cisplatin (NCT02365766), nivolumab plus ipilimumab (NABUCCO; NCT03387761), nivolumab plus urelumab vs. nivolumab alone (NCT02845323), and avelumab (AURA Trial; NCT03674424). There are currently 3 phase III trials awaiting results looking at MIBC in the adjuvant setting, namely atezolizumab (IMvigor010; NCT02450331), nivolumab (CheckMate 274; NCT02632409), and pembrolizumab (AMBASSADOR; NCT03244384).

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Whilst SCC of the bladder is uncommon (~2-5%) worldwide, it is the predominant bladder cancer type in Egypt due to *Schistosoma haematobium* infection in up to 50% of MIBC (122). Moreover, it is the next most common histological subtype in MIBC after urothelial transitional cell carcinoma. Unfortunately, this is a poorly studied entity, with the gold standard treatment of radical cystectomy only. Population based studies have reported that SCC tends to present with more advanced disease compared to transitional cell carcinoma (72% vs 52%) (123). Although perioperative or postoperative radiotherapy may improve the incidence of local recurrence, the evidence remains limited (124). Systemic chemotherapy has not been effective in improving disease free survival (125).

1.2.3.3 Metastatic

Whilst early diagnosis and multimodality therapy in NMIBC and MIBC results in optimal clinical outcomes, patients with metastatic disease is generally incurable, with a relative 5-year OS rate of 15% (126). For over thirty years, cisplatin-based chemotherapy such as MVAC (methotrexate, vinblastine, doxorubicin and cisplatin) or gemcitabine and cisplatin, has been the gold standard first-line treatment for patients with metastatic bladder carcinoma and results in a median OS of 13 – 19 months (127-129). Even though metastatic bladder cancer is relatively chemo-sensitive with an objective response rates of 36 – 65%, almost all patients eventually develop recurrence (110, 130). Moreover, cisplatin-based chemotherapy has a number of toxicities, which in an elderly population with metastatic bladder cancer and significant comorbidities, this could limit tolerability which probably explains why it is estimated that only about 50% of patients receive cisplatin-based chemotherapy (131). In the second-line setting or beyond, taxanes are the first-choice chemotherapy. Paclitaxel has shown a 5 – 19% response rates, with a median survival of 6.5 – 7.2 months, and although docetaxel has a similar clinical activity, it is less tolerated compared to paclitaxel (126).

Durable responses to CPI targeting T cell checkpoint molecules and their ligands have been seen in patients with metastatic bladder urothelial carcinoma. Since 2016, there has been five immune checkpoint inhibitors approved for the treatment of metastatic bladder cancer, all targeting the PD-1 (pembrolizumab and nivolumab) and PD-L1 axis (atezolizumab, durvalumab and avelumab) (45-50) by the European Medical Agency (EMA) and the US Food and Drug Administration (FDA) as second-line therapy or as first-line therapy in patients ineligible for platinum based chemotherapy with PD-L1 positivity only in pembrolizumab and atezolizumab.
<table>
<thead>
<tr>
<th>Trial</th>
<th>IO Agent</th>
<th>Trial details</th>
<th>Arms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KEYNOTE 361</strong></td>
<td>Pembrolizumab (anti-PD-1)</td>
<td>N = 990 PS (\leq 2)</td>
<td>1. Pembrolizumab</td>
</tr>
<tr>
<td>(NCT02853305)</td>
<td></td>
<td></td>
<td>2. Pembrolizumab + Cisplatin/Carboplatin-gemcitabine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3. Cisplatin/Carboplatin-gemcitabine</td>
</tr>
<tr>
<td></td>
<td><strong>Active, not recruiting</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CheckMate 901</strong></td>
<td>Nivolumab (anti-PD-1),</td>
<td>N = 897 PS (\leq 1)</td>
<td>1. Nivolumab + Ipilimumab</td>
</tr>
<tr>
<td>(NCT03036098)</td>
<td>Ipilimumab (anti-CTLA-4)</td>
<td></td>
<td>2. Nivolumab + cisplatin-gemcitabine</td>
</tr>
<tr>
<td></td>
<td><strong>Recruiting</strong></td>
<td></td>
<td>3. Cisplatin/Carboplatin-gemcitabine</td>
</tr>
<tr>
<td><strong>DANUBE</strong></td>
<td>Durvalumab (anti-PD-L1),</td>
<td>N = 1200</td>
<td>1. Durvalumab</td>
</tr>
<tr>
<td>(NCT02516241)</td>
<td>Tremelimumab (anti-CTLA-4)</td>
<td></td>
<td>2. Durvalumab + Tremelimumab</td>
</tr>
<tr>
<td></td>
<td><strong>Recruiting</strong></td>
<td></td>
<td>3. Cisplatin/Carboplatin-gemcitabine</td>
</tr>
<tr>
<td><strong>NILE</strong></td>
<td>Durvalumab (anti-PD-L1),</td>
<td>N = 885 PS (\leq 1)</td>
<td>1. Durvalumab + Cisplatin/Carboplatin-gemcitabine</td>
</tr>
<tr>
<td>(NCT03682068)</td>
<td>Tremelimumab (anti-CTLA-4)</td>
<td></td>
<td>2. Durvalumab + Tremelimumab +</td>
</tr>
<tr>
<td></td>
<td><strong>Recruiting</strong></td>
<td></td>
<td>Cisplatin/Carboplatin-gemcitabine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3. Cisplatin/Carboplatin-gemcitabine</td>
</tr>
</tbody>
</table>

Table 1.1: Key first-line phase III trials awaiting results, with an anti-PD-1/PD-L1 checkpoint inhibitor backbone in first-line metastatic bladder cancer.

Legend: IO, immune-oncology; N, number; PS, performance status
<table>
<thead>
<tr>
<th>Trial</th>
<th>Agents</th>
<th>Trial details</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMvigor211 (50)</td>
<td>Atezolizumab (anti-PD-L1) compared to chemotherapy (Vinflunine 320 mg/m²; Paclitaxel 175 mg/m²; Docetaxel 75 mg/m²)</td>
<td>Phase III N = 931</td>
<td>OS did not differ between treatment arms (stratified HR: 0.87; 95% CI: 0.63–1.21) in IC2/3 population with high PD-L1 positivity. Atezolizumab had a similar ORR to chemotherapy (23% vs 22%). The duration of response in atezolizumab was 15.9 months compared to 8.3 months in the chemotherapy arm (HR 0.57; 95% CI 0.26 – 1.26).</td>
</tr>
<tr>
<td>KEYNOTE 045 (47)</td>
<td>Pembrolizumab (anti-PD-L1) compared to chemotherapy (Vinflunine 320 mg/m²; Paclitaxel 175 mg/m²; Docetaxel 75 mg/m²)</td>
<td>Phase III N = 542</td>
<td>Pembrolizumab improved OS compared with chemotherapy independent of PD-L1 expression (10.3 vs 7.4 months; HR: 0.73; 95% CI: 0.59 – 0.91; P = 0.002). Pembrolizumab had a higher ORR to chemotherapy (21.1% vs 11.4%) 43.9% of patients receiving pembrolizumab were still alive after a year, compared with 31% of those given chemotherapy.</td>
</tr>
<tr>
<td>CheckMate 275 (46)</td>
<td>Nivolumab (anti-PD-1)</td>
<td>Phase II N = 270</td>
<td>ORR was 19.6% for the whole population and 28.4% for patients whose tumours had PD-L1 expression of ≥5%</td>
</tr>
<tr>
<td>JAVELIN (45)</td>
<td>Avelumab</td>
<td>Phase I N = 249</td>
<td>ORR was 17% (95% CI: 11-24), including nine (6%) complete responses and 18 (11%) partial responses.</td>
</tr>
<tr>
<td>NCT01693562 (49)</td>
<td>Durvalumab (anti-PD-L1)</td>
<td>Phase I/II N = 191</td>
<td>ORR was 17.8% (95% CI: 12.7 – 24) including seven complete responses in all evaluable patients. Responses were observed regardless of the PD-L1 expression.</td>
</tr>
</tbody>
</table>

Table 1.2: Key second-line phase III trials with an anti-PD-1/PD-L1 checkpoint inhibitor backbone in patients with platinum refractory metastatic bladder cancer.
Legend: CI, confidence interval; HR, hazard ratio; IC, immune cells; IO, immune-oncology; N, number; ORR, objective response rate; OS, overall survival; PS, performance status

Several large phase III trials are underway to assess the optimal first-line treatment for platinum eligible patients (Table 1.1). The first to be reported is the IMvigor130 is a large phase III trial looking at 1213 platinum eligible patients in the first-line setting (132). Patients were randomised 1:1:1 to Arm A (atezolizumab + platinum-based chemotherapy), Arm B (atezolizumab) or Arm C (placebo + platinum-based chemotherapy). The median PFS was 8.2 months in Arm A vs 6.3 months in Arm C (HR = 0.82; 95% confidence interval (CI): 0.70 – 0.96; P = 0.007). The interim overall survival
revealed a benefit to the Arm A combination therapy with a HR of 0.83 (95% CI: 0.69 – 1.00; P = 0.027). For Arm B vs C, the median OS in the intention to treat (ITT) was 15.7 and 13.1 months respectively (HR = 1.02; 95% CI: 0.83 – 1.24); and in the PD-L1 IC2/3 patients, not estimable and 17.8 months respectively (HR = 0.68; 95% CI: 0.43 – 1.08). The objective response rates were 47%, 23% and 44% and complete response rates were 13%, 6% and 7% for Arms A, B and C, respectively. Adverse events leading to treatment discontinuation occurred in 34%, 6% and 34% of patients in Arms A, B and C respectively. Overall, there is a benefit in PFS when adding atezolizumab to chemotherapy in all comers. However, the benefit of atezolizumab monotherapy seems to be limited to patients with PD-L1 highly expressing immune cells. We are still awaiting the full report of this trial and the other first-line trials in Table 1.1.

In the second-line setting, atezolizumab led the pack to be approved by FDA in 2016 based on results from its phase II trial IMvigor210 (133). However, the large phase III trial, IMvigor211 failed to prove superiority over second-line chemotherapy (50). IMvigor211 was a biomarker driven hierarchical statistical design, first evaluating differences in overall survival in the high PD-L1 positive group, a rational developed in their earlier phase II trial, IMvigor210 (133). KEYNOTE-045, on the other hand, included evaluated all patients irrespective of PD-L1 status. There are distinct differences between IMvigor211 and KEYNOTE-045 (47), from statistical design and patient populations to different PD-L1 antibodies used. For PD-L1 positivity, KEYNOTE-045 used a PD-L1 combine positive score (CPS) of ≥10, while IMvigor211 used an immune cell (IC) score of 2/3 to classify as positive.

The objective response rates (ORR) based on PD-L1 positivity was 21.6% in KEYNOTE-045, with a comparable 23% in IMvigor 211. There was a stark difference in the ORR based on PD-L1 positivity in the chemotherapy arm for KEYNOTE-045 was 6.7%, while IMvigor 211 was 21.6%, which also did better when compared to the trial’s unselected patients (13.4%). This unexpected favourable outcome of PD-L1 expression with improved ORR and overall survival (OS) in both the chemotherapy (particularly vinflunine) and atezolizumab arms resulted in a non-superiority trial. This result does not necessarily suggest that there was less activity with atezolizumab, but that conventional chemotherapy improved the outcomes in patients with high PD-L1 expression too. Importantly, IMvigor 211 looked specifically at PD-L1 expression on tumour infiltrating immune cells only, while KEYNOTE-045 looked at both PD-L1–expressing tumour and infiltrating immune cells. Moreover, PD-L1 expression on tumour infiltrating IC has been associated with improved OS in a group of patients with bladder cancer (134), indicating
that these patients may already have a better prognosis and that perhaps the inflamed tumour microenvironment is more susceptible to the effects of cytotoxic chemotherapy.

Another key differences between both trials is the chemotherapy distributions in the comparator arms. In KEYNOTE-045, the distribution was well balanced. However, in IMvigor 211, majority of patients received vinflunine (55%), followed by paclitaxel (33%) and docetaxel (12%). Interestingly, when stratified by the choice of second line chemotherapy, atezolizumab showed a survival benefit compared to taxane-based chemotherapy (docetaxel, paclitaxel) with a median OS of 8.3 vs. 7.5 months (HR 0.73; 95% CI: 0.58–0.92), while there was no difference with vinflunine with a median OS of 9.2 vs. 8.3 months (HR 0.97; 95% CI: 0.78–1.19).

Additionally, there were more patients who progressed on neoadjuvant or adjuvant treatment within 12 months in IMvigor211’s unselected population – 25% in the atezolizumab arm and 23% in the chemotherapy arm, with a higher percentage in the IC2/3 population of 32% in the atezolizumab arm and 31% in the chemotherapy arm. In KEYNOTE-045, the pembrolizumab arm was 11.5% and 19.5% in the chemotherapy arm. This raises the question whether this group of patients inherently have a more aggressive disease and hence more unlikely to respond to any systemic therapy. Finally, more patients underwent radical cystectomy of the primary tumour in IMvigor211 compared to KEYNOTE-045, with 42.9% vs. 20.7%, respectively. Whether this signifies the importance of the primary tumour being present, i.e. more tumour antigens, in response to CPI remains unclear.

It is naturally easy to make informal comparisons between these trials. However, due to differences in the patient populations, comparisons between a biomarker-selected and unselected trials are challenging with a risk of data overinterpretation. Therefore, it is essential to interpret these with caution. A summary of the key second-line trials that led to the approval of the five CPIs for the use in platinum refractory metastatic bladder cancer is summarised in Table 1.2.

There is a significant number of patients that do not receive second-line treatment due to decline in performance status and given the short PFS after first-line treatment, maintenance CPI has been explored in patients that had at least stable disease following first-line platinum based chemotherapy. Maintenance pembrolizumab led to a 2.6 month improvement in PFS (8.2 vs 5.6 months, p=0.023) compared to placebo (135). Although the JAVELIN Bladder 100 has not yet been presented, Pfizer have announced following the planned interim analysis that avelumab maintenance met its primary endpoint or
prolonging OS compared to standard of care. Maintenance CPI may become a new standard of care in this setting following these two studies.

There are other drugs being tested in this group of patients such as FGFR inhibitors/monoclonal antibodies like erdafitinib (136), BGJ398 (137), B-701 (138). Erdafitinib was granted FDA approval for the use in platinum refractory advanced bladder cancer patients who has an FGFR3 or FGFR2 alteration. A trial of B-701 in combination with pembrolizumab for bladder cancer patients who previously had platinum is underway (NCT03123055).

The most exciting treatment that has recently been granted breakthrough by the FDA for patients who has previously received CPI is an antibody-drug conjugate, enfortumab vedotin. An antibody-drug conjugate (ADC) comprises of a monoclonal antibody against a highly expressed cancer cell target, in this case nectin-4, with a protease-cleavable linker bound to a chemotherapeutic agent, in this case monomethyl auristatin E, a microtubule-disrupting agent. The cytotoxic agent is only internally released in select cells that express nectin-4 after internalising the ADC and lysosomal cleavage. Enfortumab vedotin monotherapy data from EV-201 were encouraging, demonstrating a 42% response rate, with 9% complete response (139). EV-103 is the phase 1b study looking at cisplatin ineligible patients receiving first-line combination enfortumab vedotin with pembrolizumab (140). 29 patients were reported receiving this combination, with a confirmed ORR of 62%, including a 14% complete response rate. The most common treatment-related side effects were fatigue (66%; Grade 3 ≥ 14%), peripheral neuropathy (52%; Grade 3 ≥ 3%), decreased appetite (52%; Grade 3 ≥ 0%), alopecia (45%), rash of any type (45%; Grade 3 ≥ 14%) and diarrhoea (41%; Grade 3 ≥ 3%). 17% experienced immune-mediated events that required systemic steroid treatment (Grade 3 ≥ 10%).

There are currently two phase III underway looking at enfortumab vedotin in the first-line setting with pembrolizumab, with or without platinum based chemotherapy (NCT04223856) and in the second-line setting compared to investigator’s choice of chemotherapy (NCT03474107).

An improved understanding of immunoregulation and molecular characterisation in bladder cancer has drastically changed the treatment landscape in patients with metastatic bladder cancer over the last 5 years. With many more therapeutic options, there is an unmet need to better understanding the mechanisms of these drugs and the biology of the disease, to help clinicians find the most optimal sequential and/or combination therapies for these patients.
1.2.4 Non-invasive immunological biomarkers in response to immunotherapy in bladder cancer

Recent clinical trial results underscore the need for effective biomarker-based patient selection. For example, as discussed earlier, despite equally promising phase I/II trial results using antibodies that target the PD-1/PD-L1 axis with atezolizumab (133) and pembrolizumab (48), using predefined biomarkers in those trials, the phase III results showed a statistically significant benefit only with pembrolizumab (47) and not atezolizumab (50) in the second-line setting for metastatic urothelial cancer. The differing PD-L1 assays, differences in the criteria for assessing intratumoural PD-L1 expression and in patients accrued in the chemotherapy arm as a biomarker for patient selection may have resulted in this unexpected discrepancy.

Despite the recent excitement and clinical successes of CPI in bladder cancer, only about a quarter of patients derive benefit (46, 47, 50). Therefore, there has been an immense interest in research aiming towards the identification and development of predictive biomarkers to CPI response and to better understand and overcome the mechanisms of drug resistance. This will allow precision medicine with better selection of patients, minimising unwanted adverse events and cost, as well as guide clinicians in determining the best rational sequential and combinatory therapies. Moreover, precise and accurate biomarkers may help guide clinical trial design.

One of the strongest predictive evidence for CPI response was found to be a high density of tumour infiltrating CD8+ lymphocytes in immunohistochemistry of lung cancer and melanoma, although this is not yet clinically validated (141). Bladder cancer with mismatch repair (MMR) deficiency, as seen in genetic syndromes such as Lynch syndrome and tumours involving the upper tract, appear to be particularly responsive to CPIs (142, 143). Currently, much attention has been focused towards PD-L1 expression, tumour mutational load, and intra-tumoural T cell infiltration within tumour biopsy specimens taken before and during therapeutic intervention (11-14, 144, 145). However, the main limitation to this is the invasiveness of these procedures in accessing longitudinal tumour biopsies prior to and during the course of therapy (146-149). There exist a number of advantages and disadvantages for using both tumour and liquid biopsies, such as blood and urine, for biomarker analysis. A summary of the advantages and disadvantages are summarised below in Table 1.3.
Table 1.3: Advantages and disadvantages in tumour and liquid biopsies.

Legends: TME, tumour microenvironment.

Routine clinical biomarkers should ideally be assessed in a minimally or non-invasive way. Hence, there is an immense interest in developing liquid biopsies from whole blood, serum, or other bodily fluids to be used as a predictive or prognostic biomarkers of CPI response. Among bodily fluids, urine and saliva are most appealing for liquid biopsy due to the non-invasive nature and easy accessibility of these fluids. However, a comprehensive and longitudinal sampling of tumour tissues and bodily fluid through the patients’ treatment history and evolution of their tumour progression may be the best approach that reflect the evolving nature of the systemic and tumour immune microenvironment. This would lead to a precision evolution tracking of the patient’s tumour and aid clinicians in the management of these patients. On the whole, with new tools in tissue and computational analysis, rigorous validation and collaborative initiatives from multidisciplinary teams, this will help researchers better understand the dynamic nature of the interaction between the tumour antigens and immune system, and thereby help better serve our patients. For the remaining of my introduction, I will focus on non-invasive, mainly urine and blood, immunological biomarkers in response to immunotherapy.

1.2.4.1 Blood biomarkers

To date, most of the data around response to CPI in blood have largely been focused on intratumoural characteristics, particularly using genomics data, such as circulating free DNA (cfDNA), tumour mutation burden (150, 151), chromosomal instability (152) and PD-L1 on circulating tumour cells (CTCs) (153). Many of these studies are in melanoma and non-small cell lung cancer, demonstrating tantalising results and their potential as
an indicator of response to CPI. However, these need to be validated in large prospective clinical studies. Unsurprisingly, there are also several blood-based immunological markers looking at various immune cells including exhausted T cells, myeloid derived suppressor cells (MDSCs), NK cells and neutrophil-to-lymphocyte ratio (NLR). Data on patients’ response to CPI in metastatic bladder cancer in peripheral blood are sparse.

The neutrophil-to-lymphocyte ratio (NLR) appears to be a prognostic marker in 87.5% of the studies in upper tract urothelial cancer, 80% of urothelial bladder cancer, and 60% of metastatic and advanced disease (154). In a group of metastatic urothelial cancer patients treated with durvalumab, a low NLR and high albumin levels are associated with tumour shrinkage and high OS (155). NLR is a widely available, easily accessible, low-cost prognostic marker that may be in broadly used in urothelial cancers. However, its clinical use still needs validation in large cohort studies. Several studies used different NLR cut-off value ranging between 2 and 5 and there is a need to establish a widely accepted NLR value threshold, particularly in different subsets of patients (154).

Circulating myeloid-derived suppressor cells (MDSCs) have been shown to be associated with poor response to ipilimumab in patients with melanoma (156). In patients with metastatic urothelial cancer, low baselines levels of circulating MDSCs were correlated with longer OS in the Checkmate 275 trial testing nivolumab (157). Furthermore, in patients undergoing neoadjuvant chemotherapy for MIBC, circulating MDSCs were negatively associated with pathological complete response rate at cystectomy (158).

One unique opportunity to tract T cell clones following anti-cancer treatment is by TCR sequencing. Several groups have investigated the effect of CPI on the TCR repertoire, both in tumours (147, 159) and peripheries, shedding light onto the mechanism of response and resistance to CPI and thereby highlighting the importance of TCR sequencing derived metrics as potential predictive markers to CPI response. Accordingly, groups have found that post treatment with ipilimumab, there is an increase in TCR diversification (160) and new CD8+ antigen specific TCR (161) in patients with melanoma. Patients with advanced prostate cancer and melanoma who maintained highly abundant circulating TCR that was detected prior to starting ipilimumab had improved survival (162). A study of 29 patients with metastatic urothelial cancer treated with atezolizumab found that patients with lower circulating TCR clonality (diverse TCR repertoire) at baseline was associated with improved PFS and OS (163). This indicates that a diverse TCR repertoire in the peripheral blood at baseline might increase the probability of broader tumour-specific T cell population that recognises several tumour
clones, whether these are neoantigens or TAA. Interestingly, the authors found that intratumoural T cell clonality at baseline had no association with outcome, although the combination of high baseline TIL clonality and low baseline circulating TCR clonality were predictive of durable responses compared to circulating TCR clonality or TIL clonality alone. Additionally, patients with durable response was also found to have a significant expansion of tumour-associated TCR clones in the blood 3 weeks after starting treatment, suggesting that anti-tumour T cells may home from the periphery to the tumour microenvironment before recirculating back. Together, these data suggest that analysis of the TCR repertoire may be helpful in providing insight into the antitumoural immune response prior and after CPI, supporting its use as a potential predictive marker of response to immunotherapy.

Taken together, these studies suggest that peripheral blood immune analysis in patients with urothelial cancer may be feasible in predicting immunotherapeutic outcomes, although larger studies are required to validate these findings.

1.2.4.2 Urinary biomarkers

The urine is a rich source of tumour-derived material that could potentially serve as a window to bladder tumour immune microenvironment in patients with bladder cancer. Urinary cell-free DNA has previously been shown to mirror the bladder tumour genomic microenvironment (164) and urinary circulating tumour DNA (ctDNA) (165) has been correlated to metastatic relapse in bladder cancer. There are several urinary biomarkers used in bladder cancer, with currently six FDA commercially approved urinary tests. These urinary biomarkers are aimed to detect bladder cancer as a primary diagnosis rather than for monitoring recurrence. They have an overall sensitivity of between 57-82% and specificity of between 74-88% (166), and are not licensed for use without cystoscopy (167). Moreover, these are all using genomics analysis, with no FDA approved immunological biomarker.

Most urinary immunological biomarker studies have largely been studies in patients with NMIBC on BCG treatment, with urinary cytokines being the most explored immunological parameter. Zuiverloon et al. showed in a systematic review that measurement of urinary interleukin (IL)-8, IL-18, and tumour necrosis factor apoptosis-inducing ligand (TRAIL) is promising in assessing response to BCG treatment if the thresholds for these levels are standardised. However, by far the most promising marker in predicting BCG response is the measurement of urinary IL-2 (168). IL-2, a 15 kDa four-α-helix bundle cytokine, is secreted as a soluble molecule mainly by activated CD4⁺ T cells in secondary lymphoid
organs and as well as by CD8+ T cells, NK cells and NKT cells during steady state resting conditions (169). While IL-2 may be secreted by activated dendritic cells and mast cells in small quantities, activated CD4+ and CD8+ T cells produce large quantities of IL-2 following an immune response. Additionally, IL-2 is a vital cytokine for T cell proliferation, differentiation and survival of cytotoxic T cells (169). Although urinary T lymphocytes were not specially examined in these studies, one can infer that the high levels of urinary IL-2 detected post BCG, would likely be secreted by T cells, suggesting that at least in part, the importance of T lymphocytes in response to BCG, with several studies demonstrating that high levels of urinary IL-2 post BCG therapy were associated with an improved recurrence free survival (RFS) (170-172).

Saint et al found a correlation between high levels of urinary white blood cells (leukocyturia) and improved RFS post BCG treatment (173). However, the subsets of these urinary white bloods cells in this study were not further explored. A small number of studies have demonstrated an increased number of urinary T lymphocytes after intravesical BCG immunotherapy administration in patients with NMIBC (N = 7-11) (174-176). Moreover, De Boer et al. showed that the CD8/CD4 ratio increase after BCG instillation, with a significantly decrease in the CD4+ T cell population (176). Urinary lymphocytes have also been identified in other autoimmune diseases (177, 178), but there have been no reports identifying urinary lymphocytes in MIBC nor in metastatic bladder cancer.

To date, I have not found any published work on urinary lymphocytes in MIBC or the metastatic setting of bladder cancer prior to this work. There are no urinary immunological biomarkers aiming to identify potential T cell biomarkers of response and resistance to checkpoint inhibitors (CPI) in bladder cancer. Comprehensive profiling of the expression of actionable immuno-oncology (IO) targets or their association with clinical outcome was not performed in any of these studies and patients with MIBC were not studied. Additionally, the extent to which urinary derived markers recognise or reflect in the tumour immune microenvironment remains unknown.

The field of cancer immunology continues to evolve at a rapid pace, and the developments in cancer immunotherapy have exceeded the understanding and scientific knowledge in this domain. While the area of cancer immunotherapy is an exciting time and offers hope in some patients with metastatic solid cancers with durable responses, this is a critical time to improve our understanding and develop biomarker to better select patients who will benefit from this treatment. Hence it is essential for all subsequent trials using CPI or in combination to integrate biomarker analysis, particularly liquid biopsies,
which are easily accessible, convenient and minimally invasive. Moreover, future research will unravel whether composite biomarkers derived from baseline and on-treatment tumour and liquid biopsies will have the best framework in helping clinician identify the most appropriate patients for CPI therapy and ultimately in predicting response.
Chapter 2: Material and Methods

2.1 Patient selection

Collection of human tissue for this study was agreed by the institutional review board as part of the University College London/ University College London Hospital BioBank for Health and Human Disease (REC 15/YH/0311) study. All patients (or their legal representatives) provided written informed consent before enrolment.

2.1.1 Cystectomy samples

Matched bladder tumour, non-tumour tissue (NT) urothelium, urine and peripheral blood mononuclear cells (PBMC) were collected from patients undergoing radical cystectomy. The patient cohort comprised of those with a transurethral resection of the bladder tumour (TURBT) histological diagnosis of non-muscle invasive bladder cancer (NMIBC; N = 6) and muscle invasive bladder cancer (MIBC; N = 32).

2.1.2 Metastatic patients

Patients with the following criteria were recruited into the study.

1. Histological diagnosis of metastatic urothelial carcinoma
2. No previous cystectomy
3. Starting on a new line of systemic therapy

Matched urine and peripheral blood mononuclear cells (PBMC) were collected from patients who were starting on systemic therapy with either chemotherapy (N = 5) or immunotherapy (N = 4). Samples were collected prior to every cycle, up to their first scan, and subsequently coinciding with their response scan.

2.2 Sample processing

2.2.1 Isolation of lymphocytes from peripheral blood and urine

On the day of radical cystectomy or systemic treatment, blood and urine samples were obtained prior to the start of surgery or administration of systemic therapy. Peripheral blood was collected into 10ml EDTA tubes. Urine was into a universal container. All urine samples were tested by dipstick for the presence of infection. Any sample that tested positive for nitrites was excluded from further analyses. Peripheral blood mononuclear cells (PBMC) and urine derived lymphocytes (UDL) were isolated through density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). Live cells were stained
fresh following isolation. Excess sample not used for staining was cryopreserved at -80°C and stored in liquid nitrogen.

2.2.2 Isolation of lymphocytes from tumour and non-tumour tissue samples
Following radical cystectomy, the bladder specimen was dissected and NT tissue was sampled from the contralateral side to the tumour as identified macroscopically. All tissue samples were placed into conical tubes containing plain RPMI culture medium (Sigma) and transferred locally on the day of collection for processing. Tumour samples were micro dissected manually and digested with Collagenase (2.5mg/mL, Gibco) and DNAse I (0.2 mg/mL, Roche) at 37°C for 1 hour, homogenized using gentleMACS Octo Dissociator (Miltenyi Biotech; program 37-h-TDK-2) and filtered through a 0.7 μm cell mesh. Leukocytes were enriched by gradient centrifugation with Ficoll-paque (GE Healthcare). Once isolated, single cell suspensions of lymphocytes were stained fresh on the day of processing. Excess sample not used for staining was cryopreserved at -80°C and stored in liquid nitrogen. All tissue samples from cystectomy were verified using immunohistochemistry by a Consultant Histopathologist specialising in Uro-oncology.

A summary of the samples collected is shown in Table 2.1 below.

<table>
<thead>
<tr>
<th>SPECIMEN TYPE</th>
<th>PURPOSE OF ANALYSIS</th>
<th>COLLECTION METHOD</th>
<th>STORAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>Immunology</td>
<td>Place into 1 x 10mls EDTA tubes</td>
<td>On room temperature; transfer to UCL for processing</td>
</tr>
<tr>
<td>Urine</td>
<td>Immunology</td>
<td>Place into universal container</td>
<td>On room temperature; transfer to UCL for processing</td>
</tr>
<tr>
<td>Tumour and non-tumour tissue</td>
<td>Histopathology</td>
<td>Histology cassette/formalin</td>
<td>Room temperature</td>
</tr>
<tr>
<td></td>
<td>Immunology</td>
<td>Place into conical tube containing plain RPMI culture medium</td>
<td>On wet ice; transfer to UCL for processing</td>
</tr>
</tbody>
</table>

Table 2.1: A summary of specimen type and samples collected.
Table depicts the specimen type collected, using various collection and storage method.
2.3 Multi-parametric flow cytometry

2.3.1 BD LSRFortessa staining panel
Multi-parametric flow cytometry was used to analyse urine, tumour, NT tissue and PBMC samples from these patients. Acquisition was performed with a BD LSR II Fortessa (BD Biosciences). The flow cytometry antibodies, clones and fluorescent labels used in the flow cytometry experiments are shown in Table 2.2. Intranuclear staining of Granzyme B, CTLA-4, FoxP3 and Ki67 was performed using the FoxP3 Transcription Factor Staining Buffer Set (eBioscience).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Supplier</th>
<th>Staining surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granzyme B</td>
<td>V450</td>
<td>GB11</td>
<td>BD Biosciences</td>
<td>Intracellular</td>
</tr>
<tr>
<td>CD8</td>
<td>V510</td>
<td>SK1</td>
<td>BD Biosciences</td>
<td>Extracellular</td>
</tr>
<tr>
<td>TIM-3</td>
<td>Q650</td>
<td>7D3</td>
<td>BD Biosciences</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Ki67</td>
<td>FITC</td>
<td>B56</td>
<td>BD Biosciences</td>
<td>Intracellular</td>
</tr>
<tr>
<td>PD-1</td>
<td>Q605</td>
<td>EH12.2H7</td>
<td>Biolegend</td>
<td>Extracellular</td>
</tr>
<tr>
<td>CD25</td>
<td>BV711</td>
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<td>Biolegend</td>
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</tr>
<tr>
<td>CD3</td>
<td>BV785</td>
<td>OKT3</td>
<td>Biolegend</td>
<td>Extracellular</td>
</tr>
<tr>
<td>41BB</td>
<td>PE</td>
<td>4B4-1</td>
<td>Biolegend</td>
<td>Extracellular</td>
</tr>
<tr>
<td>ICOS</td>
<td>PE-Cy7</td>
<td>C398.4A</td>
<td>Biolegend</td>
<td>Extracellular</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>APC</td>
<td>L3D10</td>
<td>Biolegend</td>
<td>Intracellular</td>
</tr>
<tr>
<td>FoxP3</td>
<td>PerCP-Cy5.5</td>
<td>PCH101</td>
<td>eBioscience</td>
<td>Intracellular</td>
</tr>
<tr>
<td>CD4</td>
<td>AF700</td>
<td>OKT4</td>
<td>eBioscience</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Viability Dye</td>
<td>APC-Cy7</td>
<td>-</td>
<td>eBioscience</td>
<td>Extracellular</td>
</tr>
</tbody>
</table>

Table 2.2: Antibodies used for flow cytometry staining.
Table depicts the anti-human flow cytometry antibodies used in staining panel with corresponding clones and fluorophore.

2.4 Flow cytometry data analysis

Flow cytometry data analysis was performed in FlowJo version 10.0.8 (Tree Star Inc.). Statistical analyses were performed in Prism 6 (GraphPad Software, Inc.); P values were calculated using Kruskall-Wallis analysis of variance and Dunn’s post-hoc test with error bars represent mean values with standard error of the mean (SEM), unless otherwise indicated in the figure legends (ns = p > 0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001; **** = p < 0.0001). Fewer than 200 viable CD3+ cells were defined as inadequate number of cells for analysis. Analysis of Kaplan-Meier survival curves was performed with use of the log-rank test.
2.5 T cell receptor (TCR) sequencing

The alpha and beta chains of the TCR repertoire were sequenced using a method which starts with total RNA isolated from unfractionated tissue/cells, and introduces unique molecular identifiers attached to individual cDNA molecules to provide a quantitative and reproducible method of library preparation. RNA was extracted from tissue or PBMC samples using standard silica membrane columns (AllPrep DNA/RNA Mini kit or RNeasy Micro or Mini kits, Qiagen). Full details for both the experimental library preparation, and the subsequent computational analysis using Decombinator is published in Oakes et al (179). For each sample, the output provides a list of CDR3 sequences and the number of times each CDR3 is observed.

The Jaccard index between two repertoires is calculated as the intersection between two sets of TCRs (i.e. the number of shared CDR3 sequences) divided by the union (the sum of the number of unique sequences in both sets). The similarity between two samples was measured using a standard similarity metric, calculated as the dot product between the vector of CDR3 frequencies in each sample. In Figure 3D we subsampled the combined repertoires from each compartment. Each sample contained 1000 unique CDR3s. Each sample from tumour, non-tumour tissue and blood was compared to an equal sized sample from urine. The sampling was repeated 100 times, and the figure shows the average and standard deviation. Statistical analysis was performed using a one-way Anova.

2.6 Neoantigen reactive T cell (NARTs) detection

Accurate MHC binding affinity prediction models are data-driven, based upon methods trained on peptide data with experimental information about the binding affinity to the MHC molecule. The NetMHCpan method predicts peptides binding to any MHC molecule of known sequence using artificial neural networks. This method is trained on over 180,000 quantitative binding data covering 172 MHC molecules from human (HLA-A, B, C, E), mouse (H-2), primates (Patr, Mamu, Gogo), cattle (BoLA) and swine (SLA). The authors found that when a percentile rank score of 2 % was used, 91 % of the MHC class I ligands would be recovered with a specificity of 98 %. This sensitivity value drops to 82 % if the screening were based on affinity values. Strong binders are defined as having percentile rank score of <0.5, and weak binders with percentile rank score of <2 (180).
Neoantigen prediction from whole exome sequencing data was performed by Dr Rachel Rosenthal from the Swanton laboratory. Identified non-silent mutations were used to generate a comprehensive list of peptides that are 9-11 amino acids in length with the mutated amino acid represented in each possible position. The binding affinity of each mutant peptide and its corresponding wild-type peptide to MHC was performed using NetMHCpan 3.0 (180). Candidate neo-antigens were identified as those with a predicted binding strength of <500 nM or predicted RANK score <= 2.

Synthetic peptides were purchased at Pepscan Presto, NL and a corresponding library of MHC multimers holding these predicted peptides were produced in-house by the Hadrup laboratory at Technical University of Denmark. Dr Amalie Kai Bentzen from the Hadrup laboratory subsequently applied these peptide-MHC multimers labelled with DNA barcodes for large-scale detection of patient specific neoantigens using the same published method (181). HLA molecules matching the HLA expression of BL 5 (HLA-A0201, A2601, B0702 and B4402) were screened. MHC multimers were generated with two different streptavidin-conjugates for each peptide-specificity to allow a combinatorial encoding of each antigen responsive T cell, allowing analyses for reactivity against up to 36 different peptides in parallel (182).

2.7 In-vitro expansion of lymphocytes using pre-Rapid Expansion Protocol (pREP) and REP

Single cell suspensions from urine, tumour, NT and PBMC were expanded from cryopreservation with a modified version of the pre-Rapid Expansion Protocol (pREP), followed by the Rapid Expansion Protocol (REP) using the Dudley's method (183). The modified version of the pREP was set up using unexpanded lymphocytes from each tissue-specific sample on 24 well-plates, with or without 50 Gy irradiated autologous tumour samples in the presence of 6000IU/mL IL-2 and 25ng/mL IL-21 for three weeks. Some of these pREP lymphocytes were subsequently taken for a round of REP. The REP method consists of incubating the pREP lymphocytes with 50 Gy irradiated allogeneic feeder cells from three healthy donors with 0.6ug/mL of soluble anti-CD3 (aCD3) antibody and 6000IU/mL IL-2 for three weeks, initially in T25 flasks. All cells were grown in X-VIVO 15 media (Lonza) supplemented with 5% human AB serum (Sigma).

2.8 FluoroSpot Assays

All FluoroSpot assays were performed using FluoroSpot kits from Mabtech, according to the manufacturer’s instructions. The FluoroSpot kit used for my assay was the dual
human interferon gamma (IFNg) and granzyme B (GzmB) detection kit (FSP-0110-2). Briefly, the pre-coated plate was prepared with 5 times phosphate-buffered saline (PBS) washes initially, followed by incubation of X-VIVO 15 media and 10% fetal bovine serum (FBS) under sterile conditions and in the dark. After removing the media, the expanded lymphocytes were added to each well with or without stimuli. The plate was subsequently incubated in a 37°C humidified incubator with 5% CO₂ for 20 hours to allow for cytokine secretion. After the 20-hour incubation, the plate was emptied and washed with PBS. To avoid fluorescent artefacts, all 0.2μM detection reagents were filtered just before use. After the 5 PBS washes, 100μL of a detection antibody mix of FITC-conjugated anti-IFNg and biotinylated GzmB in PBS containing 0.1% bovine serum albumin (BSA) were added for 2 hours at room temperature in the dark. After the incubation, followed by 5 PBS washes, 100μL of an antibody mix of anti-FITC-490 (IFNg) and streptavidin-550 (GzmB) in PBS 0.1% BSA were added to each well to amplify the detection and incubated at room temperature for 1 hour in the dark. Plates were washed 5 times with PBS before adding 50μl of Fluorescence enhancer solution to each well for 15 min to augment the signal. The underdrain was removed and the plate was left to dry in the dark before image analysis. The plates were analysed using the Bio-Sys Bioreader 6000, in which fluorescent spots were counted utilising separate wavelength-specific filters. Filters used were for FITC and Cy3 in order to detect fluorophores absorbing and emitting light at 490/520 and 550/570 respectively.

2.9 In vitro functional assays

2.9.1 Autologous tumour sample screen
100,000 expanded lymphocytes from each tissue-specific sample were plated into 96 well plates and incubated in media alone, soluble human anti-CD3/CD28/CD2 (Stemcell), cytomegalovirus, epstein barr virus and influenza virus (CEF) pool mix or autologous target tumour cells in X-VIVO 15 media (Lonza) for 16 hours with GolgiPlug protein transport inhibitor (Brefeldin A) (BD Biosciences). After the incubation, the UDLs were stained intracellularly for IFNg and tumour necrosis factor (TNFa).

2.9.2 Peptide screen
Lyophilised peptides were reconstituted in DMSO to 10nmols/μL. 100,000 expanded urine derived lymphocytes (UDLs) were plated into 96 well plates and incubated in media alone, single peptide, soluble human anti-CD3/CD28/CD2 (Stemcell), CEF pool mix in X-VIVO 15 media (Lonza) for 16 hours in the presence of a GolgiPlug protein transport inhibitor (Brefeldin A) (BD Biosciences). After the incubation, the UDLs were stained intracellularly for IFNg and TNFa.
2.10 Multiplex triple immunohistochemistry

The multiplex triple immunohistochemistry was performed by Ayse Akacar from the Marafioti laboratory. 2-5µm tissue sections of formalin-fixed and paraffin-embedded tumour samples were cut and stained with the following antibodies: anti-CD8 (SP239), anti-FoxP3 (236A/E7) (a gift from Dr. G. Roncador, CNIO, Madrid, Spain) and anti-CD4 (4B12; Leica Microsystems, Newcastle-upon-Tyne, UK). Dilutions were optimized with single conventional immunostaining of human tonsil sections. For multiple staining, tissue sections were incubated with the primary antibodies for 30 min after antigen retrieval by heating and endogenous peroxidase blocking (DakoCytomation). Detection was performed using the peroxidase-based detection reagent conjugate (OptiView DAB IHC Detection Kit) followed by the alkaline phosphatase detection kit (UltraView Universal Alkaline Phosphatase Red Detection Kit), both from Ventana Medical Systems, Inc.

For the automated image analysis, segmentation of CD4⁺ and FOXP3⁺ cells was performed using the Definiens Tissue Phenomics software platform utilizing a previously described machine learning-based parameter-free cell segmentation (184). Next, CD8⁺ cells were segmented and both results combined. Finally, the results were post-processed for accurate splitting of cells using a watershed transform based on the distance map of the segmentation result weighted with the original gray values of the source image. Following this, the cell segmentation results were combined with a binary mask of the tumour region obtained by manual annotation of a human expert to discard regions outside of the tumour. For quantitative evaluation, the number of cells per area was determined.
Chapter 3: Mapping the immune landscape of NMIBC and MIBC

3.1 Chapter introduction

BCG remains the gold standard for patients with high risk NMIBC (100), showing that immunotherapy remains an effective management for bladder cancer. However, about 40% of patients will develop recurrence and approximately 20% of patients will progress to MIBC within 5 years despite maintenance BCG (105). Currently in the BCG unresponsive group, the recommendation is for radical cystectomy (106) which has significant morbidities. The peak age incidence of bladder cancer is between 60 – 70 years old and is rare in patients under the age of 40 years old (84). Therefore, it is imperative to find other treatment options for this elderly group of patients, for example other forms of immunotherapy like checkpoint inhibitors (CPI) which has shown their successes in the metastatic settings. A phase I trial with monotherapy ipilimumab showed that 67% of patients with downstaging of tumour post therapy (107), while KEYNOTE-057 showed a 3-month complete response of 40.2% (108), giving a rationale behind administration of CPI in this setting.

In patients with MIBC, the 5-year survival after radical cystectomy for clinically localised MIBC is only about 50% (109). Over the last 30 years, survival rates have remained mostly unchanged indicating a need for new effective treatments (112). In three meta-analyses, the addition of neo-adjuvant platinum-based chemotherapy have shown an increase in overall survival of 5% (176). This is now the gold standard treatment of patients who are fit for chemotherapy (185). Despite this, only 10-20% of eligible patients received neoadjuvant chemotherapy and of these over a third of patients received non-cisplatin based therapy (117-119). The reason for this is unclear and could be one or a combination of the following reasons: poor renal function, neuropathy co-morbidities and patients’ preference. Therefore, it is important to identify new therapies in this setting. Neo-adjuvant use of CPI has been associated with pathological complete responses in MIBC (120, 186) and therefore would be a good option to explore more extensively in this setting.

Finally, in patients with bladder cancer, the urine is a rich source of tumour-derived cells that could theoretically serve as a window to bladder tumour immune microenvironment. Urinary cell-free DNA has previously been shown to reflect the bladder tumour genomic microenvironment (164). Moreover, increased numbers of urinary lymphocytes have been seen after intravesical BCG immunotherapy in patients with NMIBC (174-176). However, little is known about urinary lymphocytes in MIBC or the metastatic setting. I
hypothesised that characterising the lymphocytes within different tissue compartments of bladder cancer would provide a greater understanding of the tumour immune microenvironment and may provide insights for therapeutic targets and treatment strategies.

In this first results chapter, I set out to establish the following objectives:

i. Identify key differences in the immune tumour microenvironment between NMIBC and MIBC

ii. Map the immune checkpoint landscape of NMIBC and MIBC, focusing my analysis on the expression of key co-inhibitory and co-stimulatory immune checkpoints including PD-1, CTLA-4, ICOS and 4-1BB, in order to identify potential immune-oncology targets

iii. Detect viable CD3\(^+\) T lymphocytes in the urine of both NMIBC and MIBC
3.2 Results

3.2.1 Baseline characteristics and clinical variables of pilot patient cohort
To determine the immune landscape of NMIBC and MIBC, I collected blood, urine, tumour and non-tumour (NT) samples from 6 patients with NMIBC and 8 patients with treatment naïve transitional cell carcinoma (TCC) MIBC undergoing surgery with curative intent on the day of cystectomy. The median age group was matched between NMIBC (72 years old, range 65 – 80) and MIBC (71 years old, range 31 – 82). The tumour stage for NMIBC range from CIS to T1c, and T2b to T4a in the MIBC group. Two patients in the NMIBC cohort had received previous BCG treatment, 3 months (BL 34) and 6 months (BL 11) prior to cystectomy in the NMIBC group (Table 3.1).

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Histology</th>
<th>Sex</th>
<th>Age</th>
<th>Stage (Post Op)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL 4</td>
<td>NMIBC</td>
<td>M</td>
<td>70</td>
<td>CIS</td>
<td>Naïve</td>
</tr>
<tr>
<td>BL 8</td>
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<td>M</td>
<td>71</td>
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<td>75</td>
<td>T1b</td>
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</tr>
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<td>73</td>
<td>Ta</td>
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<tr>
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<td>BCG</td>
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<tr>
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<tr>
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<td>M</td>
<td>68</td>
<td>T4a N2</td>
<td>Naïve</td>
</tr>
</tbody>
</table>

Table 3.1: Clinical characteristics of NMIBC and MIBC patient cohort.
Baseline tumour characteristics and treatment history of the pilot cohort. Legend: BCG, Bacillus Calmette-Guerin; CIS, carcinoma in situ; NMIBC, non-muscle invasive bladder cancer MIBC, muscle invasive bladder cancer.

Following radical cystectomy, the bladder specimen was dissected and macroscopic bladder tumours were identified and sampled. NT tissue was sampled from the contralateral side to the tumour. Some NMIBC bladder only had inflammatory changes which was sampled and matched with the patient’s pre-cystectomy cystoscopy report. Similar to previous studies (187), tissue, urine or peripheral blood mononuclear cells (PBMC) samples containing fewer than 200 viable CD3+ cells (as determined by flow
cytometry; Figure 3.1) were considered insufficient for subsequent T cell subsets and checkpoint analyses and therefore classified as “not evaluable”. In total, 11 urine, 14 tumour, 14 NT bladder tissue (NT) and 14 PBMC samples were analysed in this chapter.

Figure 3.1: Flow cytometry gating strategy for CD3+ T lymphocyte cells.
Multi-parametric flow cytometry was used to identify T cell subsets in the urine, tumour, non-tumour and PBMC samples of patients with NMIBC and MIBC. Dot plots and gating strategies are shown from a representative patient. (A) Viable, singlet CD3+ cells were gated for within the lymphocyte gate. (B) CD8+ and CD4+ cells were identified within this population of cells. (C) Regulatory T cells (Treg) and effector CD4+ cells (CD4eff) were identified within the CD4+ population as CD3+CD4+FoxP3+ and CD3+CD4+FoxP3- cells respectively.

T cell subsets were gated based upon CD3+ within the lymphocyte gate (FSC-A by SSC-A), excluding doublets and non-viable cells (Figure 3.1A). CD3+ T cells were further divided into CD8+, CD4+FoxP3+ (CD4eff) and CD4+FoxP3+ (Treg) subsets, with matched urine lymphocytes, tumour infiltrating lymphocytes (TILs), NT tissue lymphocytes and PBMC as shown in Figure 3.1B and Figure 3.1C. To exclude the possibility that urine lymphocyte counts were associated with urinary infection, patients recruited to the study with a urine dipstick test positive for nitrites were excluded from the cohort.
3.2.2 The activation and proliferation landscape of the bladder immune microenvironment is not distinct to PBMC in both NMIBC and MIBC

TILs have previously been shown to be associated with survival in various solid tumours (188). In both humans and mice, a high effector/Treg ratio has been associated with favourable responses to immunotherapy (189, 190). Therefore, I postulated that there would be a lower effector/Treg ratio in the tumour compared to non-tumour tissue, as well as in more advanced disease like MIBC.

**Figure 3.2:** There is a higher CD8/Treg ratio in non-tumour tissue compared to tumour in the MIBC group.

Displayed are two bar graphs showing the effector/regulatory T (Treg) ratio between each tissue compartment in the NMIBC group (n=6) and MIBC group (n=8). Bars represent mean values with SEM.

**Figure 3.3:** Quadruple multiplex IHC showing a higher CD8/Treg distribution in non-tumour urothelium compared to adjacent tumour in a patient with MIBC.

Quadruple multiplex immunohistochemistry demonstrates a higher CD8/Treg distribution in non-tumour urothelium compared to adjacent tumour. Pan-cytokeratin (green), CD8 (red), CD4 (brown), FoxP3 (blue).
There was no significance with the effector/Treg ratio in the NMIBC between the tissue compartment (Figure 3.2). In the MIBC cohort, there was no difference in the CD4eff/Treg ratio. The mean CD8/Treg ratio in PBMC is 28.4 (range 1.55 – 44.5), tumour is 23.1 (range 0.76 – 43.7) and urine is 22.2 (range 4.96 – 11.7), with no significant difference between these compartments in the MIBC group. However, the mean CD8/Treg ratio in the NT urothelium tissue is 42.3 (range 3.67 – 271.0), which was significantly higher when compared to tumour (P = <0.05; Figure 3.2). The higher CD8/Treg distribution is also spatially demonstrated in the NT urothelium compared to tumour of a same patient, as demonstrated by quadruple multiplex IHC (Figure 3.3).

I next hypothesised that there would be more activation and proliferation in the tumour compared to NT and PBMC. Therefore, I looked into key activation and proliferation markers – Ki67, Granzyme B (GzmB) and CD25. Ki67 is an intracellular marker for proliferation, GzmB is a serine protease most commonly found in cytotoxic T cells and natural killer cells (191), while CD25 is present on activated T cells (169).

Figure 3.4: The activation and proliferation landscape of the bladder immune microenvironment is not distinct to PBMC in both MIBC and NMIBC. Displayed is the relative expression of activation and proliferation markers on T cell subsets within urine, tumour, non-tumour tissue and PBMC in NMIBC (n=6) and MIBC (n=8). Violin plots represent median values with quartiles. *P < 0.05.
Remarkably in the MIBC cohort, there were no significant differences between the bladder immune landscape and PBMC, except an increase in CD25 expression in CD4eff in tumour compared to urine (Figure 3.4). In NMIBC, this is much the same, except a significantly higher Ki67 expression in urine compared PBMC (Figure 3.4). When I compared the activation and proliferation phenotype between NMIBC and MIBC, only a significant increased (P = <0.05) in Ki67 and CD25 on CD8⁺ cells were seen in the urine of NMIBC and a trend towards CD4eff Ki67 in urine of NMIBC (P = 0.06) was seen (Figure 3.5). Overall there was no significant distinction between the T cell subsets in the respective tissue compartments.

Figure 3.5: The activation and proliferation landscape of the bladder immune microenvironment in NMIBC is not distinct to MIBC.
Displayed is the relative expression of activation and proliferation markers on T cell subsets, comparing NMIBC (n=6) and MIBC (n=8) within each tissue compartment. Bars represent mean values with SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

3.2.3 The immune checkpoint landscape of the bladder immune microenvironment is distinct to PBMC in NMIBC and MIBC
Having shown that there was no distinction in the activation and proliferation immune landscape in both NMIBC and MIBC in the tissue compartments, I next sought to determine whether the immune checkpoint landscape of CD8⁺, CD4eff, and Treg in
NMIBC and MIBC differs based upon the gating strategy in Figure 3.6. Representative histograms depicting the gating of positive and negative populations of cells expressing these checkpoint molecules are demonstrated. Due to their immediate clinical relevance, I focused my analysis on the expression of key co-inhibitory and co-stimulatory immune checkpoints including PD-1, CTLA-4, ICOS and 4-1BB.

![Flow cytometry representative histograms of relevant checkpoint markers in T cell subsets between NMIBC and MIBC.](image)

Representative histograms depicting the gating of positive and negative populations of cells expressing PD-1, ICOS, CTLA-4 and 4-1BB within urine, tumour, NT tissue and PBMC according to bladder subtypes.

Figure 3.6: Flow cytometry representative histograms of relevant checkpoint markers in T cell subsets between NMIBC and MIBC.
Figure 3.7: The mean percentage immune checkpoint expression in urine, tumour and non-tumour differs from PBMC in the NMIBC and MIBC cohort. Heatmap depicts the mean percentage of CD8+, CD4eff and Treg cells expressing individual immune checkpoint molecules in urine, tumour, non-tumour and PBMC samples obtained from NMIBC (left; N = 6) and MIBC (right; N = 8) patients.

Single level expression and co-expression of B7 and TNFR superfamily co-inhibitory and co-stimulatory molecules on T cell subsets were quantified by flow cytometry in matched urine, tumour, NT tissue and PBMC obtained from all patients. Flow cytometric analysis revealed remarkable similarities in the mean distribution of checkpoints amongst effector CD8+ and CD4+ cells and Treg cells present in UDLs, tumour and NT tissue compared to PBMC in both NMIBC and MIBC (Figure 3.7).

Significantly higher levels of PD-1 were found on all T cell subsets within the urine, tumour and NT compared to PBMC in both NMIBC and MIBC. The co-stimulatory receptor 4-1BB was upregulated on Treg cells in urine and tumour compared to PBMC in both bladder subtypes. Moreover, increased ICOS and CTLA-4 expression on CD4eff and Treg cells in urine, tumour and NT was detected, as compared with PBMC (Figure 3.7 & 3.8) consistent with previous studies (192). However, ICOS is upregulated on CD8+ in NT and tumour compared to PBMC in NMIBC, while ICOS and CTLA-4 is upregulated on CD4eff in tumour compared to NT in MIBC. Of relevance, the overall pattern of immune checkpoint distribution between urine, tumour and NT tissue was consistent between NMIBC and MIBC (Figure 3.7 & 3.8).
Figure 3.8: The immune checkpoint landscape of the bladder immune microenvironment is significantly distinct to PBMC in NMIBC and MIBC. Displayed is the relative expression of immune checkpoint molecules on T cell subsets within urine, tumour, non-tumour tissue and PBMC in NMIBC (n=6) and MIBC (n=8). Violin plots represent median values with quartiles. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Having found key differences in the checkpoint distribution between urine, tumour and NT compared to PBMC, I compared the expression of multiple checkpoints overlay on CD8+ and CD4+ effector T cells to interrogate the phenotypic overlap between the bladder immune microenvironment. Data obtained from two representative patients of the different histological staging subtypes of NMIBC (BL 12) and MIBC (BL 33) was analysed using the visualization software: ‘Simplified Presentation of Incredibly Complex Evaluations’ (SPICE) (193). This mode of analysis showed a high degree of concordance in the co-expression phenotype of the T cell subsets in urine, tumour and NT compared to PBMC in NMIBC (Figure 3.9). However, the co-expression overlap in the T cell subsets of MIBC was higher in urine and tumour compared to NT, then PBMC (Figure 3.9).
Overall, the intra-tumoural T cell subsets in both NMIBC and MIBC exhibit a characteristic T cell checkpoint phenotype that are distinct from PBMC.

Figure 3.9: SPICE analysis demonstrating that co-expression overlay of the checkpoint markers are distinct in the tumour microenvironment compared to PBMC. SPICE analysis of all T cells subsets displaying the co-expression of checkpoint molecules in urine, tumour, NT and PBMC in a representative patient of NMIBC (BL 12) and MIBC (BL 33). Pie charts depict qualitative distribution of checkpoint expressions on each T subset. Arcs show checkpoint makeup and overlap within pie slice.
3.2.4 ICOS is upregulated in the non-tumour tissue in NMIBC compared to MIBC in all T cell subsets

Having demonstrated that the bladder immune microenvironment is distinct from the PBMC, I set out to see if there are checkpoint markers that are characteristic of the NMIBC and MIBC bladder subtypes that could serve as an immune-oncology target.

PD-1 is also significantly upregulated in PBMC of CD4eff and Treg in patients with MIBC. This important co-inhibitory checkpoint molecule, alongside CTLA-4, is typically expressed on chronically stimulated CD8⁺ T cells (63, 194, 195). 4-1BB was significantly higher in the urine of NMIBC, while there was a trend towards 4-1BB Treg expression in the NT tissue (Figure 3.10). However, the most striking checkpoint was ICOS, which was significantly upregulated in the non-tumour tissue in NMIBC compared to MIBC in all T cell subsets. Interestingly, there was a trend towards increased CD8⁺ ICOS⁺ in urine and tumour in NMIBC (Figure 3.10).

ICOS is a co-stimulatory checkpoint and plays an important role in regulation of cell proliferation and immune responses (52, 196). The higher levels of ICOS, which are associated with T-cell mediated anti-tumour response, seen in non-tumour tissue of NMIBC compared to MIBC may explain why only 20% of NMIBC progresses to MIBC (197).

ICOS has previously been described to be seen on CD4⁺ in bladder cancer (192), but not on non-tumour bladder urothelium. Figure 3.11A shows representative histograms of ICOS gating on the same patient above, NMIBC (BL 12) and MIBC (BL 33). Although the mean fluorescence intensity (MFI) of ICOS across NMIBC is seemingly higher compared to MIBC (Figure 3.11B), the compensation for both patients were done on different days and therefore cannot be formally compared. However, the MFI of ICOS in non-tumour tissue of the MIBC patient is strikingly lower compared to tumour and urine on all T cell subsets. Representative dot plots of ICOS expression in NT tissue in these two patients are shown in Figure 3.11B.
Figure 3.10: ICOS is upregulated in NMIBC compared to MIBC in NT tissue in all T cell subsets. Displayed is the relative expression of activation and proliferation markers on each T cell subsets, (A) CD8^+; (B) CD4eff and (C) Treg, comparing NMIBC (n=6) and MIBC (n=8) within each tissue compartment. Bars represent mean values with SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001
Figure 3.11: ICOS gating in non-tumour tissue in NMIBC and MIBC.

(A) Representative histograms depicting the gating of positive and negative populations of cells expressing ICOS within urine, tumour, non-tumour (NT) tissue and PBMC according to bladder subtypes. (B) ICOS expression in NT tissue in NMIBC (BL 12) and MIBC (BL 33) in each T cell subset. Dot plots display the phenotype of each T cell subset from a representative patient in the NMIBC and MIBC group.
3.2.5 Detection of viable CD3⁺ T lymphocytes in the urine of NMIBC and MIBC

While there are several published studies on urinary lymphocytes in patients with NMIBC following intravesical BCG immunotherapy (174-176), little is known about MIBC. Here, I also detected urine derived lymphocytes (UDL) not only in 5 of 6 patients with NMIBC, but also in 6 of 8 patients with MIBC (Figure 3.12). The median urine count/ml was 34.9 (range 2.57 – 870.0) in the NMIBC cohort and 96.4 (range 0.66 – 4164.8) in the MIBC cohort.

![Figure 3.12: Detection of viable CD3⁺ T lymphocytes in the urine of NMIBC and MIBC.](image)

The proportion of CD8⁺ (blue), CD4⁺ FoxP3⁻ (CD4eff; green) and CD4⁺ FoxP3⁺ (Treg; yellow) cells present within the viable CD3⁺ gate in the tumour, non-tumour tissue (NT) and peripheral blood mononuclear cells (PBMC) is shown. Each individual patient’s histological diagnosis, pathological tumour stage and urine count/ml is shown. Legend: BCG, Bacillus Calmette-Guerin; CIS, carcinoma in situ; NMIBC, non-muscle invasive bladder cancer MIBC, muscle invasive bladder cancer.

As a control, I also analysed the urine of an age-matched cohort of six healthy volunteers being investigated for haematuria (Table 3.2). Importantly, there was an absence of lymphocytes within the urine of these healthy volunteers with no known bladder pathology, with a median urine count/ml was 1.11 (range 0.2 – 2.76).
<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Amount (ml)</th>
<th>Number of viable CD3</th>
<th>Urine count/ml</th>
</tr>
</thead>
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<tr>
<td>Female</td>
<td>28</td>
<td>40</td>
<td>8</td>
<td>0.2</td>
</tr>
<tr>
<td>Male</td>
<td>67</td>
<td>50</td>
<td>26</td>
<td>0.52</td>
</tr>
<tr>
<td>Male</td>
<td>68</td>
<td>40</td>
<td>24</td>
<td>0.6</td>
</tr>
<tr>
<td>Female</td>
<td>67</td>
<td>40</td>
<td>65</td>
<td>1.625</td>
</tr>
<tr>
<td>Male</td>
<td>49</td>
<td>50</td>
<td>134</td>
<td>2.68</td>
</tr>
<tr>
<td>Male</td>
<td>37</td>
<td>50</td>
<td>138</td>
<td>2.76</td>
</tr>
</tbody>
</table>

Table 3.2: UDLs are found at very low levels in age-matched patients with no known bladder pathology.

UDL analysis of a selected group of age-matched healthy patients being investigated for haematuria with no known bladder pathology as determined at cystoscopy. The number of viable CD3 is the number of events recorded within the viable CD3\(^+\) gate on the flow cytometer. UDL count per ml is the number of viable CD3 per ml of urine collected.

Finally, to optimise the quantity and viability of UDL for T cell analysis, I demonstrated in one patient that UDLs are best obtained as soon as possible (Figure 3.13A), preferably within 2 hours of collection as previously described (198). Stachowski and co also demonstrated that collection of urine directly into 30% fetal bovine serum (FBS) yield the best UDL. I confirmed this finding, as well as an additional quick spin of 400G for 5 minutes is essential to remove the supernatant for best viable UDL (Figure 3.13B & 3.13C). This is important for enriching for total number and percentage of UDL (Figure 3.13C). Lastly, I showed that staining fresh UDL compared to frozen is vital in T cell flow cytometric analysis (Figure 3.13D). Due to limited samples of UDLs from patients, I could only perform this experiment on one patient where I had access UDLs.
Figure 3.13: UDLs are best isolated within 2 hours of collection, in 30% FBS with a fast spin step and fresh.

(A) UDL CD3 count from the same patient (n=1) processed within 2 hours or after 7 hours showed a dropped in count by almost 50% (N = 1).  

(B) Displayed bar graph of each T cell subset counts in each condition, i) no addition of FBS with a quick spin step, removing supernatant before the ficoll step; ii) addition of 30% FBS media with no spin step before ficoll; and iii) addition of FBS direct into urine with a quick spin step, removing supernatant before ficoll. FBS, fetal bovine serum.  

(C, D) Dot plots displayed in each condition with or without FBS or a spin step (C) and in fresh or frozen staining (D).
3.3 Chapter discussion

It is widely accepted that depletion of Treg cells in the tumour results in preferential expansion of effector T cells, thereby increasing the intra-tumoural effector/Treg ratio (189, 190, 199). In this first result chapter, I find a significantly higher CD8/Treg ratio in the non-tumour tissue compared to tumour in the MIBC, which is evident on IHC as well. The lower CD8/Treg ratio in the tumour signifies the imbalance between effector CD8+ and Treg in a proliferating tumour, highlighting the importance of Tregs regulating the anti-tumour activity. Interestingly, there was no difference in the effector/Treg ratio for NMIBC. The mean CD8/Treg ratio in NMIBC was 46.5 in NT compared to 30.8 in tumour, while in MIBC, the ratio was 42.3 in NT and 23.1 in tumour (Figure 3.2). Whilst the NT CD8/Treg ratio is comparable in both groups, the higher CD8/Treg ratio in tumour of NMIBC could be explained by a less aggressive antigenic tumour as well as a sampling issue as the tumour in NMIBC is generally smaller than MIBC, and more NT tissue could have been sampled.

Using granzyme B as a surrogate for cytotoxicity, CD25 for activation and Ki67 for proliferation, I hypothesise that I would see more activation and cytotoxic activity in the tumour microenvironment due to recent T cell to cancer antigen recognition. However, in this small group of patients, there was no difference in all the tissue compartments, including PBMC in both NMIBC and MIBC. When cytotoxic T cells eliminate cancer target cells, they release contents of secretory lysosomes containing perforin and GzmB (191). Therefore, GzmB could have been degranulated when these experiments were done. Moreover, there are several other granzyme molecules, including granzyme A which have possible function significance in cancer immunity (191), which was not examined in this study. Effector T cells transiently upregulate CD25 over a short few days when activated (169), which may not be present when samples were taken. Interestingly, CD25 expression is highest on regulatory T cells which is consistent with work published by our group (200), showing that CD25 is a good immuno-oncology target in both NMIBC and MIBC. Lastly, this was in a very small group of patients and will need larger numbers to conclude if there was truly no difference between the tissue compartments.

Despite the small cohort of patients studied and the variability in histological subtypes, infiltrating T cell subsets within the bladder tumour microenvironment were found to exhibit a different immune checkpoint phenotype compared to PBMC. Moreover, high levels of PD-1 were found on all T cell subsets within the bladder tumour microenvironment, suggesting chronic antigenic stimulation and impaired T cell function (65). I also observed an increased in ICOS and CTLA-4 expression on CD4eff and Treg
cells within the bladder tumour microenvironment. This similar expression pattern has been previously documented in bladder cancer CD4⁺ TILs following anti-CTLA-4 therapy (192). This vital checkpoint molecule profiling may identify potential checkpoint targets that may be clinically relevant for the use in NMIBC and MIBC, and also enables clinicians and pharmaceutical companies to design rational combinatory therapeutic strategies with these CPIs. Hence from my data, there is strong indication to use a CPI targeting the PD-1 and CTLA-4 axis in both NMIBC and MIBC.

ICOS expression is upregulated on CD8⁺ and CD4⁺ T cells through TCR engagement and co-stimulation of CD28 upon activation, and ICOS plays a key role in T cell activation and proliferation of these cells (52, 196). Therefore, local treatment to the bladder like intravesical BCG could affect the expression on NT tissue. The ICOS expression on NT tissue of the 2 NMIBC patients who had recent BCG was 20.4% and 43% on CD8, 54% and 60.1% on CD4eff, while in the naïve patients, there was a range from 12.1% to 43.7% on CD8 and 34.7% to 85.6%. This suggest that the high ICOS expression on NT tissue is unlikely due to local intravesical BCG treatment, in this very small subset of patients. The high differential expression of ICOS in the NT tissue of NMIBC compared to MIBC may be due to a biological effect. Although some MIBC came from NMIBC, it is likely that they develop secondary to different genetic molecular alterations, with NMIBC described as arising from epithelial hyperplasia, whereas MIBC may have developed from hyperplasia, associated with genetic instability (201), where there may be more T cell regulation in the tumour microenvironment in MIBC, and thereby promoting tumourigenesis. However, these results above warrant further validation in a larger cohort of patients.

Finally, I was able to detect urinary lymphocytes in patients with NMIBC, confirming studies in this group of patients (174-176). I was also able to detect viable urinary CD3⁺ T cells in patients with treatment naïve MIBC. In depth characterization of checkpoint molecule characterisation has not been performed prior to my work in patients with MIBC. Moreover, the extent to which urinary derived markers reflect the tumour immune microenvironment remains unknown in MIBC. Therefore, I decided to focus on the MIBC group to answer these questions, as well as to increase the power of the study. Additionally, the sampling of MIBC tumours was more available and feasible as the sampling of the NMIBC tumour is more challenging due to the small amount of tumour tissue present compared to MIBC. In the next chapter, I will evaluate the phenotype of urinary derived lymphocytes (UDLs) in order to determine if the T cell landscape is reflective of the bladder tumour microenvironment.
Chapter 4: Deciphering the immune landscape of the bladder immune microenvironment in MIBC

4.1 Chapter introduction

This chapter’s results were generated as part of a collaboration with Dr Kroopa Joshi from Professor Benny Chain and Professor Sergio Quezada’s lab. The work from this chapter was published in Journal of Experimental Medicine in September 2018 as co-first authors (YNS Wong*, K Joshi*) (202).

Despite recent clinical successes and advances of checkpoint inhibition in bladder urothelial carcinoma, only about 25% of patients respond to these therapies (46, 47, 50). Research have mainly been aimed towards the characterisation of the tumour immune microenvironment to help better understanding of drug resistance, determine the best rational combinatory therapeutic strategies with immunotherapeutic drugs and discover novel immuno-oncology (IO) targets. At present, much attention has focused towards tumour mutational load, PD-L1 expression, and intra-tumoural T cell infiltration within tumour biopsy specimens taken before and during therapeutic intervention (11-14, 144, 145). Nonetheless, access to longitudinal tumour biopsies prior to and during the course of therapy in the most patients, remains a key limitation due to the invasiveness of such procedures (146-149).

As discussed in Chapter 3, the urine is a rich source of tumour-derived material that could potentially serve as a window to bladder tumour immune microenvironment in patients with bladder cancer. Urinary cell-free DNA has previously been shown to mirror the bladder tumour genomic microenvironment (164) and urinary circulating tumour DNA (ctDNA) (165) has been correlated to metastatic relapse in bladder cancer. Moreover, increased numbers of urinary lymphocytes have been demonstrated after intravesical BCG immunotherapy administration in patients with NMIBC (174-176). Comprehensive profiling of the expression of actionable immuno-oncology (IO) targets or their association with clinical outcome was not performed in any of these studies and patients with MIBC were not studied. Additionally, the extent to which urinary derived markers reflect the tumour immune microenvironment remains unknown.

In this chapter, I set out to establish the following objectives:

i. Detect viable CD3+ T lymphocytes in the urine of both treatment naïve and patients who has had prior therapeutic intervention in MIBC
ii. Map the same immune checkpoint molecules in Chapter 3 (PD-1, CTLA-4, ICOS, 4-1BB), in addition to another co-inhibitory checkpoint molecule, T cell immunoglobulin and mucin-domain-containing-3 (TIM-3) in order to identify more potential IO targets as well as discovering key checkpoint phenotype to identify the source of urine derived lymphocytes (UDLs)

iii. Evaluate the T cell receptor (TCR) repertoire of UDLs in patients with MIBC

iv. Correlate identified immune checkpoint phenotype to survival outcome in this cohort of patients
4.2 Results

4.2.1 Viable CD3\(^+\) T lymphocytes are detected in the urine of a heterogeneous cohort of patients with MIBC

I collected pre-cystectomy urine sample from 32 patients undergoing surgery with curative intent on the day of cystectomy. The most common histological subtype in the patients studied (27/32) was transitional cell carcinoma (TCC) and 5/32 patients were diagnosed with squamous cell carcinoma (SCC) (Figure 4.1). In this cohort, 13 patients were treatment naïve (all with primary bladder tumours at surgery) and 19 patients received previous systemic (neo-adjuvant chemotherapy or anti PD-L1) or intravesical therapy in the preceding six months. Of the 19 patients who received prior therapy, 7 patients developed a complete pathological response that had been downstaged to pT0 disease (6/7) or carcinoma in situ (CIS; 1/7). The remaining 12 patients had remaining pT2 – T4 disease at cystectomy (no complete pathological response; Figure 4.1 & 4.2).

![Figure 4.1: Viable urinary CD3\(^+\) lymphocytes are detected in a heterogeneous cohort of MIBC patients](image-url)

The proportion of CD8\(^+\) (blue), CD4\(^\text{eff}\) (green) and Treg (yellow) cells present within the viable CD3\(^+\) gate in the urine is shown. Samples containing fewer than 200 viable CD3\(^+\) on flow cytometry were deemed unevaulable for further T cell subset analysis and are highlighted in grey (not evaluable). Each patient’s prior treatment history, histological diagnosis, complete pathological response to therapy, clinical outcome (recurrence or death), pathological tumour stage and urine count per millilitres (ml) is shown. No complete pathological response was defined as presence of pT2 – T4 disease. A complete pathological response was defined as pT0 disease. Legend: BCG, Bacillus Calmette-Guerin; ml, milliliters; NA, not applicable; NAC, neo-adjuvant chemotherapy; PD-L1, programmed death-ligand 1; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma.
Figure 4.2: Proportion of T cell subsets within CD3⁺ T lymphocytes in matched tumour, non-tumour tissue and peripheral blood mononuclear cells.

The proportion of CD8⁺ (blue), CD4eff (green) and Treg (yellow) cells present within the viable CD3⁺ gate in the tumour, non-tumour tissue (NT) and peripheral blood mononuclear cells (PBMC) is shown. Each individual patient’s prior treatment history, histological diagnosis, pathological response to therapy, clinical outcome (recurrence or death) and pathological tumour stage is shown. No pathological response is defined as having stable disease or progressive disease at time of cystectomy. Patients with a pathological response are defined as either partial or complete responders. Legend: BCG, Bacillus Calmette-Guerin; NA, not applicable; NAC, neo-adjuvant chemotherapy; PD-L1, programmed death-ligand 1; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma.
Tumour specimens from a total of 25 patients with primary bladder tumours present at cystectomy were analysed (13 treatment naïve and 12 patients following previous therapy). As mentioned in Chapter 3, samples containing fewer than 200 viable CD3$^+$ cells (as determined by flow cytometry; Figure 4.1 & 4.2) were considered non-evaluable for subsequent T cell subsets and checkpoint analyses. In total, 24 urine, 25 tumour, 31 non-tumour bladder tissue (NT) and 32 PBMC samples were analysed in the study.

The median volume of urine collected from this cohort was 50 milliliters (ml) (range 15 – 250ml), with a median viable CD3$^+$ count per ml of 48.3 (range 0.1 – 4928.9) (Figure 4.1). I was able to identify 200 or more viable CD3$^+$ UDLs in 20/25 (80%) of patients with primary bladder tumours present (at least pT2) and 4/7 (57.1%) patients who developed a complete pathological response, irrespective of prior therapy, histology, complete pathological response and stage (Figure 4.1 & 4.2). CD3$^+$ UDLs were further divided into CD8$^+$, CD4eff and Treg subsets as shown in Chapter 3 (Figure 3.1), with matched TILs, non-tumour tissue lymphocytes and PBMC as shown in Figure 4.2. These T cell subsets were gated based upon CD3$^+$ within the lymphocyte gate, excluding doublets and non-viable cells as previously shown in Chapter 3 (Figure 3.1). Likewise, to exclude the possibility that UDL counts were associated with urinary infection, patients recruited to the study with a urine dipstick test positive for nitrites were excluded from the cohort.

4.2.2 The single immune checkpoint landscape of the bladder immune microenvironment is not largely unique between tissue compartments

I next sought to determine whether the immune checkpoint landscape of CD8$^+$, CD4eff, and Treg in UDLs would faithfully represent that of lymphocytes found within bladder tumour microenvironment and NT based upon the gating strategy in Figure 3.1. In addition to the key co-inhibitory and co-stimulatory immune checkpoints of PD-1, CTLA-4, ICOS and 4-1BB that was analysed in Chapter 3, I added a vital co-inhibitory checkpoint, TIM-3 in this chapter’s analysis as it has been associated with impaired T cell function and chronic antigenic stimulation (64).

Flow cytometric analysis of these five checkpoint molecules revealed largely remarkable similarities in the distribution of checkpoints amongst effector CD8$^+$ and CD4$^+$ cells and Treg cells present in UDLs, tumour and NT tissue that were significantly different to PBMC independent of whether the patients had prior treatment, which is similar to the description of NMIBC and MIBC in Chapter 3 (Figure 4.3). Therefore, moving forward, I will only discuss the results in the bladder microenvironment as the PBMC is distinctly different.
**Figure 4.3:** The mean percentage immune checkpoint expression in urine, tumour and non-tumour differs from PBMC independent of prior therapy.

Heatmap depicts the mean percentage of CD8$^+$, CD4eff and CD4$^+$ FoxP3$^+$ cells expressing individual immune checkpoint molecules in urine, tumour, non-tumour and PBMC samples obtained from (A) treatment naïve patients (n=13), and (B) patients that received prior systemic therapy (n=19).

**Figure 4.4:** The single checkpoint landscape of the bladder immune microenvironment is not unique between each tissue compartments.

Displayed is the relative expression of immune checkpoint molecules on T cell subsets within urine, tumour and non-tumour tissue according to treatment history. Violin plots represent median values with quartiles.

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Of relevance, the overall pattern of immune checkpoint distribution between UDLs, tumour and NT tissue was consistently similar regardless of prior therapeutic intervention (Figure 4.3 & 4.4), except a significantly higher levels of PD-1 expression on the effector CD4\(^+\) and CD8\(^+\) T cells within the tumour compared to NT in patients who had prior therapy. Moreover, high levels of ICOS and CTLA-4 expression on CD4\text{eff} and Treg cells in UDLs, TILs and NT were detected (Figure 4.3 & 4.4), consistent with previous studies (192). Notably, ICOS was significantly higher in tumour compared to NT in the effector T cells. There was no difference in the checkpoint expression on Treg, except the co-stimulatory receptor 4-1BB, which was significantly upregulated in tumour compared to NT in treatment naïve patients. Lastly, TIM-3 was upregulated in both Treg and CD8\(^+\) cells predominantly within TILs and UDLs (Figure 4.3 & 4.4). I also observed high levels of TIM-3, ICOS and CTLA-4 in addition to PD-1, on UDLs and TILs from patients with SCC (Figure 4.5), although this was largely insignificant.

![Diagram](image_url)

**Figure 4.5: The immune checkpoint landscape of bladder squamous cell carcinoma.**
Relative expression of T cell subset immune checkpoint molecules within urine, tumour and non-tumour tissue in patients with SCC (n=5). Bars represent mean values with SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001
To further delineate between the tissue compartments, principal component analysis (PCA) was used as an unsupervised technique to further analyse the tumour and urine. The PCA values represent a summary of the frequencies of immune checkpoints for the T cell subsets studied in the matched tumour and urine samples for each patient. These PCA values showed a positive correlation with a trend towards statistical significance (Spearman's Rank correlation co-efficient 0.53; P = 0.057; Figure 4.6).

![Principal component analysis showed a trend towards positive correlation in the immune checkpoints for all T cell subsets in matched tumour and urine samples.](image)

*Figure 4.6: Principal component analysis showed a trend towards positive correlation in the immune checkpoints for all T cell subsets in matched tumour and urine samples.*

Displayed is a principal component analysis (PCA) using an unsupervised technique to analyse the relationship between the immune checkpoint phenotypes observed in the tumour and urine. The PCA values represent a summary of immune checkpoint frequencies for each of the T cell subsets studied in matched tumour and urine samples for each patient (N=14). Spearman rank correlation co-efficient (R) and P values shown.

### 4.2.3 The effector UDLs exhibit a similar co-expression checkpoint landscape to TILs independent of prior therapy

Having found no distinct differences in the checkpoint landscape within the bladder immune microenvironment, I compared the expression of multiple checkpoints on CD8$^+$ and CD4$^+$ effector T cells to interrogate the phenotypic overlap between TILs and UDLs, compared to NT tissue. Using all possible co-expression checkpoint phenotypes of CD8$^+$ and CD4eff, an unsupervised hierarchical clustering analysis of CD8$^+$ and CD4eff checkpoint expression showed that UDLs and TILs had a more similar co-expression checkpoint landscape compared to NT (Figure 4.7). To ascertain the checkpoint phenotype most likely to account for the clustering of TILs and UDLs, I focused on the two checkpoint co-expression phenotypes with the largest delta in mean frequency between TILs and NT (Table 4.1). Within CD8$^+$ T cells, I found that the hierarchical clustering of UDLs and TILs was predominantly driven by PD-1/TIM-3, followed by PD-1/ICOS co-expression on CD8$^+$ T cells (Table 4.1A). In contrast, the largest delta observed between effector CD4$^+$ T cells within TILs and UDLs was largely accounted for by ICOS/CTLA-4, followed by CTLA-4/TIM-3 co-expression (Table 4.1B).
Figure 4.7: Effector UDLs and TILs have a similar in co-expression checkpoint phenotypes. Displayed is an unsupervised clustering heatmap of the mean frequencies of CD8⁺ (left) and CD4eff (right) T cells demonstrating each of all possible permutations of co-expression immune checkpoint phenotypes found most commonly in tumour, urine and non-tumour tissue. Each row within the heatmap represents a different immune checkpoint co-expression phenotype. Key represents the markers that define each of the co-expression phenotypes; presence of marker (black filled circles) or absence of marker (white circle). Only CD8⁺ and CD4eff co-expression phenotypes found at a frequency of 1% or more (mean across all patients) in any of tumour, urine or non-tumour are shown.

Table 4.1: The largest delta difference between tumour and non-tumour (NT) checkpoint co-expression phenotypes within the effector T cells in descending order. Displayed are the top five co-expression phenotypes that have the highest mean percentage difference between tumour and NT on CD8⁺ (A) and CD4eff T cells (B). The differences in the frequencies of CD8⁺ and CD4eff T cells with each of these phenotypes are displayed in descending order.

In line with the above, the frequency of CD8⁺ cells co-expressing PD-1/TIM-3 was significantly higher in UDLs and TILs as compared with NT tissue (Figure 4.8). Importantly, no significant difference was observed in the frequency of CD8⁺ co-expressing PD-1/TIM-3 between UDLs and TILs (Figure 4.8) suggestive of chronic antigenic stimulation of CD8⁺ cells found within the tumour and urine, compared to NT. Amongst the effector CD4⁺ T cell compartment, the frequency of cells co-expressing ICOS and CTLA-4 was similar in UDLs and TILs (Figure 4.8).
Figure 4.8: Effector UDLs and TILs are not significantly different in the most common co-expression checkpoint phenotypes.

Graph depicts the frequency of CD8$^+$ T cells that co-express PD-1 and TIM-3 (left) and CD4eff T cells that co-express ICOS and CTLA-4 (right) in tumour, non-tumour and PBMC samples. P values were calculated using paired t-test. Bars represent mean values with SD. *P <0.05; **P <0.005.

Figure 4.9: Graphical analysis of effector UDLs exhibit a similar co-expression checkpoint landscape to TILs independent of prior therapy.

SPICE analysis of all CD8$^+$ (A) and CD4eff (C) T cells displaying co-expression of checkpoint molecules in each compartment in a representative group of patients (BL 33 - TCC treatment naïve; BL 55 - SCC treatment naïve; BL 42 - TCC neo-adjuvant immunotherapy). Pie charts depict qualitative distribution of checkpoint expressions on CD8$^+$ and CD4eff. Arcs show checkpoint makeup and overlap within pie slice. Dot plots display the co-expression phenotype of (B) CD8$^+$ PD-1/TIM-3 and (D) CD4eff ICOS/CTLA-4 from the same patients. The percentage of cells expressing each combination of checkpoint molecules is shown.
The data obtained from three patients selected as representative of the different histological subtypes and treatment histories in this cohort (BL 33, transitional cell carcinoma (TCC) treatment naïve; BL 55, squamous cell carcinoma (SCC) treatment naïve; BL 42, TCC neo-adjuvant anti-PD-L1) was analysed using the visualization software: ‘Simplified Presentation of Incredibly Complex Evaluations’ (SPICE) (193). This different mode of analysis also showed a high degree of concordance in the co-expression phenotype of CD8+ (Figure 4.9A) and CD4eff (Figure 4.9C) amongst UDLs and tumour with remarkably high levels of PD-1/TIM-3 co-expression on CD8+ (Figure 4.8 & 4.9B) and ICOS/CTLA-4 co-expression on CD4eff (Figure 4.8 & 4.9D).

The findings above suggest that the UDL population may derived from cells shed from the tumour into the urine. However, whether they are transcriptomically or functionally similar is to be investigated.

4.2.4 The T cell receptor (TCR) repertoire of UDLs reflects the intra-tumoural repertoire

Two T cells expressing the same TCR sequence are likely to be part of the same clone, since each TCR sequence is produced at very low probability (203). The TCR repertoire therefore provides a powerful way to investigate how two T cell populations are related. We therefore sequenced the TCR repertoires of UDLs, tumour, NT tissue and PBMC in a subgroup of patients with matched RNA available. These experiments were done by Dr Kroopa Joshi and supervised by Professor Benjamin Chain. A strong correlation was observed between the number of alpha and beta TCRs sequenced, reflecting the reproducibility of the sequencing method (Spearman’s Rank correlation co-efficient 0.96, P<0.0001). Therefore, only beta TCR data will be shown in this chapter.

The beta TCR abundance distribution (i.e. the proportion of the repertoire which is made up of TCRs which occur only once, twice, three times etc.) of the four tissue compartments combined from all the patients is shown (Figure 4.10). The clonal structure of the TCR repertoire was similar across tumour, urine, non-tumour and PBMC samples, therefore any differences observed in the TCR landscape of these compartments was unlikely due to differences in the distribution of the repertoire of T cells.
Figure 4.10: The clonal structure of the TCR repertoire is similar across the tissue compartments. The distribution of beta chain TCRs in the urine is similar to tumour, non-tumour and PBMC. The proportion of TCRs found with different abundances (x axis) in urine, tumour, non-tumour (NT) and PBMC is shown.

Figure 4.11: Hierarchical clustering of CDR3s demonstrates similarity in the TCR repertoire of urine and tumour. CDR3s were filtered based on abundance (present at least 8 times) and detected in at least one of urine, tumour or NT tissue. Colour key represents the proportion of each CDR3. Legend: B, blood; NT, non-tumour; T, tumour; U, urine.

Having noted a similar TCR distribution across all of the compartments studied, we next examined the relationship between the repertoires at the level of individual complementary determining region 3 (CDR3) sequences. Unsupervised hierarchical clustering of CDR3 frequencies for each individual patient reproducibly clustered the urine TCR sequences with the tumour TCR sequences (Figure 4.11).
We modelled the urine as a subsample of one of the other compartments, in order to confirm the statistical significance of this relationship. We repeatedly sampled from each repertoire, and measured the similarity of each sample with an equal sized sample from the urine repertoire (see Material and Methods for details of the similarity metric). This bootstrap approach allowed us to compare the similarity between UDL and the other samples quantitatively. The similarity of urine to the tumour was much larger than to either NT tissue or blood (p < 0.0001), whether using the Jaccard index, which measures the number of shared sequences without regard to abundance, or the similarity metric, which considers the abundance of the shared CDR3s (Figure 4.12).

![Figure 4.12: High Jaccard index and similarity metric between the TCR repertoire of urine and tumour.](image)

The overlap, quantified by the Jaccard index (left), between the set of CDR3s found in urine and in each of the other compartments is shown. Similarity index (right) between samples of the urine TCR repertoire and the repertoire of each other compartment. The results show the mean plus standard deviation. The significance was measured by one-way Anova. ****: P <0.0001.

An important source for cellular immunotherapy of cancer are the expanded T cell clones within tumour. We therefore examined whether TCRs found at high abundance within the tumour could also be found within urine, since urine would potentially provide an easier source of such cells for adoptive therapy protocols. A number of the 10 most abundant TCRs in tumour were indeed found within urine. Strikingly, few of the expanded CDR3 sequences in urine were found amongst the 10 most expanded CDR3 sequences in NT tissue and PBMCs (Figure 4.13).

![Figure 4.13: High Jaccard index and similarity metric between the TCR repertoire of urine and tumour.](image)

Taken together, these data demonstrate that the urine contains a population of lymphocytes that closely reflect the tumour infiltrating TCR repertoire and are suggestive of similar antigenic specificities of UDLs and TILs.
4.2.5 Increased urinary CD3⁺CD8⁺PD-1⁺ lymphocytes are associated with a worse clinical outcome in patients with MIBC

Given that the T cell checkpoint phenotype and TCR repertoire highlights the role of UDLs as a surrogate for TIL phenotype and TCR repertoire, and that TILs have previously been associated with survival in various solid tumours (188), I sought to determine whether the presence of T cells in the urine was associated with tumour stage, response and/or recurrence-free survival in this cohort of patients. The 32 patients were followed up for a median time of 8 months, with 3-monthly scans in the first year post-operatively.

Firstly, I assessed whether the UDL count, taken on the day of cystectomy (number of events recorded within viable CD3⁺ gate on the flow cytometer) correlated with pathological tumour stage and treatment response as a low pathological stage and response to neo-adjuvant therapy are associated with improved recurrence free survival (204). I utilized a standardized measure of UDL count (per ml of urine) given the variation of urine volumes collected within the patient cohort. Although a similar UDL concentration was found for patients with T0, T2 or T3 disease, a significantly higher UDL concentration was found in patients with T4 tumours compared to T0 tumours (median 150.6 vs 5.45 UDL count per/ml, P = 0.009) suggesting that locally advanced tumours may be more likely to generate UDLs (Figure 4.14A). I also observed that those patients that achieved a complete pathological response to systemic therapy had a significantly lower UDL count on the day of cystectomy as compared with patients that were treatment naïve (median 5.45 vs 194.1 UDL count per/ml, P = 0.03; Figure 4.14B). However, there was no
statistically significant difference in the UDL count amongst patients that had primary tumours present at surgery (treatment naïve versus non-responding patients). In addition, no significant difference in UDL count was observed between patients that achieved a complete pathological response and those that did not. I next evaluated whether UDL count could be associated with disease recurrence. I found that disease recurrence was significantly associated with a higher UDL concentration detected at the time of cystectomy (1003 vs 17.82 UDL count/ml, P = <0.0001) (Figure 4.14C).

![Figure 4.14: High UDL count/ml is present in higher disease stage and recurrence.](image)

Displayed is the association of urinary lymphocyte count with clinical outcome. (A and B) UDL count/ml and relationship with pathological tumour stage (A) and response to treatment (B) is shown. (C) The association of disease recurrence and UDL count is shown. Mann-Whitney U test used for statistical analysis. Error bars represent mean values with SD.

When dividing patients into two groups based on the median UDL count, I observed that patients with a low UDL count (below the median) did not develop recurrence of their disease (Figure 4.15), in contrast to patients with a high UDL concentration that had a poorer recurrence free survival (UDL count above the median; P = 0.0004). Next, when considering UDL count as a continuous variable and performing a natural log transformation (Figure 4.16), the unadjusted hazard ratio (HR) was calculated as 1.80 (95% confidence interval (CI) 1.22 – 2.63, P = 0.003). Given the potential confounding effect of tumour stage and treatment response on the UDL count, I performed statistical adjustment for these variables using a Cox regression model (Figure 4.16). Notably the association between higher UDL count/ml and a poorer recurrence free survival remained statistically significant when adjusted for pathological tumour stage (adjusted HR 1.49; 95% CI 1.02 – 2.17, P = 0.04), or treatment response with an adjusted HR of 1.61 (95% CI 1.06 – 2.46, P = 0.026). The multivariate analysis when stratified for both tumour stage and response was not statistically significant, with an adjusted HR of 1.43 (95% CI 0.92 – 2.21, P = 0.108).
Figure 4.15: Increased UDL count/ml is associated with a worse outcome in patients with MIBC.
Recurrence-free survival over a median follow up of 8 months is shown according to whether patients were found to have a high UDL count (above the median) or a low UDL count (below the median).

<table>
<thead>
<tr>
<th>UDL count/ml (natural log)</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDL count/ml unadjusted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall effect</td>
<td>1.80 (1.22;2.66)</td>
<td>0.003</td>
</tr>
<tr>
<td>UDL count/ml adjusted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted by tumour stage</td>
<td>1.49 (1.02;2.17)</td>
<td>0.040</td>
</tr>
<tr>
<td>Adjusted by response</td>
<td>1.61 (1.06;2.46)</td>
<td>0.026</td>
</tr>
<tr>
<td>Adjusted by tumour stage &amp; response</td>
<td>1.43 (0.92;2.21)</td>
<td>0.108</td>
</tr>
</tbody>
</table>

Figure 4.16: Forest plot showing high UDL count/ml is associated with increased risk of recurrence.
A forest plot using a natural log transformed UDL count/ml as a continuous variable is shown (unadjusted). A cox regression model is used with UDL count/ml adjusted for pathological tumour stage and treatment response is demonstrated. Legend: CI, confidence interval; HR, hazard ratio; ml, millilitres.

Having observed that patients with an increased urinary CD3+ count were more likely to experience disease relapse, I next assessed whether there were any checkpoint phenotypes that may account for this result. I did not observe any significant differences in survival when considering urinary CD3+PD-1+ or CD8+PD-1+TIM-3+ cells. Previous work has shown that CD8+ TILs with the highest expression of PD-1 is associated with
tumour reactivity, reflective of chronic antigenic stimulation resulting in a dysfunctional and exhaustive state (37-41). In line with previous work (39), I classified CD8⁺PD-1⁺ T cells into high and low categories; cells considered to be PD-1 hi were those with expression levels of PD-1 expression greater than that observed in matched PBMC samples (Figure 4.17). I next considered whether an increased frequency of levels of PD-1 hi CD8⁺ UDLs was related to survival.

Interestingly, I observed that the frequency of CD8⁺PD-1 hi cells was significantly higher in UDLs (P = 0.01) and TILs (P = 0.0004) as compared with NT tissue (Figure 4.18A). Importantly, no significant difference was observed in the frequency of CD8⁺PD-1 hi cells between UDLs and TILs (Figure 4.18A) suggestive of chronic antigenic stimulation of these cells. The natural log UDL count/ml significantly correlated with CD8⁺PD-1 hi expression in both UDLs (Spearman’s Rank correlation co-efficient 0.62; P = 0.0012; Figure 4.18B) and TILs (Spearman’s Rank correlation co-efficient 0.68; P = 0.0002; Figure 4.18C).

Finally, I demonstrated that patients with increased levels of CD8⁺PD-1 hi cells (above the median) were more likely to develop recurrence of their disease compared to those with low levels of CD8⁺ PD-1 hi (below the median), with a highly significant P-value of 0.0009 (Figure 4.19), with an unadjusted HR of 4.3 (95% CI 1.02 – 18.23, P = 0.048).

Taken together, these data highlight the potential prognostic role of the UDL count and CD8⁺ phenotype in patients with MIBC that requires validation in a prospective study.
Figure 4.18: CD3+CD8+PD-1hi is present more in urine and tumour with high correlation to UDL CD3 count/ml.

(A) Graph depicts the frequency of CD8+PD-1hi T cells in tumour, urine, and NT tissue samples. Mann-Whitney U test used for statistical analysis. Error bars represent mean values with SD. (B) Displayed is a Spearman rank correlation of the frequency of CD3+CD8+PD-1hi in UDL (%) and the natural log UDL CD3 count/ml in urine samples. Spearman rank correlation coefficient and P values shown. (C) Displayed is a Spearman rank correlation of the frequency of CD3+CD8+PD-1hi in TIL (%) and the natural log UDL CD3 count/ml in urine samples. Spearman rank correlation co-efficient (R) and P values shown.

Figure 4.19: Increased urinary CD3+CD8+PD-1hi lymphocytes are associated with a worse clinical outcome in patients with MIBC.

Recurrence-free survival (%) over a median follow up of 8 months is shown according to whether patients had a high frequency of PD-1hi (above the median) or a low frequency of PD-1hi (below the median). *, P < 0.05; **, P < 0.005; ***, P < 0.0005; ****, P < 0.0001; NS, not significant for A–C and E.
4.3 Chapter discussion

My results show that UDLs in patients with MIBC exhibit a T cell checkpoint phenotype and TCR repertoire that overlaps with lymphocytes infiltrating the bladder tumour microenvironment. Strikingly, this resemblance was seen in a heterogeneous group of 32 patients undergoing cystectomy that included patients with different stages of disease, treatment histories and histological subtypes. Furthermore, I demonstrated that patients with a high UDL count/ml were more likely to relapse of bladder cancer, reflecting the potential prognostic value of UDL analysis.

It is possible that UDLs are derived from the tumour tissue itself, given the resemblance in the co-expression T cell checkpoint landscape and TCR repertoire between UDLs and TILs, as well as the absence of UDLs in the urine of healthy individuals (Table 3.2), although the mechanisms by which lymphocytes enter the urine remain elusive. Interestingly, the density of intratumoural tissue resident memory CD103+ T cells was inversely associated with tumour size and was not an independent predictor for OS or RFS in patients with MIBC compared to NMIBC (205); whether this was associated with UDL count/ml remains undetermined. Factors that may explain the release of TILs into the urine include the tumour invasion extent, necrosis or microvessel density, resulting in exfoliation tumour cells, and thereby leakage of infiltrating lymphocytes into the urine, all of which would be worthy of future study.

The collection of peripheral blood samples in an attempt to gain insight into the evolving bladder tumour microenvironment is typically included in current clinical studies. Remarkably, my data demonstrate minimal phenotypic and repertoire overlap between PBMC and TILs and instead suggest that UDLs better recapitulate the bladder T cell and immune checkpoint landscape that may be relevant for future clinical studies. However, note that chemotherapy is known to cause lymphopenia in peripheral blood, which may indirectly influence UDL count. The correlation between absolute lymphocyte count in peripheral blood and UDL count was not studied in this chapter as data was not collected.

The overlap between TILs and UDLs was well exemplified by similarly high levels of PD-1/TIM-3 CD8+ co-expression in both compartments; a phenotype associated with impaired T cell function and chronic antigenic stimulation (64, 65). Similar levels of ICOS and CTLA-4 co-expression on CD4eff within TILs and UDLs was also observed. This pattern of expression has been documented previously in urothelial cancer CD4+ TILs following anti-CTLA-4 therapy (192). The phenotypic and repertoire concordances between TILs and UDLs across treated and untreated patients support the use of UDLs
in the longitudinal evaluation of the bladder tumour immune microenvironment, for example in the context of immunotherapeutic clinical trials.

UDL analysis may also be used to explore the checkpoint landscape throughout therapy, given the array of checkpoint molecules expressed on the surface of lymphocytes within the urine, with the potential to inform subsequent actionable IO targets in patients with MIBC. Given the high percentage of TIM-3, CTLA-4 and ICOS co-expressed with PD-1 on effector T cell subsets within the tumour microenvironment, further investigation exploring the therapeutic targeting of these molecules, either alone or in conjunction with PD-1/PD-L1 blockade is warranted. A high level of TIM-3, ICOS and CTLA-4, in addition to PD-1 was observed on UDLs and TILs from patients with SCC. Of relevance, this is a histological subtype largely excluded from clinical trials of CPI, with limited treatment options in the clinical setting. In a neoadjuvant study looking at pembrolizumab, which included patients with predominant variant histology involving >50% of the tumour specimens, found that 6/7 patients with predominant SCC had downstaging to pT1 or less (121). Taken together with my findings, this suggest that patients with SCC may benefit from immunotherapy directed against these targets and may warrant inclusion in future clinical trials.

In addition to checkpoint molecules, the use of TCR repertoire analysis of UDLs may be used as a tool to track T cell dynamics throughout immunotherapy, providing insight into the specificity of immune response. Whether RNA from UDLs could also be used to characterize and unveil changes in the transcriptional program of tumour reactive T cells through therapy remains to be determined.

The demonstration that the UDL phenotype and repertoire overlaps with tumour lymphocyte populations, and that the number of UDLs is related to disease outcome suggests that UDL analysis may complement the use of urinary ctDNA in patients with bladder cancer, in combination providing insight into both the immune and genetic landscape within the tumour microenvironment.

My findings also demonstrated that patients were 1.8 times more likely to relapse of bladder cancer for every log unit increase in UDL count. Moreover, I found that an increased UDL count was associated with pathological tumour stage and response to systemic therapy highlighting the intricate relationship between UDL count, tumour stage and treatment response. Notably the association between higher UDL count/ml and a poorer RFS remained statistically significant when adjusted for pathological tumour stage or treatment response. Whilst increased intra-tumoural T cell infiltration has
previously been associated with improved clinical outcome in a variety of tumour types including MIBC (206), my data suggest that the presence of UDLs are associated with a worse clinical outcome. Importantly, UDL count and TIL count are two separate entities, and an increased TIL count may be reflective of an ongoing anti-tumoural immune response as previously described thus associated with a favourable clinical outcome (188).

The biological significance of an increased UDL count was subsequently explored to gain a better understanding of the possible underlying mechanism(s) that may explain the observed relationship between increased UDL count and poorer clinical outcomes. Given that PD-1 expression has previously shown to identify tumour reactive T-cells (14, 65, 207) and T cells with the highest level of PD-1 expression are associated with distinct transcriptomic, phenotypic and functional properties (38-40, 208), we next assessed the PD-1 expression levels of UDLs and correlated this with clinical outcome. I observed a similar frequency of CD8\(^+\)PD-1\(^{hi}\) cells in urine and tumour samples, which was significantly higher than non-tumour tissue. Additionally, a significant positive correlation was observed between UDL count/ml and the frequency of CD8\(^+\)PD-1\(^{hi}\) UDLs and TILs. The mechanism underlying this observation currently remains unclear, but may be reflective of i) tumour inflammation, ii) expansion and/or differentiation of CD8\(^+\)PD-1\(^{hi}\) cells, in response to chronic tumour antigen exposure, and iii) loss of basement membrane integrity during antigen specific T cells responses in the bladder.

Importantly, I found that patients with increased frequency of CD8\(^+\)PD-1\(^{hi}\) UDLs were more likely to experience disease relapse, highlighting the potential clinical relevance of CD8\(^+\)PD-1\(^{hi}\) UDLs that requires further prospective validation. Moreover, increased PD-1 expression consistent with chronic antigenic exposure and T cell dysfunction has previously been correlated with worse disease-free survival in head and neck cancer (209). Whilst it is plausible that CD8\(^+\)PD-1\(^{hi}\) UDLs may point towards tumour reactivity, it is not possible to fully conclude this. Accordingly, further phenotypic and functional studies, including bulk RNA or single cell RNA sequencing aimed at evaluating the antigenic specificity and biological mechanisms leading to UDL generation warrant further investigation.

I recognize that my findings are based on a relatively small sample size of a heterogeneous cohort of patients in terms of histological subtype, treatment histories and treatment response. However, despite the marked heterogeneity, the flow phenotype and TCR repertoire remains similar between urine and tumour, suggesting that urinary lymphocytes are dynamic and may be able to recapitulate the tumour microenvironment.
at that time point. Further prospective studies in a larger cohort of patients, considering
the above, are required to corroborate my preliminary findings and validate the
hypothesis that UDL count and phenotype is prognostic in MIBC.
Chapter 5: Development of methods to evaluate tumour reactivity and functionality of UDLs

5.1 Chapter introduction

I have identified UDLs as a readily accessible source of T cells from patients with MIBC that accurately map the immune landscape and repertoire of lymphocytes within the tumour microenvironment within a heterogeneous cohort of patients. These findings warrant a trial in a larger independent cohort of patients to validate the use of UDL analysis as a novel, clinically useful liquid biopsy with potential prognostic implications. However, a key question remains, regarding the antigenic reactivity of UDLs. It is unknown as to whether they recognise tumour antigens and whether UDLs have any anti-tumour potential, specifically neoantigen reactive T cells (NARTs).

TILs contain a heterogeneous mixture of bystander and tumour-reactive T cells (210-212) and prospective clinical studies have suggested that enriching for tumour-reactive cells could enhance anti-cancer therapeutic responses (213-215). Several early phase clinical trials have confirmed objective responses in patients with metastatic melanoma when treated with TILs in combination with high dose IL-2, in lymphodepleting preconditioning (216-218). While there is evidence to demonstrate that UDLs have potential tumour reactivity with similar flow phenotype and TCR repertoire to TILs as set out in Chapter 4, these UDLs could also be bystander T cells. Therefore, it is vital to explore this further and test the reactivities of UDLs as they may provide a convenient and easily accessible source of cells for \textit{in vitro} expansion and subsequent adoptive cellular immunotherapy for patients with bladder cancer.

Cancer arises as a result of the accumulation of somatic mutations (93), which can be recognised by the immune system as being foreign. Over the last decade, there has been a paradigm shift in studies targeting neoantigens, as they are exclusively expressed in tumour cells. This makes them extremely appealing targets for immunotherapy as they should not lead to autoimmunity, compared to tumour associated antigens (TAAs), which are differentially expressed in tumour compared to normal tissues, as well as in the thymus resulting in central tolerance. Targeting neoantigens may result in anti-tumoural immune responses and have been shown to correlate with increased patient survival (10). Moreover, there has been several studies demonstrating that these neoantigens are critical for an effective anti-tumour T cell response to checkpoint inhibitors (CPI) (11-14). Therefore, if it is proven that expanded UDLs are
able to recognised tumour neoantigens, this would make UDLs a highly attractive and non-invasive source for cellular immunotherapy in urothelial cancer.

The presence of increased numbers of TILs has previously been associated with improved survival in various solid tumours (188), including improved clinical outcome in MIBC, in patients with increased intra-tumoural T cell infiltration as determined by IHC (206). In Chapter 4 of this thesis, the data suggested that the presence of UDLs is associated with a worse clinical outcome. Therefore, it is imperative to investigate whether there is a correlation with T cell infiltration on IHC and matched UDL count/ml in the group of patients studied in this thesis.

Finally, whilst \textit{in vitro} TIL expansion with high dose IL-2 is well established (216-218), whether these methods could be replicated to expand UDLs \textit{in vitro} is unknown. Hence, I set out to develop methods to expand these UDLs from patients with bladder cancer. Various conditions for \textit{in vitro} expansion were tested, importantly whether irradiated autologous tumour samples improved the expansion of UDLs. Following incubation either with or without autologous tumour samples, the UDLs were subjected to a modified version of the pre-Rapid Expansion Protocol (pREP), followed by the Rapid Expansion Protocol (REP) as described in Chapter 2.7, a method that has widely been used to expand TILs \textit{in vitro} (183). I set out to establish the following objectives:

i. To determine whether UDLs can be expanded with or without irradiated autologous tumour samples

ii. To test the antigen reactivity of UDLs using the following assays:
   a. FluoroSpot assay
   b. \textit{In vitro} functional assay
   c. Neoantigen peptide screen

iii. To evaluate the numbers of CD8^+ /CD4^+ /FoxP3^+ T cells using immunohistochemistry (IHC) in a group of patients with high UDL count/ml and low UDL count/ml
5.2 Results

5.2.1 Expansion of UDLs in the presence of autologous irradiated tumour results in an increased number of viable cells compared to media alone

Multi-parametric flow cytometric analysis on frozen UDLs unable to recapitulate the results seen in fresh samples due to insufficient viable cell numbers (see Chapter 3). However, whether these UDLs would revive and expand under high doses of IL-2 is yet to be evaluated. As discussed previously, urinary material mirrors the bladder tumour genomic microenvironment (164), and hence contains a tumour antigen enriched source of material for T cell recognition.

Patient BL 33’s tissue-specific lymphocytes from PBMC, TIL, UDL and non-tumour (NT) were used for my initial evaluation of expansion method. Equal number of viable cells (400,000) from each tissue-specific sample were plated on 24 well-plates with and without autologous irradiated 50 Gy tumour sample in a 1:5 ratio of irradiated tumour to lymphocytes. These samples were subsequently taken through a pre-rapid expansion (pREP) protocol (Methods section 2.7) with 6000 units of IL-2 and 25ng/ml of IL-21 for 3 weeks, with the aim to enrich for tumour reactive lymphocytes without using anti-CD3 in conventional REP expansion (183). Although in media alone, viable lymphocytes were expanded in PBMC and urine, with $1.06 \times 10^6$ and $9.74 \times 10^4$ of viable lymphocytes grown respectively, TIL and NT tissue lymphocytes failed to expand (Figure 5.1). However, expansion occurred in all tissue compartments when grown against autologous irradiated tumour sample. Therefore, I focussed on expanding UDLs against autologous irradiated tumour for all other patients’ samples going forth.

A total of five patients’ cryopreserved urine derived lymphocytes (UDL), alongside their matched tissue-specific samples, were thawed and taken through pREP with autologous irradiated tumour sample in a 1:5 ratio with IL-2 and IL-21 for 3 weeks. 60% of the patient’s UDLs (3/5) were successfully expanded (Table 5.1). Additionally, one patient (BL 5) had three timepoints (Cycle 1, Cycle 3 and end of treatment) taken through pREP. However, only one time point (Cycle 1) was successful in expanding lymphocytes. Although the aim of pREP is to enrich for tumour reactive lymphocytes, the median number of UDL expanded following pREP was $3.68 \times 10^5$ (range $9.7 \times 10^4$ – $1 \times 10^6$). Therefore, to ensure there are sufficient number of lymphocytes for further functional assays, the subsequent patients (BL 38 and BL 5) were taken through a round of rapid expansion protocol (REP) with high dose IL-2 of 6000 units and anti-CD3 (Methods section 2.7) for another three weeks (pREP/REP). This resulted in a total number of $5 \times 10^7$ of UDL expanded in BL 38 and $6 \times 10^7$ in BL 5.
Figure 5.1: Expansion against autologous irradiated tumour result in increased number of viable cells compared to media alone.

Graph displayed are matched samples from peripheral blood mononuclear cells (PBMC), tumour infiltrating lymphocytes (TIL), urine derived lymphocytes (UDL) and non-tumour lymphocytes expanded with or without autologous irradiated tumour sample (N = 1).

Table 5.1: pREP successfully expanded UDLs in some patients.
Table shown are patients’ UDLs that were successfully (✓) or unsuccessfully (x) expanded through the pre-rapid expansion (pREP) protocol with the total number of viable cells displayed at the end of the 3-week period. Legend: EOT, end of treatment; NA, not applicable; pREP, pre-rapid expansion protocol; REP, rapid expansion protocol
5.2.2 pREP and REP results in a heterogeneous expansion of CD3\(^-\) & CD3\(^+\) cells (including CD8\(^+\)/CD4\(^+\) T cells)

Both methods of pREP and REP against irradiated autologous tumour sample result in a heterogeneous expansion of CD3\(^-\) & CD3\(^+\) cells, including CD8\(^+\) and CD4\(^+\) lymphocytes (Figure 5.2) in the different tissue compartments. A similar finding has been reported by several groups in various solid tumours using high dose IL-2 expansion (183, 219, 220). pREP/REP preferentially expands CD8, with minimum CD4 expansion both in BL 5 and BL 38 in all tissue compartments. IL-21 was added in pREP, with a view to select for tumour specific effector T cells, to enhance CD8 cytotoxicity but also for the proliferation of CD4 T cells (221). In REP, only high dosage of IL-2 was used to maintain T cell proliferation and growth. When focussing on just UDL, the proportion of the total viable lymphocytes in the unexpanded sample does not determine the proportion of the final expanded populations in these three patients (Figure 5.3), with a Spearman’s Rank correlation co-efficient of 0.04 and a P value of 0.92. This is similar in PBMC (Spearman’s Rank correlation co-efficient of -0.02 and a P value of 0.62), TIL (Spearman’s Rank correlation co-efficient of 0.71 and a P value of 0.06) and NT tissue samples (Spearman’s Rank correlation co-efficient of 0.48 and a P value of 0.24).

![Graph showing pREP and REP results](image)

**Figure 5.2:** pREP and REP against irradiated autologous tumour sample results in a heterogeneous expansion of CD3\(^-\) & CD3\(^+\) cells (including CD8/CD4).

The proportion of CD8\(^+\) (blue), CD4\(^+\) (green), CD3\(^-\) double negative (DN; orange) and CD3\(^+\) (yellow) cells present within the total viable cells gate is shown in 3 patients. Legend: DN, double negative; NA, not applicable; PBMC, peripheral blood mononuclear cells; pREP, pre-rapid expansion protocol; REP, rapid expansion protocol; TIL, tumour infiltrating lymphocytes; UDL, urine derived lymphocytes.
Figure 5.3: The matched unexpanded viable lymphocyte subsets in UDL does not significantly correlate with the expanded subsets. The percentage of CD8+ (blue), CD4+ (green), CD3+ double negative (DN; orange) and CD3- (yellow) cells within the total viable cells gate in the matched unexpanded and expanded UDL are shown in 3 patients.

5.2.3 Expanded UDLs produced IFNg and GzmB when restimulated with autologous tumour sample in FluoroSpot assay

After 3 weeks of pREP, the expanded lymphocytes from BL 33 were starved of cytokines for 24 hours and a FluoroSpot assay was performed using a dual interferon gamma (IFNg) and Granzyme B (GzmB) capture plate using method described in section 2.8. 30,000 cells of each expanded tissue compartment were restimulated with media alone, anti-CD3 (aCD3), and patient’s autologous tumour sample (Figure 5.4). The FluoroSpot plate was read and images were scanned using the Bioreader 6000. Each fluorescent spot captured on the plate was counted utilising two filters in FITC and Cy3 wavelengths.

The GzmB interpretation was difficult as almost all expanded lymphocytes in all conditions were producing GzmB, including in the media alone condition. This is likely due to the effect of expansion resulting in high GzmB production as opposed to a true GzmB response due to T cells responding to stimuli. The interferon gamma plate did not have the same effect as GzmB, with expanded UDL producing the most IFNg compared to the other tissue compartments (Figure 5.5A).

Even in media alone with no stimuli, there were some IFNg, which is likely a sequela from the expansion. Therefore, to overcome this, I accounted for background IFNg production in media alone condition by looking at the delta change between each condition and background. Despite this confounding factor, UDL still produced the most IFNg when restimulated with autologous tumour (Figure 5.5B).
Figure 5.4: FluoroSpot of BL 33 using a dual detection IFNg and GzmB plate.

Figure above shows a FluoroSpot readout with interferon gamma (green dots; left) and granzyme B (orange dots; right) production. Each tissue type is labelled below each FluoroSpot image. The conditions for each tissue-specific sample are labelled on the far right: media alone, anti-CD3 (aCD3) and autologous target tumour. Legend: PBMC, peripheral mononuclear cells; TILs, tumour infiltrating lymphocytes; UDLs, urine-derived lymphocytes.
Figure 5.5: Expanded UDL produced the most interferon gamma when restimulated with autologous tumour in BL 33.

(A) Bar graph showing interferon gamma (left) and granzyme B (right) production by BL 33 (no cryopreservation) from each tissue compartment, restimulated in different conditions. (B) Bar graph showing IFNg production in each tissue-specific expanded lymphocytes after restimulation with autologous target tumour of BL 33 after correcting for background media alone production. Legend: aCD3, anti-CD3; IFNg, interferon gamma; PBMC, peripheral blood mononuclear cells; TIL, tumour infiltrating lymphocytes; UDL, urine derived lymphocytes.

To account for the high production of GzmB following expansion, the expanded lymphocytes of BL 38 were starved of cytokines for 24 hours and cryopreserved in -80°C for a month before the FluoroSpot assay was set up (Figure 5.6). There is still IFNg production even after cryopreservation when restimulated with autologous tumour, while the process of cryopreservation reduces GzmB production (Figure 5.7A). In order to overcome the background IFNg and GzmB production, likewise to BL 33, I looked at the delta change between each condition and background in BL 38. Whilst UDL produced the second most IFNg and GzmB when restimulated with autologous tumour, the dual production of IFNg and GzmB was seen most in expanded UDL (Figure 5.7B).

However, as discussed above, the expansion against irradiated autologous tumour sample resulted in a heterogeneous expansion of CD3⁺ & CD3⁺ cells (including CD8/CD4) as demonstrated in Figure 5.2. 30,000 viable cells were plated per well while plating the FluoroSpot plate not accounting if they were CD3⁺ & CD3⁺. Taken together from these two experiments, although expanded UDLs are able to produced IFNg and
GzmB when restimulated with autologous tumour, I was unable to ascertain if the IFNg and GzmB responses are truly T cell specific.

**Figure 5.6: FluoroSpot of BL 38 using a dual detection IFNg and GzmB plate.**
Figure above shows a FluoroSpot readout with interferon gamma (green dots; left) and granzyme B (red dots; right) production. The conditions for each tissue-specific sample are labelled on the far right. Legend: aCD3, anti-CD3, B, blood/peripheral mononuclear cells; N, non-tumour tissue; T, tumour infiltrating lymphocytes; U, urine-derived lymphocytes.
Figure 5.7: Expanded UDL produced the most dual expression of interferon gamma and granzyme B when restimulated with autologous tumour in BL 38.

(A) Bar graph showing interferon gamma (left) and granzyme B (right) production by BL 38 (cryopreservation) from each tissue compartment, restimulated in different conditions. (B) Bar graph showing IFNg, GzmB and IFNg/GzmB production in each tissue-specific expanded lymphocytes, restimulated with autologous target tumour of BL 38, and after correcting for background media alone production. Legend: aCD3, anti-CD3 GzmB, granzyme B; IFNg, interferon gamma; PBMC, peripheral blood mononuclear cells; TIL, tumour infiltrating lymphocytes; UDL, urine derived lymphocytes
5.2.4 Expanded CD8\(^+\) UDLs produced increased amounts of IFNg and TNFa when restimulated with autologous tumour samples

As I did not account for CD3\(^-\) & CD3\(^+\) viable cells while setting up the FluoroSpot assays, I set up another functional assay for BL 38 alongside the FluoroSpot for comparison as I had more expanded lymphocytes post pREP/REP. I incubated 100,000 cells of each expanded tissue-specific lymphocytes in media alone, soluble human anti-CD3/CD28/CD2, cytomegalovirus, epstein barr virus and influenza virus (CEF) mix and autologous target tumour cells for 16 hours with GolgiPlug protein transport inhibitor. My readout for this assay was IFNg and tumour necrosis factor alpha (TNFa). Expanded lymphocytes produce IFNg and TNFa when restimulated with autologous target tumour (Figure 5.8). Media alone and CEF condition did not produce any IFNg nor TNFa. In the aCD3 condition, there were minimum IFNg or TNFa in all tissue compartments except in PBMC. This is similar to the FluoroSpot result as demonstrated in Figure 5.4. Focusing on the restimulation with autologous tumour only, CD8\(^+\) in UDL produced the most IFNg, TNFa and IFNg/TNFa, while CD4 in NT lymphocytes produced the most IFNg, TNFa and IFNg/TNFa (Figure 5.9).

Figure 5.8: Expanded lymphocytes produced IFNg and TNFa when restimulated with autologous tumour.

Dot plots showing the expression of IFNg and TNFa in CD8 after restimulation with media alone or autologous target tumour from each tissue compartment (N = 1). Legend: IFNg, interferon gamma; PBMC, peripheral blood mononuclear cells; TIL, tumour infiltrating lymphocytes; TNFa; tumour necrosis factor alpha; UDL, urine derived lymphocytes.
Figure 5.9: Expanded UDL produces most interferon gamma and tumour necrosis factor alpha in CD8 after restimulation with autologous target tumour.

Bar graph showing the expression of IFNg and TNFa in CD8 (top) and CD4 (bottom) after restimulation with autologous target tumour from each tissue compartment (N = 1). Legend: IFNg, interferon gamma; PBMC, peripheral blood mononuclear cells; TIL, tumour infiltrating lymphocytes; TNFa; tumour necrosis factor alpha; UDL, urine derived lymphocytes.
5.2.5 Expanded UDLs from patient BL 5 do not demonstrate the presence of NARTs

BL 5 is a 35 years old female patient with metastatic urothelial carcinoma. Her tumour from transurethral resection of bladder tumour (TURBT) prior to starting first-line chemotherapy was sent for whole genome sequencing. Rachel Rosenthal from the Swanton laboratory performed the bioinformatics analyses, with details on the methodology published (14). BL 5’s tumour had 235 exonic mutations, of which 137 were non-synonymous mutations. Of these, 68 were clonal mutations and 14 subclonal mutations. The current bioinformatics neoantigen pipeline is only able to predicted against MHC class I, of which 270 predicted clonal neoantigens and 61 predicted subclonal neoantigens were identified. Synthetic peptides were purchased at Pepscan Presto, NL. BL 5 received six consecutive treatments with gemcitabine and cisplatin chemotherapy.

Using the same timepoint of the sequenced tumour, BL 5’s UDL was taken for a pREP for 3 weeks against autologous tumour, and then a round of REP for 3 weeks. There were $6 \times 10^7$ expanded lymphocytes at the end of this pREP/REP before cryopreservation the cells (Table 5.1). The majority of these expanded lymphocytes were CD8 and CD3+ CD8− CD4− cells (Figure 5.1). I incubated the expanded UDL in media alone, soluble anti-CD3/2/28, CEF and the 270 predicted clonal neoantigens for 16 hours with GolgiPlug protein transport inhibitor. The assay readouts were IFNg and tumour necrosis factor alpha (TNFa).

As the neoepitope pipeline was only predicted against MHC class I, further analysis will focus on CD8+ T cells only as well as there were no expanded CD4+ T cells (Figure 5.1). There were expanded CD8 in UDLs that produced up to 3.5-fold IFNg and TNFa when restimulated with clonal neoantigens, even when you remove background cytokine production (Figure 5.10). When expanded UDL was restimulated with the 270 clonal neoantigens individually, there was a median percentage IFNg expression of 11.07 (range 5.88 – 19.9), TNFa expression of 12.2 (range 7.32 – 24.07), double positive IFNg/TNFa expression of 7.7 (range 4.12 – 16.2). The median IFNg and TNFa expression of these clonal neoantigens were similar to anti-CD3/2/28 and viral CEF responses. The background media alone expression of IFNg and TNFa was the lowest (Figure 5.11).
Figure 5.10: Expanded UDL produces interferon gamma and tumour necrosis alpha when restimulated with clonal neoantigens.

Dot plots showing the expression of IFNg and TNFa in CD8 after restimulation with media alone, CEF, anti-CD3/2/28 and clonal neoantigens. A representative neoantigen IFNg and TNFa response shown. Legend: CEF, cytomegalovirus, epstein barr virus and influenza virus; IFNg, interferon gamma; TNFa; tumour necrosis factor alpha.

Figure 5.11: The median interferon gamma and tumour necrosis alpha expression are similar when restimulated with clonal neoantigens, CEF and anti-CD3/2/28.

Violin plots showing the expression of IFNg and TNFa in CD8 after restimulation with the 270 clonal neoantigens from BL 5, media alone, CEF and anti-CD3/2/28. Bars represent median (line) and quartiles (dash line). Legend: CEF, cytomegalovirus, epstein barr virus and influenza virus; IFNg, interferon gamma; TNFa; tumour necrosis factor alpha.

Although the median for the 270 clonal neoantigens are similar to the positive and viral controls, it is important to look at the neoantigens that produces the most IFNg or TNFa, as they could represent the key tumour neoantigens that T cells react to. After subtracting the background media alone expression of IFNg and TNFa, I looked further into the clonal neoantigens that produced the most IFNg and/or TNFa (Table 5.2). Interestingly, there were three peptides in the top 10 responses that were previously found either in BL 5’s TILs (LEMLDFFDMLW) or PBMCs (ILCLLEMSFEV, MTCELHLICSV). Moreover, peptide ILCLLEMSFEV and MTCELHLICSV were detected at several timepoints in her...
PBMC post chemotherapy (See Chapter 6). Therefore, to confirm if these were real responses, I sent the expanded UDLs to the Hadrup’s laboratory for NART screening. Only HLA A02.01, HLA A26.0, HLA B07.02 and HLA B44.02 were screened (n = 179), HLA C07.02 and HLA C05.01 were not screened (n = 91). Unfortunately, no NARTs were detected from this screen.

<table>
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</table>

Table 5.2: Top neoantigens IFNg and TNFa responses.
Table shown are the top neoantigens IFNg, TNFa and IFNg/TNFa responses and the ranking of the other respective responses. Highlighted in grey are the peptides previously detected in PBMC and TILs by the Hadrup group (See Chapter 6).

5.2.6 Patients with high UDL had significant number of CD8 and CD8/CD4 ratio per mmSq compared to patients with low UDL
I found that high UDL count/ml and CD8\(^{+}\) PD-1\(^{+}\) were associated with worse outcome in Chapter 4. On the contrary, groups have found that the presence of TILs is associated with improved survival (188, 206). As UDLs and TILs have very similar phenotypic markers and TCR repertoire, it is crucial to understand the origins and biology of UDLs in relation to TILs. Therefore, I set out to look at a group of patients with high UDL count/ml (N = 4) and recurrence, and patients with low UDL count/ml (N = 2) and no recurrence from the same cohort. Multiplex triple immunohistochemistry (IHC) staining of CD8/CD4/FoxP3 were done by Ayse Akarca from the Marafioti lab (Figure 5.12).
Definiens Tissue Phenomics software platform was used for automated counting of the triple IHC staining using a previously described machine learning-based parameter-free cell segmentation (184). The whole tumour was segmented out using a matched pan cytokeratin stained slide. The stromal, peri- and intra-tumoural segments were not separated from this analysis. I found that patients in the high UDL group had significantly highly number of CD8 compared to the low UDL group (P <0.005, Figure 5.1). There was no difference in the CD4 nor FoxP3 in both groups, with a trend towards higher total T cells in the high UDL group which is likely due to CD8 T cells. A significant high CD8/CD4 ratio (P<0.005, Figure 5.14) is seen in the high UDL group compared to the low UDL group, and interestingly there was no significant difference in the CD8/FoxP3 ratio between both groups (Figure 5.14).

Figure 5.12: Triple multiplex immunohistochemistry showing T cell distribution in a patient with low UDL count and high UDL count.
Triple multiplex IHC demonstrating the CD8, CD4 and Foxp3 distribution in a patient with low UDL count (A) and another patient with high UDL count (B). CD8 (red), CD4 (brown), FoxP3 (blue).
Figure 5.13: CD8 in the tumour was significantly higher in the high UDL group. 
Displayed shown are the total number of T cells (CD8/CD4/FoxP3), CD8, CD4 and FoxP3 per mm_Sq in 
the tumour in low UDL group (number of slides = 3) and high UDL group (number of slides = 10). Mann-
Whitney U test used for statistical analysis. Error bars represent mean values with SD, **P < 0.005.

Figure 5.14: CD8/CD4 ratio in the tumour was significantly higher in the high UDL group. 
Displayed shown are the ratios of CD8/CD4, CD8/FoxP3 and CD4/FoxP3 in the tumour in low UDL group 
(number of slides = 3) and high UDL group (number of slides = 10). Mann-Whitney U test used for statistical 
analysis. Error bars represent mean values with SD, **P < 0.005.
5.3 Chapter discussion

Expansion of UDLs have previously been published previously, although these UDLs were stimulated directly in vitro (174). In this chapter, I developed methods for expansion of UDLs from cryopreservation and was able to perform functional in vitro assays with these expanded cells. I found that expanded UDLs produced IFNg and GzmB, both via a FluoroSpot assay and an in vitro functional assay. However, whether these UDLs are truly tumour reactive remains uncertain.

Whilst using human bladder samples in in vitro experiments may be more clinically relevant than mice or established cell line models of bladder cancer, the major limitations are the limited and heterogenous patient samples, as well as the difficulty in replicating these experiments due to lack of samples. Therefore, any conclusions drawn in this chapter have to be interpreted with caution.

While cryopreservation of TILs is a commonly used laboratory method, research groups have found that this process can affect the quality of the samples following thawing (222). In Chapter 3, I showed that flow cytometric staining of fresh UDLs compared to frozen UDLs is vital in T cell flow cytometric analysis (Figure 3.13D), with only 2.18% viable T cells detected from a single cryopreserved sample. Despite this, the pREP REP method was able to expand UDLs in a select group of patients. Several groups have found expansion of TILs is possible after cryopreservation (223, 224). At present, I am unable to predict which patients’ UDLs post cryopreservation may expand and this needs to be further investigated. Also, expansion against irradiated autologous tumour samples resulted in more than 10-fold expansion in numbers, signalling how important the presence of tumour antigens is for T cell expansion.

Importantly, after accounting for background change, UDL still produced IFNg and GzmB when restimulated with autologous tumour sample, suggesting that some of these UDLs may be tumour reactive. However, as discussed above in Chapter 5.2.2, the expansion methods resulted in a heterogeneous expansion of CD3− & CD3+ cells. For the FluoroSpot assay, 30,000 viable cells were plated per well, not accounting for CD3+ specific viable cells, nor CD8+ or CD4+. Moreover, this expansion method is known to expand CD3− CD56+ natural killer (NK) cells, CD3+ CD56+ NKT cells and CD3+ CD8− CD4− gamma delta T cells (225). These cells are also known to produce both IFNg and GzmB (226, 227). Therefore, even in the same patient, the differential population of cells between the tissue compartments could lead to varying results of IFNg and GzmB production. For both BL 33 and BL 38, expanded UDLs had the least CD8+, while PBMC
had the most. Hence from the FluoroSpot assays, I cannot confidently conclude that these expanded UDLs are more tumour reactive compared to expanded lymphocytes from PBMC or NT, particular in BL 33. One of the ways that this could be overcome is to repeat this experiment either by pre-sorting CD3+ T cells or to add an MHC I blocking antibody as a control in one of my conditions, which would suggest that any IFNg and/or GzmB production in this condition is likely via the NK pathway.

Subsequently, I performed an in vitro assay with BL 38, which showed that CD8+[113] in UDLs produced the most IFNg, TNFa and IFNg/TNFa when restimulated with autologous tumour (Figure 5.7), suggesting that perhaps most of these expanded UDLs contain tumour reactive lymphocytes. Unfortunately, one of the biggest limitations with these patient-derived samples is that samples are limited meaning that systematic optimisation of the experimental conditions are restricted, making interpretation somewhat difficult sometimes. Nonetheless, more experiments utilising more patients' samples are needed to verify if UDLs are truly tumour reactive.

The objective of pREP/REP is to expand tumour reactive lymphocytes with the aim to be able to use this as an adoptive cellular therapy. By expanding UDLs against irradiated autologous tumour samples, I aimed to select for tumour reactive lymphocytes. Moreover, the addition of IL-21 in the pREP method was to select for tumour specific effector T cells, to enhance CD8 cytotoxicity but also for the proliferation of CD4 T cells (221, 228). pREP and REP methods resulted in a heterogeneous expansion of CD3- & CD3+ cells, including CD8+ and CD4+, and this does not correlate with the starting populations of lymphocytes. This heterogeneity has been shown in other studies with a differential expansion of CD8+ and CD4+, even when derived from the same patient (183, 219, 229). The variability of each patient's autologous tumour sample between each culture may allow for unpredictable or sporadic advantage to certain cell populations at the time of culture initiation, resulting in a heterogenous mix of expanded lymphocytes. Other modified versions of the expansion method that other groups have used to expand specifically tumour reactive lymphocytes include co-stimulation through 4-1BB (76, 229) or blocking PD-1 (230), which could be adopted in the future.

The phenomenon of intratumour heterogeneity within solid tumours and their metastases is well recognised (231-234). Increased responsiveness to CPI in tumours enriched for clonal neoantigens has previously been shown (14). Using a similar pipeline as described in Methods 2.6, 270 clonal neoantigens were predicted from a patient's bladder tumour (BL 5). Although the median IFNg and TNFa response from CD8+ UDL was similar to positive and viral controls, some expanded CD8+ UDLs had up to 3.5-fold
IFNg and TNFa response to specific clonal neoantigens, which could represent NARTs. However, no NARTs were detected in this patient’s urine sample, which could have been due to a variety of reasons. Firstly, approximately a third of the predicted clonal neoantigens were not screened as a number of the HLA monomers could not be synthesised as the specific HLA type could not be generated. Secondly, this expansion method may not have been optimum in expanding specifically tumour reactive T cells, particularly NARTs. Andersen and co have demonstrated that rapid expansion decreases the frequency of tumour specific T cells (235), and that perhaps unexpanded materials are needed to detect NARTs. Thirdly, the bioinformatics prediction pipeline only included strong predicted binders and perhaps some key neoantigens were not synthesised and screened. Moreover, there are many other endogenous factors including nonpeptide cleavage probability, transporter associated with antigen processing transport efficiency, peptide expression level, mutation allele fraction, and neoantigen cellular prevalence that could be affected. Finally, it remains a possibility that UDLs may not be tumour antigen reactive. Nonetheless, this is a single patient experiment and more experiments are needed to make a conclusion.

Finally, I had demonstrated in Chapter 4 that high UDL count was associated with a worse recurrence free survival in patients with MIBC undergoing a radical cystectomy. This could be seen to be in contrast to previous published data which have demonstrated that high TILs are associated with improved survival in various solid tumours (188). I therefore assessed the relationship between TIL and UDL abundance in a proportion of the MIBC cohort. Although the patient numbers were small in this analysis, high CD8+ TILs were associated with high UDL count although no relationship with UDL count was seen in with the total TIL or CD4+ TIL groups. It is critical to distinguish between i) the ratio between the effector and regulatory T cells, ii) the localisation of these immune cells to the cancer cells, and iii) the activation status of the effector immune cells. Although CD8 TILs in MIBC have been shown to be predictive of survival on IHC (206), it is worth noting that Sharma et al. looked specifically only at intratumoural TILs, while in my analysis, I did not differentiate between stromal, peritumoural, and intratumoural TILs, which is a limitation of my analysis. Finally, I identified CD8 PD-1hi as a prognostic marker in my cohort of patients. The high number of CD8 on these IHC could be PD-1hi, suggesting a chronically activated and exhausted phenotype. Therefore, further phenotyping of these CD8+ T cells and their exact proximity and location within the tumour is vital in understanding the biology and origins of UDLs. The PD-1 staining on these IHC slides are ongoing and we await the data from this cohort of patients.
Chapter 6: Using non-invasive methods to track the immune landscape of metastatic patients on therapy

6.1 Chapter introduction

Several groups are striving to identify predictive biomarkers of response to checkpoint inhibition (CPI) in many solid tumours. There have been many reports on genomic and immune predictors of response based on baseline tumour samples taken prior to systemic therapy (11, 13, 147, 236). However, these markers are not perfectly predictive with a substantial overlap between responders and non-responders to CPI. Chen and co found that immune signatures in on-treatment tumour biopsies are highly predictive of response with non-overlapping immune signatures in responders compared to non-responders (146). Nonetheless, access to longitudinal tumour biopsies prior to and during the course of therapy in the most patients, remains a key limitation due to the invasiveness of such procedures.

Given the invasive nature of these tumour biopsies, several clinical studies in solid tumours are collecting peripheral blood samples with the view to gain insight into the dynamic immune tumour microenvironment (237). Although the peripheral blood is an excellent non-invasive source of potential biomarkers, none so far has been validated as a predictive biomarkers of response to CPI in prospective studies (238). Moreover, several groups have reported that the immune profile of peripheral blood is distinct to that seen within the immune tumour microenvironment (195, 212). Taken together, it is imperative to find a non-invasive window to the tumour microenvironment that may provide insight into the dynamic and evolving nature immune tumour microenvironment.

I have demonstrated that urine derived lymphocyte (UDL) and tumour infiltrating lymphocyte (TIL) from patients with MIBC have a remarkably similar immune checkpoint phenotype and TCR repertoire. Although it is important to track these patients on neoadjuvant therapies, the treatment window of these therapies is usually completed within 9 weeks. Importantly, UDL analysis is potentially most relevant in patients who have not undergone cystectomy in the metastatic setting, as the bladder is still present. It is estimated that approximately 60% of patients with metastatic bladder cancer present with de novo metastatic disease with the primary bladder cancer in situ (239). Additionally, about 25% of patients with metastatic bladder cancer would have received bladder sparing primary therapy such as radiotherapy, with a proportion likely to have tumour relapse within the bladder. Thus, UDL analysis, is not only likely to have utility in the assessment of patients with muscle invasive localized disease but could be important
for the longitudinal evaluation patients with metastatic bladder cancer with their primary bladder cancer *in situ* undergoing systemic therapies.

For this chapter, I have collected longitudinal peripheral blood and urine samples from a group of patients with metastatic bladder cancer undergoing systemic anti-cancer therapy, including chemotherapy or CPI. I was able to correlate the immunophenotyping results from the urine and blood with re-staging CT imaging.

I set out the following objectives in this chapter:

i. To detect UDLs in patients with metastatic bladder cancer longitudinally throughout systemic anti-cancer therapy

ii. To evaluate the changes in immune checkpoint landscape with therapy and correlate this with response to treatment

iii. To correlate UDL count/ml and CD8 PD-1\(^{hi}\) as found in Chapter 4, as well as other identified immune checkpoint phenotypes with to survival outcomes in this cohort of patients

iv. To determine whether we can identify neoantigen reactive T cells (NARTs) in PBMC
6.2 Results

6.2.1 Baseline characteristics and clinical variables of the metastatic cohort

A total of 9 patients with metastatic urothelial carcinoma were followed up through the course of their treatment or until censored date (Table 6.1). There were 3 females and 6 males in this cohort, of which 5 were on Gemcitabine/Cisplatin chemotherapy and 4 were on an immuno-oncology (IO) therapy targeting the PD-1/PD-L1 axis (atezolizumab and pembrolizumab). These patients had at least two sequential PBMC and UDL samples collected over their treatment course. The median number of urine samples collected was six cycles (range 2 – 7). There were 3 responders, defined as partial response (n=2) and stable disease (n=1) after treatment and 6 patients who progressed during the course of this treatment. Of the 6 patients who progressed, 5 had a response CT scan halfway through treatment course before progression, including one patient who had a RECIST immune stable disease (iSD; Figure 6.1) (240). The median response in these 5 patients was 3 months (range 1 - 4 months). Of the 3 responders, two had local treatment to their bladder after systemic therapy, while one is still having ongoing treatment, with continued response.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Type of treatment</th>
<th>Line of treatment</th>
<th>Type of metastases</th>
<th>No. of urine samples collected</th>
<th>Treatment response</th>
<th>PFS (Months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL 5</td>
<td>F</td>
<td>Gem/Cis</td>
<td>First-line</td>
<td>Visceral</td>
<td>7</td>
<td>Progression</td>
<td>5</td>
</tr>
<tr>
<td>BL 40</td>
<td>M</td>
<td>Gem/Cis</td>
<td>First-line</td>
<td>Visceral</td>
<td>4</td>
<td>Response</td>
<td>34</td>
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<td>Gem/Cis</td>
<td>First-line</td>
<td>Visceral</td>
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<td>Progression</td>
<td>3</td>
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<tr>
<td>BL 49</td>
<td>M</td>
<td>Gem/Cis</td>
<td>First-line</td>
<td>Visceral</td>
<td>6</td>
<td>Response</td>
<td>28</td>
</tr>
<tr>
<td>BL 72</td>
<td>M</td>
<td>Gem/Cis</td>
<td>First-line</td>
<td>Visceral</td>
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<td>Progression</td>
<td>5</td>
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<tr>
<td>BL 61</td>
<td>F</td>
<td>Atezolizumab</td>
<td>Second-line</td>
<td>Visceral</td>
<td>2</td>
<td>Progression</td>
<td>6</td>
</tr>
<tr>
<td>BL 64</td>
<td>F</td>
<td>Pembrolizumab</td>
<td>First-line</td>
<td>Visceral</td>
<td>6</td>
<td>Response</td>
<td>23</td>
</tr>
<tr>
<td>BL 65</td>
<td>M</td>
<td>Pembrolizumab</td>
<td>First-line</td>
<td>Visceral</td>
<td>5</td>
<td>Progression</td>
<td>7</td>
</tr>
<tr>
<td>BL 68</td>
<td>M</td>
<td>Pembrolizumab</td>
<td>First-line</td>
<td>Visceral</td>
<td>6</td>
<td>Progression</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 6.1: Clinical characteristics of metastatic bladder patient cohort.
Baseline patient characteristics, treatment and response history of the metastatic cohort. Legend: Cis, cisplatin; Gem, gemcitabine; IO, immunotherapy; PFS, progression free survival; UDL, urine-derived lymphocytes
Figure 6.1: Swimmers plot of patients’ response to treatment.
Displayed is a swimmer’s plot demonstrating the metastatic cohort of patients’ response to treatment over time (months) since starting treatment.
6.2.2 Single immune checkpoint and activation expression in UDL is not significantly associated to treatment response or progression

Having previously described the checkpoint landscape of T lymphocytes in the urine of patients with MIBC (Chapter 4), I sought to determine whether the immune checkpoint landscape of CD8\(^+\), CD4\(^{eff}\), and Treg in UDLs would differ in the metastatic cohort. I have focused my analysis on the expression of the same key co-inhibitory and co-stimulatory immune checkpoints as set out in previous chapters including PD-1, TIM-3, CTLA-4, ICOS and 4-1BB.

Flow cytometric analysis demonstrated striking similarities in the distribution of checkpoints amongst the effector CD8\(^+\) and CD4\(^+\) cells and Treg cells present in UDLs between the metastatic and MIBC cohort (Figure 6.2), which was statistically not significant. Of relevance, the overall pattern of immune checkpoint distribution in the urine remains largely consistent regardless of therapeutic intervention (baseline compared to end of treatment (EOT) in metastatic cohort; naïve compared to treated in MIBC cohort), which was also seen in the MIBC group in Chapter 4. EOT is defined as when patient has completed their treatment, progressed or the last UDL collected while on treatment. The immune checkpoint expression in PBMC is less upregulated compared to UDLs in the metastatic cohort, likewise to the MIBC cohort in Chapter 4.

![Figure 6.2: The UDL immune checkpoint expression does not differ between metastatic or MIBC.](image)

Heatmap depicts the UDL mean percentage of CD8\(^+\), CD4\(^{eff}\) and Treg cells expressing individual immune checkpoint molecules from metastatic cohort (n=9) at baseline and treatment naïve patients (n=13), and the end of treatment (EOT) samples from the same metastatic cohort and patients that received prior systemic therapy (n=19). Legend: EOT, end of treatment; MIBC, muscle invasive bladder cancer.
Figure 6.3: Single immune checkpoint and activation expression in UDL is not associated to treatment response or progression.

Displayed are violin plots showing the relative expression of immune checkpoint markers, activation and proliferation markers on T cell subsets in response to treatment. Each dot is a representative patient. All comparisons are not significant except for those labelled. Violin plots show the median values with quartiles.

*P < 0.05
I next sought to see if the immune landscape differs between UDLs of patients who had a response or progression. The immune checkpoint landscape is largely similar between the T cell population, with a trend towards the PD-1 expression on Tregs in patients who have progressed (P = 0.06). There was also a statistically significant increase in CD25 on CD8$^+$ and Ki67 in CD4eff in patients who had a response scan.

6.2.3 Presence of increased UDL count/ml, effector PD-1$^{hi}$ T cells and PD-1/TIM-3 at end of treatment are associated with worse clinical outcome

Previously I described that patients with high urinary UDL count/ml and CD8 PD-1$^{hi}$ had a worse recurrence free survival in the MIBC cohort. I sought to see if this was the same in the metastatic cohort. At baseline, there was a trend towards CD8 PD-1$^{hi}$ in patients who has progressed (P = 0.09), with a significantly worse progression free survival (PFS) in patients with high CD8 PD-1$^{hi}$ above the median (P = <0.005) in baseline UDL samples. Otherwise, there was no statistically significant difference between UDL count/ml, PD-1$^{hi}$, TIM-3 and PD-1/TIM-3 at baseline in all other UDL T cells subsets between patients who responded or progressed, nor any significance in PFS (Table 6.2).

Focussing now on EOT UDL analysis, patients with disease progression were found to have a significantly higher UDL count/ml or CD8/CD4eff PD-1$^{hi}$ at EOT (Table 6.2 & Figure 6.4). Moreover, patients with UDL count/ml or effector PD-1$^{hi}$ above the median at EOT do significantly worse (Figure 6.4). This highlights that high UDL count/ml or high effector T cells PD-1$^{hi}$ at baseline does not predict response to treatment nor outcome, but the UDL count/ml and effector PD-1$^{hi}$ at EOT coincides with response as well as predicts outcome. Presence of PD-1/TIM-3 in CD8 or CD4eff at EOT also predicts worse outcome, while only TIM-3 in CD4eff is significant in progressive disease (Table 6.2).
Figure 6.4: Presence of high UDL count/ml and effector T cell PD-1<sup>hi</sup> at EOT<sup>*</sup> are associated with worse outcome.

Displayed is the association of urinary lymphocyte count with clinical outcome at the end of treatment (EOT) as defined by end of treatment or last collected UDL on treatment. (A, C, E) Relationship with response to treatment and UDL count/ml (A), CD8 PD-1<sup>hi</sup> (B) and CD4eff PD-1<sup>hi</sup> is shown respectively. Mann-Whitney U test used for statistical analysis. Error bars represent mean values with SD, *P < 0.05. (B, D, F) Progression-free survival (%) over a median follow up of 7 months is shown in patients were found to have a high UDL count/ml (B), high CD8 PD-1<sup>hi</sup> (D), high CD4eff PD-1<sup>hi</sup> (F) (above the median) or a low UDL count/ml, CD8 PD-1<sup>hi</sup> and CD4eff PD-1<sup>hi</sup> (below the median).
<table>
<thead>
<tr>
<th>Marker</th>
<th>T cell subset</th>
<th>Baseline</th>
<th></th>
<th></th>
<th></th>
<th>EOT</th>
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<td>NS</td>
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<td>*</td>
<td></td>
<td></td>
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<tr>
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<td>*</td>
<td>*</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD4eff</td>
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<td>NS</td>
<td></td>
<td>*</td>
<td>**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treg</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>CD4eff</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td>*</td>
<td>**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treg</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td>NS</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PD-1/TIM-3</td>
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<td>0.05</td>
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<tr>
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<td>CD4eff</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td>*</td>
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<td></td>
<td>0.07</td>
<td>0.07</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 6.2: EOT effector PD-1<sub>HI</sub> and PD-1/TIM-3 are associated with worse clinical outcomes.
Displayed is the summary of the association of urinary lymphocyte count with clinical outcome at baseline or end of treatment (EOT) as defined by end of treatment or last collected UDL on treatment. Legends: CD4eff, CD4 effector T cells; EOT, end of treatment; NS, not significant; P, progression; PD-1, programmed death-1; PFS, progression free survival; R, response; TIM-3, T cell immunoglobulin and mucin-domain containing-3; Treg, regulatory T cells.
6.2.4 UDL count/ml falls with response to treatment even in patients who eventually progressed

Having shown that high UDL count/ml and high effector PD-1<sup>hi</sup> at EOT predicts worse clinical outcome, I next sought to see if these markers are dynamic and may help to track treatment response when compared to re-staging CT imaging thereby providing a possible non-invasive method of tracking response to systemic anti-cancer therapy.

Figure 6.5 is a plot showing the UDL count/ml (log) longitudinally throughout systemic anti-cancer treatment. The 3 patients who had a response are indicated with the blue lines, and they all had a low log UDL count/ml at their corresponding last response scan as shown by the yellow triangle symbol. All 6 non-responding patients who eventually had disease progression at EOT are indicated with the red lines and had a high log UDL count/ml at their corresponding progression scan (green circle). 5/6 patients who eventually progressed, had a partial response or stable disease scan after a median of 78 days of treatment (range 26 – 141 days) as shown in Figure 6.1. 3/5 of these patients also had a low log UDL count/ml at their response scan as indicated by the yellow triangle.

![Graph showing patients' log UDL count/ml changes through treatment over time.](image)

**Figure 6.5: Log UDL count/ml changes through treatment over time.**

Graph showing patients’ log UDL count/ml through treatment over time from baseline who have progressed (red line) or responded (blue line) at the end of their treatment. Triangle (yellow) symbol represents the timepoint of response on scan and the circle (green) symbol represents time point of progression on scan. Bold line represents that this is patient’s first-line treatment and dashed line represents patient’s second-line treatment.
For each patient, a UDL/count per ml was obtained at multiple times throughout the course of treatment, including at baseline and EOT. The maximum fold change at any given point during the course of treatment was calculated against baseline or nadir for each patient and is plotted below (Figure 6.6). Given that a percentage/fold change value does not take into account absolute numbers, any individual UDL count/ml that was less than 4.7 was excluded to prevent distortion of the data. 4.7 was chosen as it was the lowest quartile of UDL/count per ml in the previously published MIBC cohort in chapter 4. Looking at the maximal UDL count/ml fold change from nadir, I found that the largest fold change from baseline matches with the patients’ eventual response scan at EOT, except for BL 72 where the biggest fold change did not match with the eventual outcome.

![Figure 6.6: Disease progression correlates with maximal increase in UDL count/ml from baseline. Waterfall plot values shown are the largest percentage change from baseline or nadir. Each bar represents one patient. Red, progression; blue, response.](image)

Taken together, our data suggest that UDL count/ml falls with response to treatment even in patients who eventually progressed. This is statistically significant when considering all the responses (P = <0.05; Figure 6.7). CD8 PD-1<sup>hi</sup> and CD8 PD-1/TIM-3 was also statistically significantly higher in patients who have progressed when taking all responses into account (P = <0.05; Figure 6.8). A representative patient (BL 72) overlay co-expression dot plots of PD-1/TIM-3 on CD8 in urine and PBMC, showed the double
co-expression PD-1/TIM-3* population disappearing at response scan and reappearing at progression (Figure 6.8).

Figure 6.7: UDL count/ml falls with response to treatment even in patients who eventually progressed. Relationship with all responses to treatment and UDL count/ml is shown. Mann-Whitney U test used for statistical analysis. Error bars represent mean values with SD, *P < 0.05.

Figure 6.8: Flow cytometric gating strategy depicting CD8 PD-1hi and PD-1/TIM-3 UDLs in treatment response and progression. Relationship with all responses to treatment, and CD8 PD-1hi and CD8 PD-1/TIM-3 is shown respectively. Mann-Whitney U test used for statistical analysis. Error bars represent mean values with SD, *P < 0.05. (top) Overlay co-expression pattern of PD-1/TIM-3 on CD8 of urine (blue) and peripheral blood mononuclear cells.
Collectively, there is an indication that tracking the fall and rise of UDL count/ml, CD8 PD-1\(^{hi}\) and CD8 PD-1/TIM-3 matches the response scans of this cohort of patients. However, with the current cohort and analysis, I am unable to conclude if tracking UDL count/ml may detect early responses to bring forward scans or intensify therapy if indicated.

6.2.5 Longitudinal tracking of the PBMC immune checkpoint landscape is not associated with treatment outcome.

Although several groups have reported that the immune profile of peripheral blood is distinct to the immune tumour microenvironment (195, 212), as a control to UDLs, I also analysed the immune profile of matched peripheral blood samples. The median expression of the key prognostic phenotype, PD-1\(^{hi}\) and PD-1/TIM-3, is not upregulated in baseline blood samples and median in all T cell subsets (Table 6.3). There is minimal, if at all, range in the expressions of these prognostic phenotypes as compared to UDLs. When focusing on the effector/Treg ratio, there was no significant changes seen at EOT or when taking all responses into account (Figure 6.9). In this small metastatic cohort, there is no association in the checkpoint landscape in PBMC longitudinally.

<table>
<thead>
<tr>
<th>PD-1(^{hi})</th>
<th>CD8</th>
<th>CD4eff</th>
<th>Treg</th>
</tr>
</thead>
<tbody>
<tr>
<td>baseline median</td>
<td>0.45 (0.23 – 0.97)</td>
<td>1.1 (0.27 – 1.66)</td>
<td>0.99 (0.05 – 1.25)</td>
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<tr>
<td>EOT median</td>
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<td>0.86 (0.02 – 2.08)</td>
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<tr>
<td>PD-1/TIM-3</td>
<td>0.34 (0.13 – 1.26)</td>
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<tr>
<td>baseline median</td>
<td>0.6 (0.06 – 1.9)</td>
<td>0.22 (0.02 – 1.42)</td>
<td>0.6 (0.02 – 4.27)</td>
</tr>
<tr>
<td>EOT median</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3: PBMC median expression of PD-1\(^{hi}\) and PD-1/TIM-3 in T cells subsets at baseline and EOT.

Displayed is a table showing the median expression of PD-1\(^{hi}\) and PD-1/TIM-3 at baseline and end of treatment (EOT) of all T cell subsets.
Figure 6.9: There is no significant difference in the ratio of effector:Treg cells in PBMC samples, between responders and progressors.

Displayed shown are the relationship with responses to treatment in PBMC of all T cell subsets. Relationship of CD8/Treg ratio and CD4 effector/Treg ratio at EOT and for all responses is shown respectively. Mann-Whitney U test used for statistical analysis. Error bars represent mean values with SD; NS, not significant.

6.2.6 Neoantigen reactive T cells (NARTs) are detected in PBMC of a patient post chemotherapy

Although the immune profile in PBMC may not recapitulate the tumour microenvironment, there have been previous reports that have shown the presence of NARTs in the peripheral blood of patients with solid tumours (241-243). This may represent a novel non-invasive method to access NARTs that may be used subsequently for adoptive T cell immunotherapy. Moreover, whether chemotherapy may change the immune landscape by inducing neoantigen recognition is unknown. The samples from BL 5, as described in Chapter 5.2.4, was used for this section’s results. To recap, BL 5 received six consecutive treatments with gemcitabine and cisplatin chemotherapy, with an initial response scan after 3 cycles of chemotherapy, but unfortunately progressed at the end of her 6th cycle of chemotherapy. We analysed each peripheral blood drawn before initiating each cycle of treatment.

Although I have shown that there was no association with the key immune checkpoint phenotype and effector to regulatory T cell ratio in these metastatic cohort, BL 5 had dynamic changes of various checkpoint molecules, proliferation and activation markers, mainly in the effector T cells. (Figure 6.10A). At day 90, there was a spike in GzmB and Ki67 on CD8, with a 2-fold increase in PD-1, ICOS, CTLA-4 in both CD8+ and CD4eff. 4-1BB also had a 2-fold increase in CD8+ only. At day 132, there was a huge spike in PD-1, ICOS, GzmB and Ki67 on CD8+, and ICOS on CD4eff. A dot plot of PD-1 and Ki67 is shown over time (Figure 6.10B)
Several groups have shown that T cell reactivity induced during CPI is directed towards neoantigens predicted through gene alterations (11-13), in particular clonal neoantigens (14). There is considerable evidence to show that in conventional chemotherapy can boost the anti-tumour immune response. Gemcitabine, that is typically used in treatment of urothelial cancer, increases dendritic cell dependent antigen presentation (244), causes upregulation of MHC class 1 expression (245), leads to a relative increase in T-cells (246), and a decrease in the immunosuppressive Treg cells (247).

Figure 6.10: Dynamic changes of checkpoint molecules expression, proliferation and activation markers in PBMC of BL 5 on chemotherapy over time. 
(A) Percentage expression of each immune checkpoint, proliferation and activation marker in each T cell subset over time. PD-1, blue; ICOS, orange; CTLA-4, purple; 4-1BB, light blue; GzmB, red; CD25, pink; Ki67, green. (B) Dot plots of PD-1 and Ki67 in BL 5 PBMC over time post chemotherapy.
Dr Bentzen from the Hadrup laboratory applied peptide-MHC multimers labelled with DNA barcodes for large-scale detection of patient specific neoantigens using the same published method (181). They screened for antigenic recognition based on BL 5’s specific mutations and tissue type and a total of 179 of 270 possible clonal and 34 of 61 possible subclonal neoantigens, restricted to HLA-A0201, A2601, B0702 and B4402 were screened. The DNA-barcode labelled MHC multimer approach allowed them to screen for responsiveness to all of the potential neoepitopes in parallel, which was vital in the limited material available. Lastly, they applied the DNA-barcode based approach and combined with staining of surface markers, enabling simultaneous assessment of target recognition and activation status of the cancer-responsive T cells.

A total of 9 different neoantigen responses were detected over the course of her treatment (Figure 6.11). The majority of these responses were detected at day 132 after chemotherapy, which corresponded to the overall changes seen in the CD8+ T cell. Among these neoantigen responses, a preferable recognition of clonally-derived antigens (7 of 179 possible) was observed over subclonally-derived antigens (2 of 34 possible), in line with previous study (14). Finally, there were 2 clonal neoantigen responses to tumour infiltrating lymphocytes (TILs) pre-starting chemotherapy as indicated by “Pre (TILs)” in Figure 6.10. These were not detected in the peripheral blood over the course of her treatment.
6.3 Chapter discussion

In this chapter, I have utilised multi-parametric flow cytometry to explore the dynamic immune response within the urine of patients with metastatic bladder cancer being treated with systemic anti-cancer therapy. In summary, I found that UDL count/ml falls in response to treatment and that patients with effector T cells of PD-1<sup>hi</sup> and PD-1/TIM-3 at EOT have a worse outcome. There are several limitations of this study, as discussed below. I studied multiple immune checkpoint molecules and the flow cytometry panel was very similar to that used for the MIBC cohort in the previously published paper containing numerous co-inhibitory immune checkpoint molecules including PD-1 and TIM-3. Whilst this checkpoint panel may be useful in helping clinician rationalised which combination CPI to give when there is evidence of de novo or acquired resistance to CPI, the panel is not exhaustive, with a number of checkpoint molecules that were not studied in this chapter including LAG-3, TIGIT, BTLA, VISTA and OX40 missing (23), with several of these currently being studied in clinical trials. Moreover, it is crucial to understand beyond T cells, with a number of key myeloid cells associated with anti-PD-1 response including an increased in natural kill cells and a decrease in macrophages (248), and with tumour-associated macrophages correlated with poor anti-PD-1 response in patients with metastatic melanoma (249). Thus, the flow cytometry panel could be improved to include myeloid markers.

In patients with MIBC, as described in Chapter 4, I found that high UDL count/ml and CD8 PD-1<sup>hi</sup> was associated with a worse clinical outcome in terms of progression free survival (PFS). In this chapter, when looking at patients with metastatic disease undergoing systemic anti-cancer therapy, there was no association between the baseline UDL count/ml and the number of CD8 PD-1<sup>hi</sup> T cells in baseline urine samples and PFS. This may be explained, at least in part, by the relatively small group of patients studied in this chapter.

Previous studies have shown that the co-expression of PD-1 and TIM-3 on effector T cells is associated with significantly higher levels of PD-1 on the surface of the T cell as well as more dysfunction as compared with effector T cells with either single or no detectable expression of these receptors (250). Interestingly, I observed that patients with an increased UDL count/ml, increased number of effector PD-1<sup>hi</sup> T cells and an increased number of PD-1/TIM-3 effector T cells at the end of treatment urine samples was associated with a poorer PFS. This finding indicates the importance of obtaining on-treatment urine samples compared to baseline in patients undergoing systemic anti-cancer therapy, in helping to determine prognosis. This may suggest how a baseline
“cold” tumour may become “hot” upon T cell activation (251). However, the number of patients studied in this cohort is very small and larger prospective studies are needed to confirm this finding. Moreover, the size and extend of the bladder tumour in situ which not usually quantified in the metastatic setting, may have had an impact on the results.

Crucially, there is also a hint that these markers are also indicative to patients’ response to treatment. In the six patients who eventually progressed, five patients had a CT scan showing a response midway through treatment. The mean days between first scan to EOT was 73.4 days (range 1 – 174), with the mean days between EOT and EOT samples collected was 13.6 days (range 0 – 40). When I looked at these markers at the time of treatment response and progression, there was a significantly higher number of UDL count/ml, and higher expression of CD8 PD-1hi and PD-1/TIM-3 at the time of disease progression as shown on CT imaging. Interestingly, BL 65 was the only patient who had an iRECIST stable disease (iSD) at the time of a re-staging CT scan with a UDL CD8 PD-1/TIM-3 of 49.2%. This patient had subsequent disease progression following a few weeks and the UDL CD8 PD-1/TIM-3 of 55.3% at progression. He unfortunately passed away shortly after cessation of therapy. This observation, albeit, in a single patient suggests the patient may not have been responding to treatment, despite having iSD on a re-staging CT scan and may have benefited from closer monitoring and a change in management. If UDL immune checkpoint monitoring and its association with response to systemic anti-cancer therapy is proven in a larger group of patients prospectively, this may provide a non-invasive biomarker of response to systemic anti-cancer therapy.

Several studies published to date have utilised the peripheral blood to identify biomarkers of response and resistance to systemic anti-cancer therapies (237). In this chapter, I did not see an association between the peripheral blood immune checkpoint landscape, either in baseline or EOT samples, and PFS or response to treatment. Moreover, there was no difference in the effector to regulatory T cell ratios between those who had responded or progressed. In general, I observed that the expression of immune checkpoint molecules in effector cells in the periphery was low as compared with the tumour perhaps reflecting the relatively small number of tumour antigen reactive cells in the periphery as compared with the tumour.

Several published reports have found the presence of NARTs in the peripheral blood of patients with solid tumours (241-243), highlighting the possibility that tracking of NARTs in the peripheral blood may represent a novel non-invasive method to develop personalised therapies in the cancer treatment. In this chapter, we have shown a case study of a patient whom we have whole genome sequencing data from the tumour,
paired with neoantigen prediction, as well as several PBMC at various timepoints of her systemic treatment. I showed that for this one patient, she had a dynamic change in her immune profile of her peripheral blood towards the end of her treatment. Interestingly, my colleague was able to detect peripheral blood lymphocytes that recognised predicted neoantigens, that wasn't present at baseline. This indicates that chemotherapy may induce neoepitope recognition and potentially assist T cell mediated tumour cell killing when combined with checkpoint inhibition. Moreover, these non-invasive NARTs could be preferentially expanded to be given as an adoptive cellular therapy. Thus, according to this small dataset, peripheral blood monitoring of the immune checkpoint landscape may not be useful in predicting clinical outcome in patients with metastatic bladder cancer undergoing systemic therapy given that T cell immune checkpoint expression is relatively low in the periphery. However, studies monitoring NARTs in the blood may be useful and warrant further study in the future.
Chapter 7: Conclusions

7.1 Concluding remarks
Given the very nature of a dynamic immune system in the context of an evolving tumour, the development of a predictive or prognostic biomarker faces considerable challenges. CPI have revolutionised the treatment in several solid cancers, including urothelial bladder cancers. However, only a small fraction of patients responds to these therapies (46, 47, 50). Therefore, researchers are focussing their attention towards identifying predictive biomarkers of response to CPI, including tumour mutational load, PD-L1 expression, intra-tumoural T cell infiltration and TCR clonality within tumour biopsy specimens that are taken at baseline prior to the commencement of systemic therapy (11-14, 144, 145). For many of these markers, there is a substantial overlap between responders and non-responders of CPI, making it even more imperative to understand this complex and dynamic cancer immune response. Moreover, one of the main limitations in these markers is the invasiveness of accessing these longitudinal tumour biopsies prior to and during the course of therapy (146-149).

Chen and colleagues demonstrated that immune signatures in on-therapy tumour biopsies are highly predictive of response with non-overlapping immune signatures in responders compared to non-responders (146). Moreover, patients may develop acquired resistance to CPI with some developing late acquired resistance after more than 6 months of tumour response (252, 253). Nonetheless, access to longitudinal tumour biopsies prior to and during the course of therapy remains a key limitation in most patients due to the invasiveness of such procedures. As patients are followed up and monitored with response scans only every few months, it is essential to have a non-invasive method of assessing response and the early detection of progression, so we are able to stop ineffective treatments early and change the course of management. Therefore, routine clinical biomarkers should be easily accessible and minimally invasive, particularly in assessing the dynamic and evolving nature of tumour growth and its interaction with the immune system.

It is challenging to rely on a single immunological biomarker to select patients for CPI therapies given the dynamic nature of the tumour-immune interaction, as well as the complex interplay between the regulation of multiple immune checkpoints and their ligands. Hence, it is vital to evaluate the tumour microenvironment in real time and longitudinally where possible, to combat the constant evolution of the immune response, from a “cold” to “hot” tumour and then adaptive resistance. Therefore, it is unlikely that a single immunological biomarker identified as a “snapshot” of the tumour
microenvironment at baseline can solely predict responses to any agent. It is likely that a combinatorial panel of relevant predictive markers may be used in future to guide clinicians. In fact, Cristescu et al recently showed that by using a combination of tumour mutational burden and inflammatory biomarkers including PD-L1 and T cell-inflamed gene expression profile, helped identify patients that were responsive to anti-PD-1 therapies in a pan cancer group of patients (254). Moreover, certain panels of markers may guide the rationale behind combination therapies, and additionally, it is important to re-examine the tumour microenvironment for changes in markers to help direct the next appropriate combinatorial therapy to further increase efficacy, and hopefully lead to durable clinical responses.

Even though the peripheral blood is an excellent form of liquid biopsy for potential biomarkers, no blood based predictive biomarker has been validated in prospective studies thus far in bladder cancer (238). Furthermore, we and other groups have reported that the immune profile of peripheral blood is remarkably unique to the immune tumour microenvironment (65, 202). These findings highlight the importance of tumour sampling to obtain the most accurate picture of the tumour-immune interface within the tumour microenvironment itself. However, for clinical utility, it will be crucial to find a non-invasive source that recapitulates a dynamic and evolving immune tumour microenvironment. The urine represents a unique opportunity to access tumour-derived material in patients with bladder cancer. It is easily accessible, non-invasive, and could serve as a window to the bladder tumour immune microenvironment in these patients.

Through my work in this thesis, I have demonstrated that
1. UDLs are found in patients with urothelial carcinoma but are absent in patients without urinary tract disease
2. UDLs are consistently found in both patients with NMIBC and MIBC
3. UDLs are also present in patients with metastatic bladder cancer with primary disease in situ
4. UDLs have a remarkably similar flow phenotype and TCR repertoire to TILs
5. There is some evidence that UDLs are functional tumour reactive T-cells. However, the numbers are small.
6. Finally, I demonstrated a poor prognostic association in patients with high UDL CD3$^+$ count and an exhaustive phenotype of CD8$^+$ PD-1$^{hi}$, which can be tracked longitudinally in patients with metastatic bladder cancer undergoing systemic therapy in a small group of patients.
Taken together, the data above may provide support for the use of UDLs as an immune biomarker to track response to systemic therapy. However, the result from this thesis is in its infancy and larger cohort of patients are needed to validate the findings.

Targeting neoantigens in adoptive cellular therapies have advantages over those that target tumour associated antigens. For instance, autoimmunity is not expected if T cells respond to neoantigens as these antigens are only present in the tumour cells. Conversely, targeting tumour associated antigens have been shown to have off-target effects on healthy tissues that is associated with autoimmune toxicities (255, 256). Furthermore, T cells that recognise neoantigens are not exposed to central tolerance, hence they are more likely to express higher affinity TCRs compared to those that recognise self-antigens (257). Therefore, if expanded UDLs are able to recognise neoantigens, this makes them a highly attractive non-invasive source for adoptive cellular immunotherapy in urothelial cancer.

7.2 Future work

7.2.1 In depth characterisation of the immune landscape of the bladder microenvironment

The findings presented in this thesis provide a proof of principle that lymphocytes within the urine may be used to track the intra-tumoural immune response. However, the analysis of more patient samples is required in a larger prospective validation cohort to confirm these findings. The 13-colour flow cytometry panel presented in this thesis is mainly a checkpoint T cell panel. Several questions pertaining to T cell differentiation, dysfunction or with regards to the myeloid compartment in UDLs remains unanswered in this limited panel.

Whilst TILs exhibit checkpoint molecules, there has been work from other groups to suggest that activation or reinvigoration of circulating exhausted T cells is associated with response to PD-1 blockade (258) and that the presence of dysfunctional TILs is linked with epigenetic reprogramming in a fixed chromatin state (259). There are various combinations of markers have been used to phenotype TILs isolated from renal, lung, melanoma, colon and ovarian cancers. These markers include T cell activation status (HLA-DR, CD38, Ki67), cytotoxicity potential (GzmB), transcription factor profile (EOMES, Tbet), tissue residency (CD69, CD103) and linear differentiation (CD45RA, CCR7, CD27, CD28) (40). Therefore, we are developing a more in-depth flow cytometric panel using the BD FACSymphony system, which is equipped with five lasers and is able to generate data from up to 30 parameters. This would allow for more in-depth analysis.
of the phenotype of immune cells through the use of more markers enabling higher dimensional analysis of immune cells. The new markers would include the current flow cytometry panels shown in this thesis and some of the markers above to distinguish between the exhausted, terminally differentiated, activated effector and naïve T cells. This may enable to us to identify early prognostic and predictive markers of response to therapy.

In the trials using atezolizumab in metastatic urothelial cancer, PD-L1 expression was evaluated using the VENTANA SP142 on tumour infiltrating immune cells including macrophages, dendritic cells and lymphocytes (260). This determined the inclusion criteria for recruitment into IMvigor 211 (50). While my panel did not specifically study the ligand, PD-L1, its receptor PD-1 was heavily examined and in fact was found to be prognostic in some patients. Therefore, future work is needed to characterise the UDL PD-L1 expression in these subsets of myeloid cells including B cells, NK cells, macrophages, neutrophils, and dendritic cells to further understand the interplay between these immune cells and T cells.

7.2.2 Gaining insight into the tumour antigen reactivity of UDLs and understanding the mechanisms leading to their presence in the urine

Although the findings support the possible tumour reactivity of UDL, there is no conclusive data in the thesis that directly indicates this. Accordingly, more patients’ samples are needed to clarify, using the same method of in vitro expansion and readout to confirm if UDLs are truly tumour antigen reactive. The transcriptomic analysis of UDLs may also provide insight into the function of UDLs. Accordingly, further phenotypic and functional studies, including bulk RNA or single cell RNA sequencing aimed at evaluating the antigenic specificity and biological mechanisms leading to UDL generation warrant further investigation.

To understand the discrepancy between high UDL and poor outcomes, further analysis is ongoing in differentiating between stromal, peritumoural, and intratumoural TILs on immunohistochemistry (IHC). The proximity of TILs to the vasculature and the regulatory role of endothelial cells are being explored. It is essential to ascertain the effector and regulatory T cells ratio and the localisation of the immune cells to the cancer cells. Finally, I found that patients with CD8 PD-1hi in UDLs have poorer clinical outcomes. Therefore, it is critical to identify if these CD8+ lymphocytes in the TILs are PD-1+ and whether they display other exhaustion markers such as TIM-3. This would enable us to better understand the origins and biology of UDLs.
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7.2.3 Tracking UDL through therapies in metastatic patients

In Chapter 6, I demonstrated that the UDL count falls in response to therapy, while the presence of PD-1hi and PD-1/TIM-3 is associated with poorer clinical outcomes. Whilst these results are tantalising, the numbers are too small to make any concrete conclusions. Moreover, there is a heterogeneous mix of patients undergoing different systemic therapies in this group. Therefore, a larger prospective study is needed to validate these findings and prospective tracking of the immune checkpoint landscape and TCR repertoire in longitudinal urine samples obtained from this group of patients. This is important in those patients undergoing systemic immunotherapy and may prove valuable in the validation of UDLs as a biomarker to predict response to CPI. A much more ambitious future application of this would be to explore the potential use of UDLs as a non-invasive source for adoptive cellular immunotherapy for patients with bladder cancer. This would require proof of concept studies and validation through clinical trials.

Finally, as a result of the findings presented in this thesis we have designed two investigator-initiated academic studies to perform a broad biomarker assessment enabling us to capture de-novo and acquired resistance mechanisms in patients with urothelial carcinoma undergoing checkpoint blockade. The first study is in a translational study in advanced urothelial cancer in patients receiving maintenance CPI following first-line platinum-based chemotherapy. Patients will undergo longitudinal urine sample collection and on disease progression, a repeat tumour biopsy, a core of which will used to establish ex-vivo immune competent cancer models. The aims of this study are to establish whether UDLs are similar to TILs, assess if UDLs are predictive markers of early response or progression and determine if UDLs can guide suitable combination therapies tested in the ex-vivo model. The second study is testing whether the combination of a TAA peptide vaccine and CPI can delay or obviate the need for cystectomy in patients with NMIBC who have failed BCG treatment. Patients will undergo longitudinal urine sample collection, which will hopefully serve as a non-invasive biomarker of treatment response or resistance.

Taken together, the data presented in this thesis provide insight into the origins and biology of UDLs through the application of high dimensional flow cytometry, TCR sequencing and functional assays. While collection of matched human tissue, urine and blood samples were challenging, we showed that they are essential in further understanding the dynamic immune biology of urothelial cancer. I hope that my efforts during this thesis and the strength of these data will sustain local collaborations, and drive national and possibly international efforts to incorporate longitudinal urine sample collection into clinical studies in patients with urothelial cancer. In this way, I hope that
my work will contribute to better personalized therapies and improved patient outcomes for urothelial cancer.
Chapter 8: Appendix

8.1 List of papers


Peer-reviewed publications under TRACERx Consortium


8.2 List of abstracts and presentations

• Wong YNS et al. Nivolumab and ipilimumab treatment in prostate cancer with an immunogenic signature (NEPTUNES). ASCO 2019, Chicago, USA – Poster


• Wong YNS et al. Urine-derived lymphocytes (UDLs) as a non-invasive surrogate marker of tumour infiltrating lymphocytes (TILs) in patients with muscle invasive bladder cancer (MIBC). ESMO 2017, Madrid, Spain – Poster

• Wong YNS*, Bentzen AK* et al. The dynamics of neoepitope recognition as response to therapy in a patient with bladder cancer. CIMT 2017, Mainz, Germany – Poster


• Khetrapal P, Dong L, Wong YNS, et al. molecular tracking of bladder cancer using mutations detected in plasma cell-free dna through radical cystectomy and chemotherapy. AUA 2017, Boston, USA – Poster

• Joshi K… Wong YNS, et al. Characterisation of the TCR repertoire in NSCLC to reveal the relationship between TCR heterogeneity and genetic heterogeneity that is influenced by mutational load and is associated with disease recurrence. ASCO 2018, Chicago, USA – Oral
• Joshi K… Wong YNS, et al. Characterisation of the TCR repertoire within the TRACERx study. ESMO Immuno-Oncology 2017, Lausanne, Switzerland – Oral


Carcinoma: Updated Results From a Phase 1/2 Open-label Study. JAMA oncology. 2017;3(9):e172411.


132. Grande E, Galsky M, Arranz Arija JA, De Santis M, Davis ID, De Giorgi UFF, et al. LBA14_PRIMvigor130: Efficacy and safety from a phase III study of atezolizumab (atezo) as monotherapy or combined with platinum-based chemotherapy (PBC) vs placebo + PBC in previously untreated locally advanced or metastatic urothelial carcinoma (mUC). Annals of Oncology. 2019;30(Supplement_5).


136. Siefker-Radtke AO, Necchi A, Park SH, Garcia-Donas JS, Huddart RA, Burgess EF, et al. First results from the primary analysis population of the phase 2 study of erdafitinib (ERDA; JNJ-42756493) in patients (pts) with metastatic or unresectable urothelial carcinoma (mUC) and FGFR alterations (FGFRalt). Journal of Clinical Oncology. 2018;36(15_suppl):4503-.


Carlo MI, Zhang L, Mandelker D, Vijai J, Cipolla CK, Robson ME, et al. Cancer predisposing germline mutations in patients (pts) with urothelial cancer (UC) of the renal pelvis (R-P), ureter (U) and bladder (B). Journal of Clinical Oncology. 2017;35(15_suppl):4510-.


228. Li Y, Bleakley M, Yee C. IL-21 Influences the Frequency, Phenotype, and Affinity of the Antigen-Specific CD8 T Cell Response. The Journal of Immunology. 2005;175(4):2261.
