INVESTIGATING THE ROLE OF NEUTROPHILS IN A LUMINAL-LIKE COLD MODEL OF MUSCLE-INVASIVE BLADDER CANCER AND ITS RESPONSE TO RADIATION THERAPY

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June 2022

A thesis submitted to McGill University in partial fulfillment of the requirement of the degree of Master of Science

Acknowledgments

This thesis and the work done surrounding it would not have been possible without the unconditional support of the people surrounding me. I am forever grateful for their encouragement, help and wisdom that have pushed me and allowed me to grow into the scientist and person that I am today.

I first want to thank my supervisor, Dr. Wassim Kassouf and my co-supervisor, Dr. Jonathan Spicer for the trust they have put in me to conduct this project and for their wisdom and patience throughout. This project was exciting and one that suited perfectly my goals as a scientist. It has been an incredible learning experience, and the environment they have created for their students is extremely supportive. I could not have asked to have a better environment to grow and learn.

I also would like to thank the chair of my research advisory committee, Dr. Alice Dragomir, and the members, Dr. Jacques Lapointe and Dr. Julia Burnier, for their time and guidance during the duration of my project. Their insight was nothing but helpful and allowed me to see the bigger picture in my own project.

An immense thank you to Dr. Jose João Mansure, who has been there, for any need that would arise throughout my project. He has been the best mentor a master student could have asked, when presented with this daunting entry into the journey of graduate studies. Thank you for teaching me, mentoring me, and most importantly supporting me. Your advice has shaped me into the scientist that I am today, and into the person that I am today. I will forever be grateful for your guidance throughout these last two years.

I would also like to thank the past and present members of the Kassouf lab team: Surashri Shinde- Jadhav, Eva Michaud, JiaMin Huang, Jaleh Ebnealian, Gautier Marcq, Ronald Kool,

Rodrigo Skowronski. The healthy work environment promoted by each and every one of you has allowed me to grow in a safe environment, that allowed me to explore my full potential. Your guidance through scientific research, but also through life as a graduate student and young researcher have been nothing but helpful. The support and help throughout the long weeks, late-nights and weekends we have spent working with the 100+ mice at the time, thank you. This would not have been possible for me without you all.

I would also like to thank members of the Spicer lab, mainly Dr. Roni Rayes and Meghan DeMeo, for their guidance with a subject I was not fully familiar with. Their insight and helpful tips allowed this project to fully evolve into what it is today and allowed me to grow in this field as an immunologist and researcher.

Finally, this would not have been possible without my entourage, without my family and friends. Thank you for being there for me in these two years and supporting me in this huge endeavour that I have undertaken. To my family, who have supported me in the early mornings and late evenings home. To my friends, who have seen the blood, sweat and tears behind this project, who have been a shoulder to cry on for the failures, and a friend to celebrate the wins. To the people who have balanced my stressful days with some quality time and a moment to breathe and forget my worries. Although you haven't directly contributed to this project, you have contributed to me, and have held down the fort for me when times were rough in these last two years. The impact you all had on the success of this project is greater than you imagine. Thank you.

STATEMENT OF INTEGRITY

I, Sabina Fehric, attest that the work submitted represents solely my own efforts. I am aware of the University rules and regulations on plagiarism and subsequent penalties.

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Abstract

Bladder cancer is the 10th most common cancer worldwide. There are two types: Non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). Approximately 30% of patient will present with the muscle-invasive form of the disease. Currently the standard of treatment is radical cystectomy, which greatly decreases the quality of life of the patient and some older patient may not be suitable for the procedure. Radiation therapy has increased in the field as a bladder-sparring treatment for MIBC patient. However, 30% of patients do not respond to treatment and progress to a metastatic form of the disease. Understanding the underlying cause behind this phenomenon could help us predict which patients will respond to the treatment and find ways to improve their clinical outcomes. Our team has shown that in a murine hot tumour model (high immune infiltration), neutrophils and neutrophil extracellular traps play an important role in radiation resistance. However, this was never tested in a cold tumor MIBC model (low immune infiltration), which has a high neutrophil infiltration. Therefore, we set out to understand the role of neutrophils in this cold, luminal-like model of MIBC and whether modulation of neutrophils, with the combination of immune checkpoint inhibitors, could improve the response to radiation therapy.

To do this we performed an *in vivo* study where luminal-like cell line (UPPL), were injected in the flank of mice. Mice were treated with every possible combination of radiation therapy, anti-PD-L1 and DNAse I (which degrades the neutrophils extracellular traps). Tumor growth and survival was monitored in each group and flow cytometry was performed on the tumor samples at endpoint and after 21 days post treatment initiation (midpoint).

Tumor growth was able to show two distinct populations in each treatment arm: some mice had a response to the treatment and some mice did not respond at all. Flow cytometry data pointed

towards neutrophil having a role to play in the response to the treatment. Midpoint data showed a very high neutrophil and low $CD8^+$ T cell infiltration in the radiation group alone, and a higher $CD8^+$ T cell infiltration in the triple combination group. This dynamic in the triple combination group changes at endpoint, as the neutrophil infiltration increases and $CD8^+$ T cells infiltration decreases over time. This may skew the survival, which is why no changes are noted in survival of the mice amongst the different groups. This could also be due to the treatment regimens that may require further optimization in the future.

Overall, the data shows clear indication that neutrophils do play a role in disease progression and response to radiation therapy, as differences can be observed among the different treatment arms. Optimization of the treatment regimen and more studies on the tumor samples (for example, immunofluorescence on tumor tissues from the endpoint mice) could help us confirm these results. These findings bring us one step closer to understanding radioresistance and could help us elucidate this phenomenon in the clinic as well. This could help improve the treatment in the clinic of MIBC. It brings us one step closer to personalized medicine, as we would be able to predict the outcomes to radiation therapy and improve the response of patient who would theoretically not respond.

Résumé

Le cancer de la vessie est le 10^{ième} cancer le plus fréquent dans le monde. Il en existe deux types : le cancer de la vessie non invasif sur le plan musculaire et le cancer de la vessie avec envahissement musculaire. Environ 30% des patients présentent la forme avec envahissement musculaire. Actuellement, la norme de traitement est la cystectomie radicale, qui diminue considérablement la qualité de vie du patient, et certains patients âgés peuvent ne pas être adaptés à cette procédure. La radiothérapie s'est développée comme alternative dans le domaine en tant que traitement. Cependant, 30% des patients ne répondent pas au traitement et évoluent vers une forme métastatique de la maladie. Comprendre la cause sous-jacente de ce phénomène pourrait nous aider à prédire quels patients répondront au traitement et à trouver des moyens d'améliorer leurs résultats cliniques. Notre équipe a déjà démontré que dans un modèle murin de tumeur chaude (forte infiltration immunitaire), les neutrophiles et les pièges extracellulaires des neutrophiles jouent un rôle important dans la résistance aux radiations. Cependant, cela n'a jamais été testé dans un modèle de tumeur froide (faible infiltration immunitaire), qui présente une forte infiltration de neutrophiles. Nous avons donc cherché à comprendre le rôle des neutrophiles dans ce modèle de tumeur froide de type luminal du cancer de la vessie avec envahissement musculaire et à savoir si la modulation des neutrophiles, avec l'association d'inhibiteurs de points de contrôle immunitaire, pouvait améliorer la réponse à la radiothérapie.

Pour ce faire, nous avons réalisé une étude in vivo dans laquelle la lignée cellulaire de type luminal (UPPL) a été injectée dans le flanc de souris. Les souris ont été traitées avec toutes les combinaisons possibles de radiothérapie, d'anti-PD-L1 et de DNAse I (qui dégrade les pièges extracellulaires des neutrophiles). La croissance tumorale et la survie ont été surveillées dans

chaque groupe et la cytométrie en flux a été réalisée sur les échantillons de tumeurs au point final et après 21 jours après le début du traitement (point médian).

La croissance tumorale a pu montrer deux populations distinctes dans chaque bras de traitement : certaines souris ont eu une réponse partielle au traitement et d'autres n'ont pas du tout répondu. Les données de cytométrie en flux ont montré que les neutrophiles avaient un rôle à jouer dans la réponse au traitement. Les données au point médian ont montré une infiltration très élevée de neutrophiles et faible de cellules T $CD8^+$ dans le groupe radiothérapie seule, et une infiltration plus élevée de cellules T $CD8^+$ dans le groupe triple combinaison. Cette dynamique dans le groupe triple association change au point final, l'infiltration des neutrophiles augmentant et celle des cellules T $CD8^+$ diminuant au fil du temps. Cela peut fausser la survie, ce qui explique pourquoi aucun changement n'est noté dans la survie des souris entre les différents groupes. Cela pourrait également être dû aux régimes de traitement qui peuvent être optimisés à l'avenir.

En résumé, les données indiquent clairement que les neutrophiles jouent un rôle dans la progression de la maladie et la réponse à la radiothérapie, car des différences peuvent être observées entre les différents groupes de traitement. L'optimisation du régime de traitement et d'autres études sur les échantillons de tumeurs (par exemple, l'immunofluorescence sur les tissus tumoraux des souris du point final) pourraient nous aider à confirmer ces résultats. Ces résultats nous rapprochent un peu plus de la compréhension de la radiorésistance et pourraient nous aider à élucider ce phénomène en clinique également. Cela pourrait contribuer à améliorer le traitement du cancer de la vessie avec envahissement musculaire en clinique. Cela nous rapproche de la médecine personnalisée, car nous pourrions prédire les résultats de la radiothérapie et améliorer la réponse des patients qui, théoriquement, ne répondraient pas.

Contributions of Authors

This project was given to me by Dr. Wassim Kassouf. The experiments were designed under the guidance of Dr. Jose João Mansure, Dr. Wassim Kassouf, as well as my co-supervisor Dr. Jonathan Spicer. The experiments were all conducted by myself, with the help and guidance of the members of Dr. Wassim Kassouf's team: Dr. Surashri Shinde-Jadhav, Dr. Eva Michaud and JiaMin Huang. Guidance from Dr. Jonathan Spicer's team on neutrophil knowledge, specifically Dr. Roni Rayes was greatly appreciated. The analysis was done by myself, under the guidance and discussions with Dr. Jose João Mansure and Dr. Eva Michaud.

CHAPTER 1: Introduction

1. Bladder Cancer Introduction

1.1 Epidemiology

Bladder cancer has been found to be the 9th most common cancer worldwide and it is estimated to be the 8th leading cause of cancer death in the USA, in men specifically [1, 2]. According to the Global Cancer Observatory (GLOBOCAN), there has been 573 278 cases of bladder cancer worldwide. In the US, it is the 4th most common cancer found in men specifically [3]. Geographically, bladder cancer has been found to have a higher prevalence in countries in Europe and North America, and the lowest prevalence in countries in Latin America, Sub-Saharan Africa and South East Asia [1].

1.2 Risk Factors

Many different risk factors are involved in the development of bladder cancer such as gender and age. Men have been found to be 3-4 times more at risk of developing bladder cancer men [1, 3]. About 80% of cases are diagnosed in patients over the age of 65, and 90% in patients over 55 [3, 4]. Data suggests that bladder cancer is more common in Caucasians, compared to African Americans [3]. However, African Americans seem to have a higher mortality rate [1].

Certain habits and exposures can also promote bladder cancer development. There is a reported 3- to 5-fold higher risk of developing bladder cancer in smokers, which increases depending on the usage intensity and duration [3, 5]. Approximately half of the bladder cancer cases in men are reported in smokers, with an increase in risk and intensity of the diseases in consumers [3, 5]. Carcinogens found in smoking cause DNA mutations and hypermethylation, which promotes tumor development [3]. Although more research is needed, a recent study on E-cigarettes has found biomarkers of carcinogen present in users which could be linked to the risk of

bladder cancer [6]. Exposure to aromatic amines in the work environment has been shown to potentially increase the risk of developing bladder cancer. These compounds, including 2-napthylamine and benzidine, are found in the manufacturing of products such as dyes, fuels and rubber [3, 5, 7]. High concentration of inorganic arsenic in drinking water has been shown to be correlated with the risk of developing bladder cancer [5]. High arsenic could potentially be found in drinking water sourced from private wells which were constructed in the 1960's, when arsenic was largely used in pesticides [8].

Some diseases are also linked to the development of bladder cancer. Cowden's syndrome predispose the patients for renal and bladder cancer, through the deletion or point mutation of *PTEN* [4, 9]. Lynch syndrome is found to increase bladder cancer through a defect in DNA mismatch repair system [4, 10]. Infection with *Schistosoma haematobium,* a parasite, has been linked to bladder cancer development in the Middle East and Africa [4, 11]. The parasite causes inflammation and tissue destruction which promotes the development of tumor in the bladder [11].

1.3 Etiology

Many factors are at play in the development of bladder cancer. The most common genetic mutations involve genes such as TP53, MDM2, PPARG, TERT, FGFR3, EGFR, ERBB2, BRAF, PIK3CA, and RAS [12]. The p53 tumor suppression is an important pathway involved in bladder cancer development, mostly through alterations in the *TP53* and *MDM2* genes [12]. *TP53* encodes for p53 protein, which is an important tumor suppressor. Multiple studies showed a 50-60% of bladder cancers had the *TP53* mutations, and is more commonly found in muscle-invasive bladder cancer [13, 14]. *MDM2* encodes for a E3 ubiquitin ligase which targets the p53 protein for degradation. This gene is often amplified through mutations such as polymorphisms or

amplification patterns [15-17]. Another frequent mutation in bladder cancer is in the *TERT* gene, which encodes for a telomerase reverse transcriptase [14, 18]. Expression of the reverse transcriptase prevents telomere shortened and is normally downregulated. In bladder cancer, the upregulation of the transcriptase allows the cancer cells to divide indefinitely [18]. Mutations in the *PPARG* gene which encodes a family of nuclear receptors also occur in bladder cancer. These receptors play a role in cell metabolism, immunity and adipogenesis and have been found to be amplified specifically in luminal molecular subtype of bladder cancer [19, 20]. Multiple genes in the RTK/RAS/PI3K pathway are also involved in the development of bladder cancer. Fibroblast growth factor 3 (*FGFR3*), epidermal growth factor receptor (*EGFR*), erb-B2 receptor tyrosine kinase (*ERBB2*), *BRAF*, *PIK3CA* and *RAS* are all commonly mutated genes in the pathway found in bladder cancer, which promote angiogenesis, cancer growth and survival [12].

1.4 Symptoms of Bladder Cancer

Most common sign of bladder cancer is haematuria, or blood in the urine [21]. Other symptoms include urgency to urinate and irritation while voiding the bladder [21]. More advance stages of bladder cancer can induce symptoms such as the inability to urinate, the loss of appetite and weight loss and lower back pain [22]. These symptoms are often related to other ailments such as urinary tract infections and can easily be mistaken for another disease by the patient.

1.5 Diagnosis of Bladder Cancer

Bladder cancer diagnosis is done through a combination of cytology and cystoscopy. Cytology consists of looking at single cell layers to determine whether they could be dysplastic. Although it has high specificity, it is not very sensitive, and therefore, not as powerful for

asymptomatic patients of bladder cancer [23]. Cystoscopy consists of inserting a camera through the urethra, to observe the bladder for any abnormalities. If cystoscopy results are abnormal, a transurethral resection of the bladder (TURBT) is performed, where a piece of the tumor is removed to be able to stage and confirm bladder cancer diagnosis [24]. These techniques are still considered the best ways to diagnose bladder cancer. However, many new technics are emerging, that allow for a better imaging and less-invasive detection methods. Recently, photodynamic diagnosis/blue-light cystoscopy and narrow-band imaging have allowed for better imaging technics. Blue-light cystoscopy consists of adding a dye (e.g. 5-ALA) to the bladder which gets absorbed by dysplastic tissues. When the bladder is exposed to blue light, any abnormal tissue will reflect a red light and will be more easily detected [25, 26]. Narrow-band imaging allows for a better visibility of blood vessels and overall allows for better imaging than standard technics, without the need of additional dyes [25, 26]. Recently, liquid biopsies have been emerging as a less-invasive method to detect bladder cancer, through blood or urine samples. Different markers such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs) and miRNAs have been shown to be linked with early diseases recurrence and correlated to tumor stages [27, 28]. Liquid biopsies could be developed into great tools for less-invasive diagnostic methods. Once bladder cancer is diagnosed, treatment will depend on the classification and the staging of the disease.

2. Bladder Cancer Classification

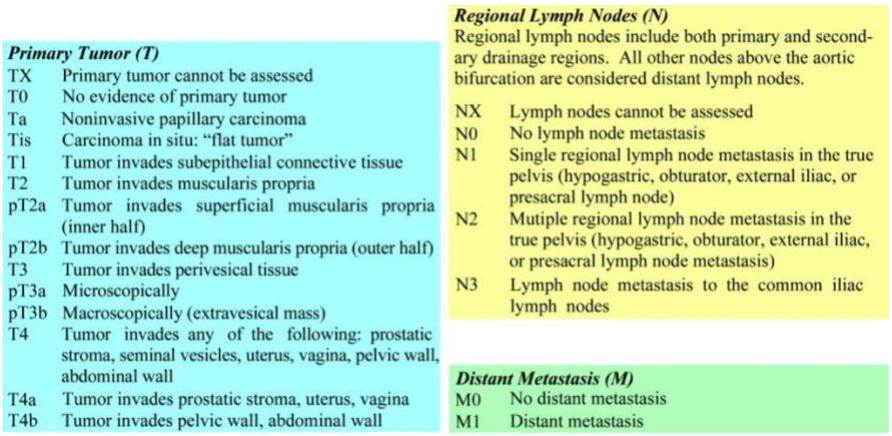
Bladder cancer is first diagnosed through the cell of origin. The three most common variations include urothelial carcinomas, squamous cell carcinomas and adenocarcinomas [29]. Urothelial carcinomas account for approximately 80-90% of bladder cancer [29]. Squamous cell carcinomas and adenocarcinomas are rarer in bladder cancer. Squamous cell carcinomas are more

common in countries which are endemic with the parasite *S. haematobium*, and is linked to chronic bladder inflammation due to the infection with the parasite [30]. Adenocarcinomas are more common in developing countries, accounting for approximately 10% of bladder cancer related radical cystectomies (compared to about 1% in developed countries) [31].

2.1 Stages of Diseases

Urothelial bladder cancer is differentiated through TNM staging and high vs low-grade tumors. TNM staging differs between different cancer types and considers the development of the primary tumor, lymph node involvement and detection of metastasis. Carcinoma *in situ*'s (stage Tis), are considered stage 1 of bladder cancer. Before this, the tumors are considered papillary and are less likely to spread when compared to the flat carcinoma *in situ* tumors [32]. As tumor develops, TNM staging increases. As soon as the primary tumor reaches T2, they are considered muscle-invasive bladder cancer as they breach the muscle layer [18]. No matter the grading of the tumor or lymph node involvement, once metastasis occurs, the bladder cancer is at Stage 4 and is considered a metastatic bladder cancer (Table 1) [18].

Tumors can also be classified by separating between high and low-grade tumors. This classification relies on how dysplastic and different the tumor cells are compared to the original cell. High-grade tumors are more aggressive than low grade tumors. In bladder cancer, low grade tumors are associated with mutations in *FGFR3* whereas high grade tumors are associated with mutations in tumor suppressor genes such as *PTEN* and *TP53* [18, 33]. Non-muscle invasive bladder cancer (NMIBC), muscle invasive bladder cancer (MIBC) or metastatic bladder cancer have different characteristics in patients and are treated and managed differently in clinic



Primary Tumor (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Noninvasive papillary carcinoma
Tis	Carcinoma in situ: "flat tumor"
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscularis propria
pT2a	Tumor invades superficial muscularis propria (inner half)
pT2b	Tumor invades deep muscularis propria (outer half)
T3	Tumor invades perivesical tissue
pT3a	Microscopically
pT3b	Macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumor invades prostatic stroma, uterus, vagina
T4b	Tumor invades pelvic wall, abdominal wall

Regional Lymph Nodes (N)

Regional lymph nodes include both primary and secondary drainage regions. All other nodes above the aortic bifurcation are considered distant lymph nodes.

NX	Lymph nodes cannot be assessed
N0	No lymph node metastasis
N1	Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node)
N2	Mutiple regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node metastasis)
N3	Lymph node metastasis to the common iliac lymph nodes

Distant Metastasis (M)

M0	No distant metastasis
M1	Distant metastasis

Table 1: Bladder Cancer grading and staging using the TNM system. TNM grading of bladder cancer tumors. These take into consideration the primary tumor, draining lymph nodes and metastasis. Figure adapted from Jacobs *et al.* [34]

2.2 Non-Muscle Invasive Bladder Cancer

NMIBC accounts for approximately 70% of bladder cancer cases and can be separated into low, intermediate and high-risk categories [35]. The 5-year disease free survival rates lower as the risk categories changes, with 43% for low-risk, 33% for intermediate-risk and 22% in high-risk NMIBC patients [35]. Patients with high-risk NMIBC have a 45% chance of progressing into MIBC and should get intravesical immunotherapy [36]. This progression usually happens within 48 months after initial diagnosis and patients who progress from NMIBC to MIBC have an overall worse prognosis than patients who diagnose directly with MIBC [37].

Treatment of NMIBC varies on the risk category of the tumor. Overall, TURBT will be performed, both for official diagnosis and for removal of the tumors. Intermediate and high-risk tumors should be continuously monitored through cystoscopies, since it has been shown that 1/3 of these patients need another TURBT later on [29, 36]. These patients often also receive intravesical chemotherapy to kill remaining tumor cells following TURBT.

Intravesical chemotherapy directly following TURBT effectively reduces the risk of recurrence [38, 39]. The most common chemotherapy agent is Mitomycin C, which causes alkylation of the DNA and in turn, inhibits DNA synthesis [35, 40]. Side effects include irritation while bladder voiding and in some extreme cases, bladder necrosis [35]. Gemcitabine has recently been studied as an alternative since it is less expensive, and shows lower levels of toxicity [35, 41].

The most common immunotherapy is bacillus Calmette-Guérin, or BCG therapy. It is the best method to prevent recurrence in intermediate and high-risk NMIBC patients [36]. Originally used as a vaccine for tuberculosis made with an attenuated strain of *Mycobacterium bovis,* it is now considered the best first-line intravesical immunotherapy for NMIBC. The exact mechanism by which this therapy functions is unclear. However, it seems to trigger an immune response, similar to the one triggered against tuberculosis, which causes immune cells to clear any remaining cancerous cells in the bladder [40]. There are two different types of BCG therapy: induction and maintenance. Induction therapy is the first exposure to BCG and is given for 6 weeks [42, 43]. Maintenance therapy is recommended for a duration of 3 years, especially in patients with high-risk NMIBC [43]. BCG therapy has shown to be overall best to prevent recurrence in intermediate and high-risk NMIBC, better then mitomycin C chemotherapy, and helps with overall survival rates [36, 42, 44]. In high-risk NMIBC, both induction and maintenance therapy are strongly recommended [44]. Certain side effects of BCG include chemical cystitis and in extreme cases, BCG sepsis [36].

Some patients do not respond to BCG therapy. BCG-unresponsiveness is characterized in patients who have received at least 1 induction therapy and maintenance

therapy and either show persistent high-grade tumor regardless or high-grade tumor recurrence 6-12 months after last BCG treatment. These patients are then treated with radical cystectomy [45].

2.3 Muscle-Invasive Bladder Cancer

MIBC accounts for 30% of bladder cancer occurrences and has a worse prognosis than NMIBC diagnosis [35]. Approximately 50% of MIBC patients will go on to develop metastatic bladder cancer [29]. Current standard of treatment is neoadjuvant chemotherapy combined with radical cystectomy, which includes pelvic lymph node dissections and urinary tract reconstruction. Different types of reconstruction include incontinent conduit diversions, continent cutaneous diversions and orthotopic neobladders [35, 46]. The addition of platinum-based neoadjuvant chemotherapy (e.g. cisplatin) is recommended as it improves 5-year overall survival when compared to radical cystectomy alone. Cisplatin-based chemotherapy stops cancer cells from growing and multiplying [29, 35, 47]. However, 50% of patients are not considered for neoadjuvant chemotherapy due to their age and comorbidities [48]. Radical cystectomy also has a high rate of complications due to the invasiveness of the procedure; approximately 30% of patients have post-operative complications 30 days after surgery, and 67% have complications within 90 days [49, 50]. Some bladder-sparring treatment options have been developed for patients who may not be suitable for radical cystectomy and neoadjuvant chemotherapy. These include unimodal therapies of radiation therapy (RT) and chemotherapy, partial cystectomy and combined therapies.

Radiation therapy as a monotherapy is done when patients are not fit for surgery. The 5-year overall survival however is still lower than in patient who are given radical cystectomy and there is a high risk for salvage cystectomy [47, 51]. Chemotherapy as a unimodal therapy is very

rarely used. Combining both chemotherapy and RT is more effective and increases the 2-year overall survival when compared to RT alone [47, 52].

Partial cystectomy consists of resecting out the tumor, while trying to preserve the bladder and its function as much as possible. Specific criteria are needed for a tumor to be considered for partial cystectomy. A single solitary tumor must have 2cm margins around it and the patient must have no previous history of metastatic disease [53]. This procedure has less complications than radical cystectomy but has a higher risk of bladder cancer recurrence. Ultimately, approximately 30% of patients end up receiving radical cystectomy regardless [52].

Currently, the most promising bladder-sparring method is trimodal therapy (TMT) which combines TURBT and chemoradiation therapy. The procedure allows for most of the tumor excised through TURBT, and treatment is followed by both chemotherapy and radiation therapy. The overall survival is comparable to radical cystectomy and people who do not respond can quickly be caught and given salvage cystectomy [47, 54].

2.4 Metastatic Bladder Cancer

Metastatic bladder cancer is the worst possible prognosis, since cancerous cells have spread to other organs. The 5-year overall survival rate of these patients is 15% [55]. Metastasis occurs mostly in the lymph nodes, bones, other urinary organs, lungs and the liver [56]. Treatments given are usually to manage or delay progression of the disease.

Standard is platinum-based chemotherapy, more specifically, cisplatin-based. These treatments are even more effective in patient who show mutations in DNA repair systems since they are sensitive to cisplatin [55]. A combination of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) was previously used. However, MVAC is less recommended due to high

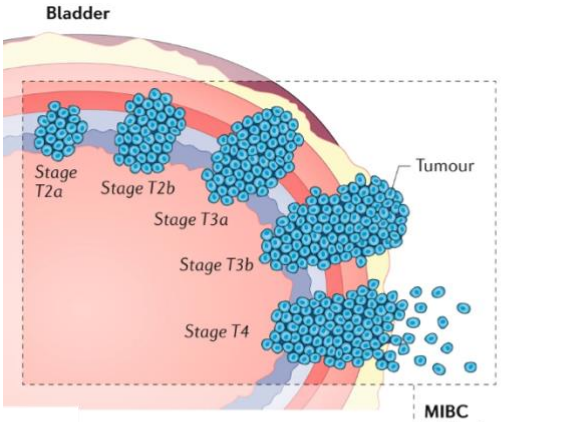
toxicity levels, which can cause harmful effects such as neutropenia and cardiac toxicity [55]. Recently, the combination of gemcitabine and cisplatin has been studied as an alternative since it's known to be less toxic in patients with metastatic disease [55]. Second-line treatment options include immune checkpoint inhibitors (ICI) and *FGFR3* inhibitors and depend on certain characteristics in patients.

ICI's are known to be effective in many different types of cancer, including bladder. The treatment works by administering antibodies that target immune checkpoint molecules, which usually promote immune suppression. By binding to these, T cells are allowed to retain their anti-tumor function. These molecules include molecules such as programmed death 1 (PD-1) and programmed death ligand-1 (PD-L1). Multiple different ICI have been approved for bladder cancer that target either PD-1 (Pembrolizumab or Nivolumab) or PD-L1 (Atezolizumab, Durvalumab and Avelumab) [55] . ICI are used as a second line of treatment in patients who do not respond to platinum-based chemotherapy with the only exception for Pembrolizumab or Atezolizumab, which can be used as a first line of treatment in patients with high levels of PD-L1 [55].

Erdafitinib, a tyrosine kinase inhibitor that targets *FGFR3* is a compound approved for use in bladder cancer in patient who exhibit *FGFR3* mutations [55]. This mutation occurs in approximately 20% of bladder cancer, which makes it an interesting target for therapies [57]. In patients, who receive this treatment, approximately 40% respond to therapy, most of them in a partial manner [57]. If all treatments offered to the patients fail, they are given the option to apply to be part of a clinical trial. More recently, enfortumab vedotin has been approved in patients with metastatic bladder cancer who progress after cisplatin-based chemotherapy and ICI.

2.5 Molecular Subtypes of Bladder Cancer

Another recent approach to classifying bladder cancer is through the different molecular subtypes. For MIBC, it was initially classified into 2 categories: luminal and basal. These have differentiated into distinct subpopulation and are now defined into 6 commonly accepted molecular subtype: luminal-papillary, luminal non-specified, luminal unstable, basal-squamous, stroma rich and neuroendocrine (Figure 1) [18, 58].



Subtype	Signatures	Mutations
LumP (24%)	• PPARG⁺ • FGFR3⁺ • CDKN2A⁻	• *FGFR3* (40%) • *KDM6A* (38%)
LumNS (8%)	PPARG⁺	*ELF3* (35%)
LumU (15%)	• PPARG⁺ • E2F3⁺ • ERBB2⁺ • Genomically unstable • Cell cycle positive • APOBEC⁺ • High TMB	• *TP53* (76%) • *ERCC2* (22%)
Stroma-rich (15%)	• Smooth muscle • Fibroblast • Myofiblast gene signatures	–
Ba/Sq (35%)	• Squamous differentiation markers • Fibroblasts and myofibroblast gene signature • EGFR⁺	• *TP53* (61%) • *RB1* (25%)
NE-like (3%)	• Neuroendocrine differentiation marker • TP53⁻ • RB1⁻ • Cell cycle positive	• *TP53* (94%) • *RB1* (39%)

Figure 1: MIBC Molecular Subtypes. Molecular subtypes of MIBC and some of their known signatures and mutations. Figure adapted from Tran. *et al* [18]

The basal molecular subtype, short for basal-squamous, is a molecular subtype where cells originate from the basement membrane of the urothelium. Specific mutations in these cells include overexpression of the epidermal growth factor receptor (EGFR), signal transducer and activator of transcription 3 (STAT3), CD44 and hypoxia-inducible factor 1-alpha (HIF1α) [18, 59, 60]. Deletions in TP53 and RB1 are also common [18]. These type of tumors are aggressive and develop into much more advanced stages, often leading to metastasis [61]. Although high levels of lymphocyte infiltration is a characteristic of these, the lymphocyte do not seem to be protective [61]. Treatment for these types of tumors consists of an aggressive treatment plan of cisplatin-based chemotherapy, as basal tumors may respond well to this type of platinum-based therapy [61, 62]. High levels of activating EGFR mutations could also be indication of possible targeting of these mutations. *In vitro* studies have shown that basal MIBC tumor cell lines are sensitive to the use of erlotinib (an anti-EGFR molecule), indicating a potential therapeutic benefit in patients with high EGFR mutations [63, 64]. Basal cells also have a reportedly high level of immune-checkpoint markers and therefore, could be good candidates for immune checkpoint inhibitors [58].

Other important molecular subtypes are the luminal subtypes, which include luminal papillary, luminal non-specified and luminal unstable. The cells originate from the cells in contact with the lumen of the bladder, also called the umbrella cells of the urothelium. Luminal subtypes, especially luminal papillary, have been shown to be the least aggressive subtype and are found in younger patients [18]. Luminal subtypes are characterized by the mutations in *FGFR3* and *ERBB2* gene, and activation of PPARγ pathways [60, 61]. Activation of estrogen receptors and it's pathway have also been found in these molecular subtypes [60]. Luminal molecular subtypes have low immune profiling and therefore, do not make good candidate for immune checkpoint therapies [65]. Luminal subtypes do not respond to neoadjuvant chemotherapy neither [62]. However,

radiation therapy could be an interesting alternative for luminal subtypes as these cells lines have been shown to be at least moderately sensitive to radiotherapy, as they are killed more efficiently by this treatment [66]. Out of the three luminal subtypes, the luminal unstable has the highest sensitivity to radiotherapy since it has the highest expression of cell cycle markers [58, 67]. Therefore, exploring radiation therapy in a luminal-like model could be of interest for these types of patients. Many mouse models and cell lines have been created to study these different molecular subtypes and their responses to treatment, in an *in vivo* setting.

2.6 Mouse Models of Bladder Cancer

Different mouse models have been used to study bladder cancer, mostly focusing on either xenograft models or allograft models most of the time. An example of a xenograft model is a patient-derived xenograft, or PDX. The use of patient derived tissue, which is then engrafted into a mouse to further study it. This method allows to recapitulate exactly the type of tissue that is seen in the clinic. PDX models in the context of MIBC were shown to retain 92-97% of the genetic aberrations once transferred from the patient into the mouse, proving this model is accurate in showing what is occurring in the clinic [68]. The allograft models are murine cells lines which are then engrafted in the mouse as well. This allows the use of immunocompetent mice, as there is no risk of rejection of the implanted tissue.

Many different murine cell lines have been developed to study bladder cancer *in vivo*. Three have been developed in the commonly used C57/B6 mice. These include MB49, BBN and UPPL. Another cell line, MBT-2, has also been developed in C3H/He mice. Out of these cell lines, MB49, BBN and MBT-2 are considered basal-like models, whereas UPPL is considered a luminal-like model.

The MBT-2 cell line is often used for orthotopic models of bladder cancer [69]. It is derived from bladder tumors in female C3H/H3 mice [70]. These tumors are induced in mice using the carcinogen N-[4-(5-nitrofuran-2-yl)-1,3-thiazol-2-yl] formamide and have epithelial-like characteristics [69]. This cell line was originally very aggressive, which did not allow for long-term studies in mice. MBT-2 was then manipulated to decrease cell proliferation through the dampening of *c-myc* activity, which allowed mice to survive longer [69]. MBT-2 cells express markers found in basal-like molecular subtype such as KRT5, KRT14 and HIF1α. These also have high PD-L1 expression, which recapitulates the basal molecular subtype as well [68].

MB49 cell line is the most commonly used cell line to study bladder cancer. It is derived from male mice bladder tumors induced through the exposure of the carcinogen 7, 12-dimethylbenz[a]anthracene (DMBA) [68]. Similar to human cells from the basal molecular subtype, the MB49 cell line has high expression of CD44. These cells also have mesenchymal characteristics, such as epithelial mesenchymal transition (EMT) signalling and fibrosis markers [68]. MB49-I is a more invasive and aggressive form of the MB49 cell line that produces metastasis. This cell line was developed through continuous passaging of the original cell line [71].

BBN cell line was produced through the exposure of C57BL/6 mice to the carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) through drinking water. This method causes high-grade and invasive orthotopic tumors in the mice. This cell line replicates the basal molecular subtypes in human through the high frequency of mutations in *TP53* [72].

Finally, the UPPL cell line, is produced through the knockout of *PTEN* and *TP53* (Upk3a-CreERT2; Trp53L/L; PtenL/L; Rosa26LSL-Luc) derived from male C57BL/6 mice [65]. *PTEN* and *TP53* are common mutations in MIBC, and have been shown to promote invasiveness [73]. These cells lines have characteristics similar to the human luminal subtype cells, such as a papillary

histology and low immune infiltration. Markers found in the luminal subtype, such as high expression of PPARγ and GATA3, are also found in the UPPL cell line [65].

3. Radiation Therapy

3.1 Introduction to Radiation Therapy

Radiation therapy consists of high-energy beams that are delivered to the tumor and target cancerous cells. The delivery of either x-rays or γ-rays allows for DNA damage in cancerous cells, since these are more due to their high proliferation rate [74]. The rays work by passing through the tissues, breaking the chemical bonds and creating ions by removing the electrons from atoms. These ions can then cause serious damage to the cancer cells [74]. Irradiation can also cause damage to the organelles themselves, and can damage the endoplasmic reticulum (ER), mitochondria, ribosomes and lysosomes [75]. Radiation therapy locally controls tumors in certain types of cancer but has yet to be efficient in all types. A secondary effect of radiation therapy has been described over the last decade called the abscopal effect. The abscopal effect is a systemic immune anti-tumour response that is induced after radiation therapy of a specific local region [76]. Different cells can be affected in the tumor microenvironment (TME), and different effects can be seen in the process such as an increase of pro-inflammatory cytokines [77]. Different dosing and regimens can be given, and each have their own effects on the system and the TME.

3.2 Radiation Therapy in Cancer

3.2.1 DNA Damage and Repair Mechanisms

Radiation therapy induces DNA damage, which causes cell death in cancer cells through apoptosis, necrosis, senescence, mitotic catastrophe and autophagy [74]. The DNA damage caused

by radiation therapy includes base damages, DNA crosslinking and DNA-protein crosslinking, single-strand breaks (SSB) and double-strand breaks (DSB) [74, 75]. DNA base damages are modification of bases, which then affects the DNA double helix structure. Base damages are play a small role in cancer cell death due to radiation since they are easily repaired [74]. SSB are caused by small nicks in the backbone of the DNA and are also easily repaired [74]. DNA protein-crosslinking are produced by the creation of covalent bonds between proteins and a DNA strands. This occurs in high doses of γ-rays and studies suggest that it does not play a large role in tumor cell killing [74]. The most harmful DNA damage and most important in cancer cell killing due to radiation therapy are DSB, which is highly toxic for cells [74, 75]. In studies DSB can be detected through the detection of a specific marker of DNA damage: γH2AX, which is a marker of histone phosphorylation. DSB are breaks in both strands and repair occurs in 3 stages: The induction of the damage, signalling pathways and finally the repair phase. [75]. DSB are repaired through either homologous recombination (HR) or non-homologous end-joining (NHEJ). The selected repair pathway depends on the complexity of the break, the chromatin formation and cell cycle [74, 75].

HR occur in heterochromatin and is usually more precise of a method of DNA repair, since it uses a template to repair the breaks. This method occurs specifically in S phase of G1 phase, since a sister chromatid is available for use as a template to complete the missing base pairs [74].

NHEJ is the main mechanism of DSB repair, since it is quick and is a higher affinity process. Repair occurs in multiple steps. Recruitment of Ku70-Ku80 heterodimers first occurs and form the DNA-PK holoenzyme. Incompatible ends of the break are cleaved and processed, followed by the engagement of the DNA ligase complex, which ligates the two ends together [78]. This method is error-prone since addition and deletion of certain base pairs occurs. No template is used compared to the HR methods to ensure the exactitude of the added base pairs.

Specific mutations can also affect the DNA repair pathway, such as mutations in *BRCA1* and *BRCA2*. *BRCA1* is an important player in the regulation of HR and in certain types of NHEJ. If mutation occurs in these genes, DNA repair is impaired, and sensitivity to radiation therapy increases [79]. Accumulation of damages that are poorly repaired will cause cell death.

3.2.2 Cell Death Induced by Radiation Therapy

Cell death is defined as the loss of replicative ability of the cell and in turn, the loss of viability. Radiation therapy can induce different types of cell death [80]. Cell death can occur in either a in controlled cell death, or an uncontrolled one, which causes more damage to surrounding tissues [80]. The different cell death types that can be induced due to radiation therapy include apoptosis, necrosis, necroptosis, senescence, mitotic catastrophe, autophagy and immunogenic cell death [74, 80-82].

Apoptosis is considered a type 1 programmed cell death and is often called "cellular suicide" as it is a controlled type of cell death. It is one of the most prevalent types of cell death induced by radiation therapy. The morphological characteristics include cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and the formation of apoptotic bodies. Apoptotic bodies are small vesicles containing the contents of the dead cells, to facilitate phagocytosis by macrophages and reduce collateral damage to surrounding tissues [74, 80]. When the damage is too large, apoptosis is triggered by three different pathways that involve caspases (proteins that degrade cellular proteins): the intrinsic pathway, the extrinsic pathway and the ceramide pathway [82]. The intrinsic pathway uses caspase 8 as an initiator caspase whereas the extrinsic pathway uses caspase 9. The ceramide pathway is an important pathway induced by radiation therapy. All three pathways converge to activate caspases 3, 6 and 7, which degrade the proteins [74, 80, 82].

Necrosis is considered and uncontrolled cell death, due to extreme environmental conditions which include radiation, hypoxia, heat and pH changes [74, 80, 82]. These conditions induce an increase in pro-inflammatory conditions such as the upregulation of nuclear factor κB (NF-κB). The cell starts to swell (oncosis) until the cell membrane ruptures and the contents of the cells are spilled into the extracellular environment, which can cause damage to surrounding tissues [80]. Necrosis is usually determined through morphological analysis at endpoint, therefore, does not give the method of cell death, but rather indicates that the contents of the cells were spilled into the extracellular environment [83]. If apoptotic bodies are not properly phagocytosed, they lose membrane integrity and can spill their contents as well and resemble necrosis at endpoint. Therefore, a more definitive term of necrosis should be oncosis followed by necrosis [80].

Necroptosis, a recently discovered cell death, is a regulated type of necrosis [82]. This process is activated by death receptors, more specifically tumor-necrosis factor receptor 1 (TNFR1). When this receptor is bound by its ligand, a caspase independent pathway is triggered, that induces the formation of a necrosome, which causes membrane permeabilization [80, 82]. High doses of radiation were shown to inhibit the caspase 8 pathway in certain cancers, which pushes the production of the necrosome and the necroptosis pathway instead [80, 84]

Senescence is the permanent cell growth arrest. This is triggered by telomere shortening, p53 and retinoblastoma protein (RB) signalling and DNA damage [74, 85]. These are morphologically enlarged and flattened and increase reactive oxygen species (ROS) production. These cells eventually die by apoptosis. Low doses of radiation increase senescence and aren't high enough to induce apoptosis, which can help reduce negative side effects [74].

Mitotic catastrophe, also called mitotic cell death occurs when there is defect in mitosis, due to bad chromosome segregation [74]. This is an important mechanism of cell death in radiation

therapy seen in solid tumors. This mechanism is a delayed type of mechanism, and is often seen later after radiation therapy has been given [86]. Cells undergoing mitotic catastrophe are morphologically large cells, with multiple nuclei [86]. There are two different known mechanisms of mitotic cell death. The first one induced due to premature entry into mitosis and a weakened G2/M checkpoint in the cell cycle. This process is thought to be due to an ineffective p53 activation [74, 86]. The second pathway triggers mitotic cell death through the hyperamplification of centrosomes [86].

Autophagy is the process by which damaged organelles in a cell are segregated in vesicle to be targeted for lysosomal degradation upon cellular stress. When this process is excessive, autophagic cell death occurs [82]. This process is seen in both normal and cancerous cells [74]. Radiation causes autophagic cell death through ER stress and the activation of the mTOR pathway. ER stress causes protein folding and triggers the unfolding protein response, triggering radiation-induced autophagy [87]. The mTOR pathway, more specifically the PI3K-Akt-mTOR pathway is the most important pathway in the radiation-induced autophagy. PI3K activation occurs through different stimuli such as hormone, growth factors, oncogenes. The pathway leads to activation of mTORC1, which inhibits autophagy [87]. Studies have conflicting findings on the role of autophagy on the response to radiation therapy. Whether autophagy promotes radiosensitivity or radioresistance is still debated in the literature [74]

Immunogenic cell death (ICD) is the process which is thought to recruit immune cells for killing of the tumor cells and induces immune memory [81]. Overall, ICD allows for an increase in dendritic cell (DC) phagocytosis of tumor cells, DC processing of the tumor antigens, release of IL-1β by the DC in the extracellular environment, and subsequent priming of CD8+ T cell lymphocytes for specific tumor killing [81]. Three events are necessary to induce DC priming and

activation for ICD: cell surface translocation of calreticulin (CRT), release of HMGB1 into the extracellular environment and release of ATP into the extracellular environment. All three event can be triggered by radiation therapy [81]. CRT translocation on the surface of tumor cells acts as an "eat me" signal for DC to phagocytose tumor cells [81]. HMGB1 found in the extracellular environment acts like a danger signal. Through binding to either RAGE, TLR2 or TLR4 receptor, HMGB1 increases pro-inflammatory pathways in immune cells. Radiation induces the release of HMGB1 from tumor cells through necrosis [81]. Finally, free ATP in the extracellular environment binds to P2XR7 purinergic receptor. This process allows the increase of production and release of IL-1β from DC's. ATP is released from dying tumor cells following radiation therapy.

3.3 Radiation Therapy Resistance

Historically, 4 factors determined the response to radiation, called the "4 R's of Radiotherapy". They are: repair, reassortment, repopulation and reoxygenation [88]. Later, a 5th R was added: radiosensitivity [88]. Finally, even more recently, reactivation of anti-tumor immune response was added as the 6th R of radiotherapy [89]. Many different cellular pathways are involved in radiation resistance of tumor cells, which include adaptive pathways, DNA damage repair pathways, adhesion pathways, inflammatory pathways, developmental pathways, hypoxic pathways and RTK-PI3K/Akt pathways. All of these have important regulators that play a role in the response of cancer cells to radiation therapy [74]. Radiation therapy induces changes in the immune system, hypoxic and fibrotic processes. Although radiation increases pro-inflammatory signals, there exists a balance in our immune system. This triggers an increase in immunosuppressive pathways, which can promote resistance to radiation [77]. Changes in the TME induced by radiation therapy can also induce hypoxia. A hypoxic environment reduces the

production of reactive oxygen species (ROS), which in turn reduces cell death. This environment prevents DNA damage from occurring and therefore, promotes radiation resistance [77]. HIF1α also independently plays a role in promoting resistance [77]. Finally, fibrotic processes occur due to chronic inflammation and trigger tissue remodeling and repair. Myofibroblast transformation that occurs during fibrotic events causes excess deposition of components in the extracellular matrix (ECM) that causes scarring of tissues and can impair organ function [77]. All three of these events can be targeted by drugs to try and prevent radiation resistance.

To promote an active immune system and decrease immunosuppressive activity, ICIs have been developed. Certain molecules, such as vascular endothelial growth factor (VEGF) or HIF1α can be targeted and blocked to reduce hypoxia. Finally, growth factor signalling and signalling pathways for TME remodelling can be targeted by certain drugs to reduce fibrotic processes [77].

3.4 Radiation Therapy and Fractioning Strategies

Different dosing and regimens of radiation therapy can be given to patients to achieve different effects. The conventional radiation therapy treatment (ConvRT) consists of 1.8-2.2 Gy fractions given daily 5 times per week, for 3 to 9 weeks. The total dosage will reach approximately 60-90 Gy [90]. A hyperfractioning regimen (HyperRT) can also be given where each fraction has a lower dosage (0.5-1.8 Gy per fraction) but is given more than once a day, over the period of 2-4 weeks. This regimen has proven superior to the convRT in head and neck cancer, small-cell lung cancer and non-small cell lung cancer, but has not proven beneficial in other cancer, showing that the location and the cancer type have different responses to different regimens [90]. The opposite, a hypofractioning regimen (HypoRT), also exists, where the patient is given doses of 3-20 Gy per day, only a few times in a single week. This regimen was proven as efficient as the convRT in

prostate cancer. However, it could be more beneficial for the patient since it is given over a shorter period of time and allows for less visits from the patient. [90]. In bladder cancer, a convRT regimen is given, with a total dose of 64Gy, given in 32 fractions. A hypoRT regimen also exists and was shown to be as efficient as the convRT, where a total of 55 Gy is given in 20 fractions [91]. Radiation therapy is known to have an effect on the immune system, and different dosages and fractioning regimens will have different effects.

4. The Immune System in Response to Cancer

4.1 Introduction

The immune system and cancer have now been studied for a long time, as they go hand in hand in disease progression. The immune system is first triggered by the response to danger signals. In theory, there are three phases the cancerous cells use to evade immune system detection, which are called "The Three E's". These are: elimination, equilibrium and escape [92].The innate immune system is the portion of the system which responds first. These are mostly non-specific responses that happen quickly and trigger the next phases of the immune response. Following priming by the innate response, the adaptive compartment gets activated. This response can take a longer period of time to develop but is much more specific to the threat at hand. The development of immune checkpoint inhibitors to counter the evasion of cancerous cells from immune detection has been increasingly interesting in cancer therapies over the last few years. However, the innate compartment is just as important as it is the one that triggers the immune response in the first place, and the interplay between both compartments can also be hijacked by cancerous cells. Defects in the innate portion of the response are also known to cause disease progression, and cancer therapies

have tried to manipulate this compartment, to improve the adaptive response to cancerous cells [93].

4.2 The Innate Immune Response

The innate immune response is the compartment of the immune system which is the first to react upon danger signal detection. Many cells are part of this response including macrophages, dendritic cells (DCs), neutrophils. A recent subset of cells in the innate response has been discovered, termed innate lymphoid cells. These include both a cytotoxic population (NK cells) and a non-cytotoxic population called innate lymphois cells (ILCs) [94]. The innate response is considered a quick and non-specific response with no memory. However, recent findings have discovered an immune memory in innate cells. Not as strong as the adaptive memory response, it still provides some sort of memory for a few weeks following injury [95].

4.2.1 Danger Signals

Specific danger signals are needed to induce an immune response, called damage-associated molecular patterns (DAMPs). They can be separated into two categories: pathogen-associated molecular patterns (PAMPs) and alarmins. PAMPs are derived from pathogens. In the context of cancer, alarmin are more at play, and are released by cells upon tissue injury. Different alarmins include AMPs (α-defensins and β-defensins), nuclear binding proteins, heat shock proteins and even certain metabolites. Nuclear binding proteins include nucleosome-binding proteins such as HMGB1 and HMGN1 and cytokines such as IL-1α and IL-33, who can also act like alarmins [96]. Heat shock proteins, which include HSP70, HSP90 and HSP96, are usually intracellular chaperones. When found extracellularly, they act as a danger signal for the immune

system [96]. Metabolites that are usually not found extracellularly, such as ATP, can also act as alarmins [96]. These alarmins trigger the chemotaxis of innate immune cells such as DCs and promote their maturation into antigen-presenting cells (APCs) [97]. APCs can then present the antigens to cells from the adaptive immune system, to trigger a specific response to the threat.

Tumor antigens presented by APCs are antigens that allow T cells to specifically attack the tumors. There are three different kinds of tumor antigens: tumor-specific antigens (TSA), cancer germline antigens (CGA) and tumor-associated antigen (TAA). TAA's can also be expressed by normal cells, but they are highly overexpressed in cancerous cells [98].

4.2.2 Macrophages

Macrophages are monocytes who have migrated into the tissues towards the danger signals and have differentiated. There are globally two distinct populations of macrophages: M1 and M2 macrophages [99]. M1 macrophages are considered classically activated macrophages. They are activated through the recognition of pathogens or dangers and develop to become APCs. They have pro-inflammatory properties, by secreting pro-inflammatory mediators such as tumor-necrosis factor alpha (TNFα) and nitric oxide (NO). They also attract other pro-inflammatory cells into the microenvironment such as other M1 macrophages and neutrophils [99]. M2 macrophages are considered alternatively activated macrophages and have the opposite effect of M1 macrophages. These are considered anti-inflammatory and promote a suppressive environment. They secrete anti-inflammatory cytokines such as IL-10, IL-13 and transforming growth factor beta (TGFβ), and thus, can suppress M1 macrophages. They also promote ECM modelling and remove cell debris in the microenvironment [99, 100].

Tumor-associated macrophages (TAMs) are macrophages who have infiltrated the TME. These macrophages have been educated by cancer cells, who promote the differentiation of these into anti-inflammatory phenotypes, and such, most TAMs are M2-like macrophages. Macrophages are the most frequent immune cell in the TME and can sometimes account for 50% of the tumor mass [99-101]. These TAMs promote tumor growth, angiogenesis, remodelling of tissues and suppress anti-tumor immunity and thus, promote cancer progression [101].

4.2.3 Dendritic Cells

DCs are the most important APCs and antigen presentation is considered their main role in immunity. Different subsets of DCs exist, and can be separated into two classes: plasmacytoid DCs (pDC) and conventional DCs (cDC) [102]. Only a small portion of total DCs are considered pDCs, and little is known about their mechanism of action. So far, they have been characterized to play a role in type 1 interferon production [102]. CDCs, the larger portion of DCs, can be subdivided into smaller population, which is differentiated by the expression of the CD11b marker. $CD11b^+$ cDCs are lymphoid resident and play an important role in $CD4^+$ T cell activation in the lymph nodes. $CD11b^-$ cDCs can either be lymphoid resident or non-lymphoid resident and are the most efficient APCs. This type of DC is the main portion that infiltrates into the TME [102]. Another smaller portion of DCs have recently been identified: cDC1, which are efficient in cross-presentation. These stimulate the $CD8^+$ cytotoxic T cells that are specific for tumor cells through the secretion of chemokine C-X-C motif ligand 9 and 10 (CXCL9/10). A defect in this cell type could promote an anti-tumorigenic TME and promote tumor growth [93].

4.2.4 Neutrophils

Neutrophils play an important role in response to infection, as they are the main defense mechanism of our body against invading pathogens. They occupy a large portion of immune cells, composing from 50% to 70% of immune cells circulating in the blood in humans. Neutrophils must constantly be replenished in the blood since they have a very short lifespan in circulation [103]. They are considered highly plastic cells and will have different actions in the immune response depending on the maturity of the cells and the location (Table 2). Immature neutrophils that may be circulating are considered to have an immunosuppressive activity [103].

A main mechanism of defense of neutrophils is the production of neutrophil extracellular traps (NETs). NETs are web-like structures mainly composed from the degranulation of neutrophils of their own DNA in the extracellular environment. This DNA is combined by multiple proteins attached to the structure, and have specific histone citrullination [104]. NETs have conflicting effects on the tumor in the tumor microenvironment. Some proteins bound to the DNA, such as myeloperoxidase (MPO) can have anti-tumorigenic effects and kill tumor cells [104]. However, some studies were able to show that NETs can also promote tumor metastasis, both by trapping circulating tumor cells, acting as an adhesion substrate, and by degrading the extracellular matrix, which allows extravasation of tumor cells from the primary tumor to distant sites [104, 105]. NETs can also act as a physical barrier for the tumors, and block any immune cells from physically reaching the tumors [106].

Neutrophils who have infiltrated the TME are known as tumor-associated neutrophils (TANs). A higher level of these in the TME are indicative of a poor prognosis in the clinic [103]. A high neutrophil to lymphocyte ratio is also a marker for a poor prognosis in patients [107, 108]. TANs can have both an anti-tumorigenic or a pro-tumorigenic effect on the TME. They possess

anti-tumorigenic effects through the production of ROS and NO, secretion of alarmins during degranulation and by becoming APC [103, 109, 110]. However, their antigen presenting capacities are not as efficient as macrophages and DC [111]. As cancer progresses, tumour cells promote the differentiation of TANs into a pro-tumorigenic phenotype, promoting tumour growth [109]. These pro-tumorigenic and immunosuppressive neutrophils can also be classified as myeloid-derived suppressor cells (MDSCs).

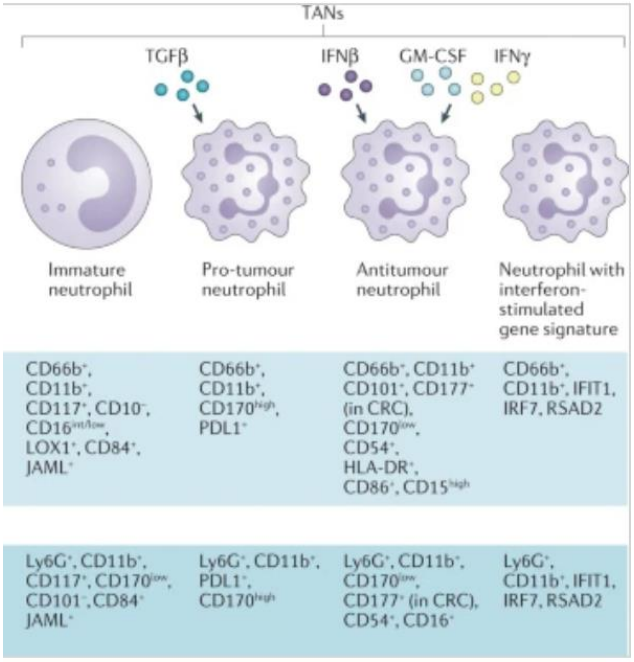


Table 2: Neutrophil Plasticity and Differentially Expressed Markers. Differences in neutrophil markers depending on maturation and location. Different neutrophils have varying anti-inflammatory or pro-inflammatory effect. Their differentiation is promoted through different mediators. Figure adapted from Jaillon. *et al.* [103].

4.2.5 Myeloid-Derived Suppressor Cells (MDSC)

MDSCs are myeloid-derived cells that expand under pathological conditions such as cancer, autoimmune diseases and inflammation. These cells are not fully matured cells that are recruited prematurely, and thus, have more of a suppressive phenotype. Their expansion occurs under the

detection of certain metabolites such as prostaglandins, stem-cell factors, vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). However, activation of these cells mostly occurs from by-products produced by T cells or tumor stromal cells, such as interferon gamma (IFNγ), TLRs, IL-4, IL-13 and TGFβ [112]. Although their main function is immunosuppression, MDSCs were shown to also promote angiogenesis and tumor metastasis [113]. MDSCs can be further subdivided into 2 distinct populations: M-MDSC ($CD11b^+$ and $Ly6C^{high}$) and PMN-MDSC ($CD11b^+$ and $Ly6G^+$). Both of these populations are suppressive and have been shown to promote the development of regulatory T cells (T_{REG}), which are immunosuppressive T cells [112]. The M-MDSC subset is most similar to macrophages and have their suppressive effect through production of nitric oxide through inducible nitric oxide synthase (iNOS). PMN-MDSC more closely resemble neutrophils and have their immunosuppressive effect through peroxynitrates products from endothelial nitric oxide synthase (eNOS) [112, 113]. These are also able to inhibit other myeloid cells and NK cell function. There is still debates in the field on whether pro-tumorigenic neutrophils and PMN-MDSCs are two distinct immunosuppressive population of neutrophils or not, and more research is necessary to elucidate these cells [114]. Some markers have been identified to differentiate PMN-MDSCs and neutrophils, such as CD84, but these have not been confirmed by usage in the overall research community, and therefore, needs to be looked into in more detail to confirm these findings [115].

4.2.6 Innate Lymphoid Cells

The innate lymphoid cells have a similar morphology to lymphocytes, but do not contain any specificity to viruses or memory. The innate lymphoid compartment can be subdivided into 2 distinct populations: NK cells and ILCs [94, 116]. NK cells are considered the cytotoxic portion

of innate lymphoid cells. These make-up 10-20% of peripheral blood mononuclear cells (PBMCs) and are important players in tumor cell killing and virus-infected cell killing [117]. Their cytotoxic activity can occur through 2 different pathways. The first involves perforin and granzyme B, which create pores in the cell membrane and induce necrosis of the targeted cell. The second pathway involved the binding of FasL to the targeted cell, and triggers induction of apoptosis [117]. Many immunotherapies have been researched and developed to target the NK cell compartment in cancer. These include strategies such as adoptive transfer, direct stimulation of NK cells, blocking inhibitory signals of NK cells, and increasing NK cell recruitment in the TME [118]. ILCs are considered the non-cytotoxic portion of innate lymphoid cells and can further be divided into ILC1, ILC2 and ILC3. ILC1s have high IFNγ signatures and high expression of T-bet transcription factor. ILC2s are important in parasitic infection clearances and produce T_{H2}-associated cytokines upon stimulation through IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). Finally, ILC3s depend on RORγt and IL-7Rα expression and produce cytokines such as IL-17A and IL-22 [94, 116]. The role of ILCs in tumor development is still up for debate. Some studies show that NK cells and ILCs can help clear tumor cells. However, other work shows that they could potentially promote tumor growth. Therefore, more research is needed to elucidate their role [94].

4.3 The Adaptive Immune Response

The adaptive immune response follows the activation from the innate immune response and is more specific to the target danger and creates a memory response for future exposure to the specific threat. There are two compartments of the adaptive response, a humoral response, induced by B cells and a cell-mediated response, induced by T cells. The humoral response allows for the production of specific antibodies by the B cells, who can target the danger. The cell-mediated

response involves T cells, either $CD4^+$ or $CD8^+$. These T cells are activated by the innate response, though APCs in the lymphoid organs.

4.3.1 Activation of T cells

The first step is T cell activation, which must occur through 3 signals. The first is antigen recognition. This occurs through the presentation of antigens on the major histocompatibility complex (MHC) on the APCs. Different T cells can have different roles and therefore, bind different MHCs. $CD4^+$ T cells will recognize antigens presented on MHCII, whereas $CD8^+$ T cells will recognize those presented on MHCI [119]. At the same time, the second signal must occur, also termed the co-stimulatory signal. Naïve T cells express CD28 on their cell membrane, which must bind to B7-1 or B7-2 on APCs to become activated and promote their expansion [119]. Finally, a cytokine signal must also be present to differentiate the T cells. Different cytokine stimulation will differentiate the T cells into different populations, specific for the response needed to the current danger [119]. Once activated, the T cells can exert their specific roles in the immune system. A specific subset of T cells will later go on to develop into memory T cells, who will allow a stronger and quicker response to the same antigen, if ever encountered again. Both $CD4^+$ T cells and $CD8^+$ T cells can create memory T cells.

4.3.2 $CD4^+$ T cells

$CD4^+$ T cells have many different roles, depending on their differentiation promoted through cytokines in the extracellular environment. Specific T cells called helper T cells promote a pro-inflammatory environment, and differentiation mainly into T_{H1}, T_{H2}, T_{H9} and T_{H17}. A subset

of anti-inflammatory, regulatory T cells (T_{REG}) also exist to counter the helper subsets. Cancer progression mostly depends on T_{H1} and T_{REG} involvement in the TME.

$CD4^+$ helper T cells promote recruitment of $CD8^+$ cytotoxic T cells into the TME, which help target and kill tumor cells. $CD8^+$ T cells are activated through the production of IL-2 into the TME from the $CD4^+$ helper T cells. $CD4^+$ helper T cells also promote an anti-inflammatory environment by maintaining DCs. This occurs through the increase in expression of CD40 on the T helper cells, which binds to DCs and allows them to continue having their effective functions [120]. T_{H1} differentiate into this subset by stimulation through IL-2 and IFNγ. They promote cytotoxic killing tumor cells through their own production of IFNγ and TNFα [120].

On the other hand, T_{REG} cells promote an immunosuppressive environment. These cells have been developed for self-maintenance and to mediate return to homeostasis following an intense immune response. These were first described to be $CD4^+CD25^+$ T cells with immunosuppressive properties [121]. Later, these cells have been characterized by their important expression of FoxP3 transcription factor and are mainly identified through expression of FoxP3 in the literature. The induction of T_{REG} cells occurs after encounter with TGFβ, and mainly produce anti-inflammatory cytokines such as IL-10 and TGFβ [120]. These cells have been shown to play an important role in cancer progression, and a higher infiltration of T_{REG} cells in the TME is considered a poor prognosis in cancer diagnosis [122]. T_{REG} cells promote an anti-tumour response through their expression of CTLA-4, which competitively binds to B7-1 and B7-2 on APCs and blocks their efficient activation of helper T cells. Thus, promoting a decrease in T cell activation and a dampening of the anti-inflammatory response [122]

4.3.3 $CD8^+$ T cells

$CD8^+$ T cells are also called cytotoxic T cells (CTLs). These are known to have a direct anti-tumour effect and are important in cell killing. A first mechanism by which CTLs promote cell killing is through the perforin and granzyme B pathway, which forms pores in the cell membrane and initiate apoptosis of the targeted cell [123, 124]. The second mechanism is through FasL-Fas binding. FasL expressed on CTLs can bind to the Fas receptor on tumor cells or target cells and trigger a downstream effect intracellularly in the target cell, that leads to the apoptosis of the cell[123, 125]. Finally, CTLs are very prominent producers of IFNγ, which can have many anti-tumour effects such as, improving antigen presentation of APCs, increasing production of ROS and NO, increasing the recruitment of APCs and T cells in the TME and help block oncogenic pathways used by tumor cells to expand [126], Neutrophil to lymphocyte ratios, more specifically neutrophil to $CD8^+$ T cell ratio has been correlated with a poorer outcome in multiple different cancer such as bladder and non-small cell lung cancer [127-129].

4.4 Immune Response in Cancer

Tumors are considered very heterogenous. There are both intertumoral and intratumoral differences in the tumors. Intertumoral heterogeneity is the differences between tumors, and can be affected by aspects such as genetics, differences among people (such as age and lifestyle) and different types and subtypes of cancer [130]. Differences in the tumor itself also exists, called intratumoral heterogeneity. These differences can be intrinsic, such as differences in the type of cancerous cells due to clonal growth, or extrinsic, such as changes in the TME. [130]. One of the differences in the TME, are immunological changes. These differences can be classified by two types: hot tumor and cold tumors.

Immunologically hot tumors are considered immune inflamed. They have high T cell infiltration, high IFNγ, increased expression of PD-L1 and higher tumor mutational burden. These types of tumors respond better to ICIs due to their higher T cell infiltration and expression of PD-L1 [131]. On the other hand, cold tumors have very low T cell infiltration. They can be subdivided into two groups: Immune excluded and immune desert. Immune excluded cold tumors have $CD8^+$ T cells on the outer edge of the tumors, but these cannot efficiently penetrate the tumor core. Immune desert cold tumors have no $CD8^+$ T cells in the periphery of the tumor [131]. Cold tumors have low mutational loads, low expression of MHCI receptors and low PD-L1 expression. Other immune cells can be found in these types of tumors such as TAMs, T_{REG}, and MDSCs. These do not respond to ICI treatments [131]. Therapeutic attempts have been made to turn a cold tumor into a hot tumor, to allow ICIs to have an effect. These attempt target poor activation of T cells, low homing of T cells and defective APCs, all aspects found in cold tumors [98].

A newer subdivision of immune differences in tumors has been suggested, called immune archetypes. These are more precise than cold and hot tumors and provide a more specific classification. They include 12 different groups, which are: immune rich $CD8^+$ T cells and macrophages, immune rich $CD8^+$ T cells and monocytes, immune rich $CD4^+$ T cells, immune stroma $CD8^+$ T cells, immune stroma $CD4^+$ T cells and macrophages, T cell centric macrophages, T cells centric DCs, myeloid centric DC 1, myeloid centric DC 2, Immune desert $CD4^+$ T cell and macrophages, immune desert monocytes and finally, immune desert $CD8^+$ T cells and macrophages [132]. These 12 groups would allow for a more specific subdivision of tumors depending on the type of cell infiltrating and in turn allow for more precise treatment of these patients with different immunotherapies.

4.5 Radiation Therapy and its Impact on the Immune System

Radiation therapy can have a dichotomous effect on the immune system. It can promote an anti-tumorigenic environment, by promoting activation of the immune system and promoting ICD of tumorous cells. On the other hand, radiation can also induce a suppressive environment and in turn, promoting a pro-tumorigenic effect in the TME [133].

Radiation therapy can trigger ICD response in many different ways and activate the immune system. First, production of type 1 IFN (IFNα and IFNβ) is increased following radiation therapy. Cytosolic DNA, which can be caused due to the effect of radiation on DNA damage, is detected in the cell by the cGAS-STING pathway. This pathway ultimately leads to the production of these type 1 IFNs, which are considered pro-inflammatory [133, 134]. Type 2 IFN can also be upregulated intratumorally, and trigger an anti-tumor response in the TME [133]. Activation of NK cells also occur following radiation, and an upregulation of MHC Class I expression allows for increased antigen presentation from APCs. Radiation also induces the release of DAMPs in the microenvironment, which allows activation of APCs as well [133]. Finally, radiation can trigger systemic effects called the abscopal effect. The abscopal effect is defined as clearance of distant tumors, that were not specifically targeted by radiation therapy. By radiating the local tumor, distant tumors also regress [135]. This effect occurs through ICD. Activation of APCs locally can provide systemic effects as these cells can migrate and activate distant T cells which can in turn, have a systemic role in distant tumor clearance. While giving radiation does trigger abscopal effect, the occurrence rate is still low in the clinic. Combining it with another therapy such as ICI, may improve the abscopal effect [81, 136].

One the other hand, radiation therapy can also induce an immunosuppressive environment as well. Radiation induces the recruitment of immune suppressive cells such as T_{REG} cells, MDSCs

and promotes the development of pro-tumorigenic TAMs and TANs [133]. T_{REG} cells were also shown to be more resistant to radiation induced death, which creates an immune selection of these cells in the TME over other immune cells, which can promote an overall immunosuppressive environment [137]. Immune suppressive cytokines are also produced in the TME upon radiation, more specifically TGFβ and IL-10 [133]. Finally, radiation also induces the upregulation of PD-L1 on tumor cells. This is done through 4 different pathways: IFNγ production, EGFR pathway, the cGAS-STING pathway and DNA damage. IFNγ production from $CD8^+$ T cells, triggers upregulation of immunosuppressive molecules such as PD-L1 on tumor cells [133, 138]. Both the cGAS-STING pathway and the EGFR pathway all induce PD-L1 expression through triggering the JAK/STAT pathway [133]. DNA damage, more specifically DSB produced by radiation therapy induces the increase of PD-L1 on tumor cells as well, through the ATM/ATR/Chk1 pathway [139].

5. Immunomodulatory Methods Used in Cancer Therapies

Immunomodulatory methods have increased in the cancer field as potential therapeutics, as this system plays in cancer clearance or cancer progression. Here, we detail two different potential targets explored: PD-1/PD-L1 pathway and NET targeting.

5.1 Immune Checkpoint Inhibitors

Immune checkpoint molecules are used by the immune system to keep it in homeostasis. After an immune response, these molecules are used to bring back the immune system to an inactivated state, once the danger is cleared. There are many different molecules used to achieve the end result. In cancer, the most important pathway is that of PD-1/PD-L1 interaction.

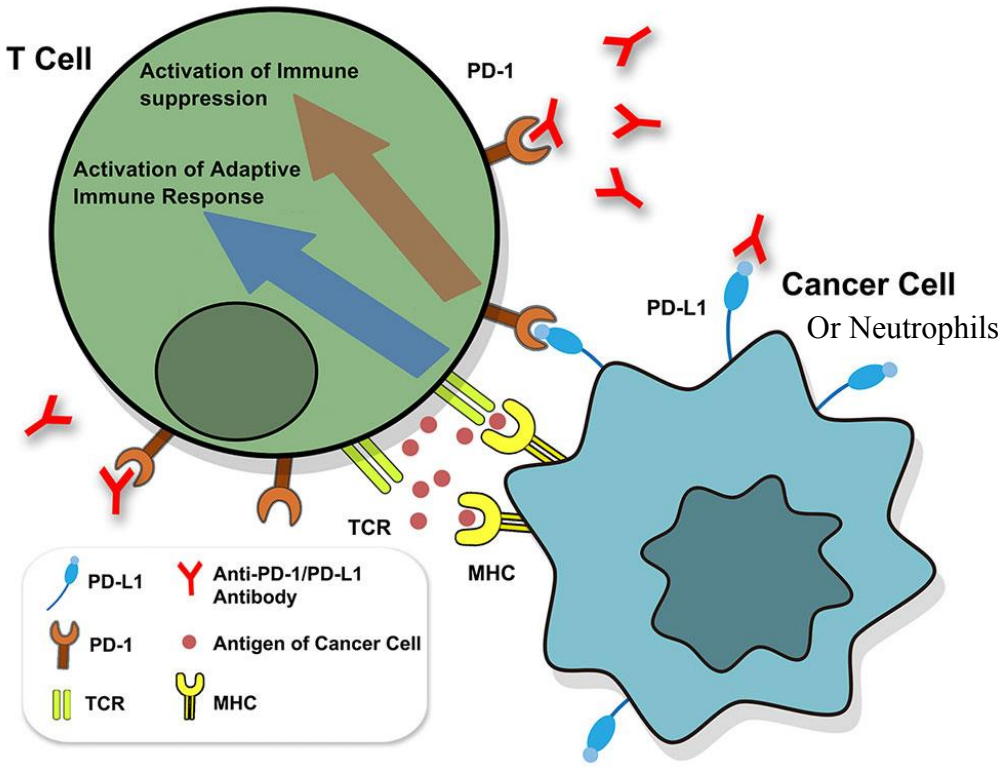


Figure 2: The PD-1/PD-L1 pathway. Schematic of the PD-1/PD-L1 pathway showing the interaction between the two molecules and the role of anti-PD-1 and anti-PD-L1 therapy blockade. Figure adapted from Zhang *et al.* [140]

The PD-1/PD-L1 pathway allows for immune tolerance once activation is not needed anymore. PD-1 binds to its receptor PD-L1 and PD-L2 [141] (Figure 2). PD-1 is expressed on T cells once they are activated. The expression of PD-1 slowly increases as a surface marker on these cells. Studies have shown that PD-1 expression is even higher in tumor-specific T cells [141]. PD-L1 on the other hand, is expressed on many different cells such as macrophages, DCs, epithelial cells and sometimes some T cells and B cells can also express it on their membrane surface. Neutrophils can also express PD-L1 on their surface to dampen the immune response and is correlated to a pro-tumorigenic phenotype [103, 142]. Tumor cells will also express PD-L1 on their surface to evade the anti-tumour response of the immune system. PD-L1 expression is upregulated upon increase in IFNγ in the TME, $CD8^+$ T cell responses and characteristically T_{H1}

immune responses [141]. Although PD-L1 is known mostly for its inhibitory signals, it can also play different roles outside of its role in the immune system. PD-L1 can induce effects such as EMT transitioning and a stem cell-like phenotype in cancer cells [141]. PD-L2, has very similar functions as PD-L1, but it is almost exclusively expressed on APCs. It was shown to bind to PD-1 with a higher affinity than PD-L1 [143, 144].

To counter the effect of cancer cells blocking immune activation through this pathway, many molecules were developed. One of these are ICIs. ICIs block the PD-1/PD-L1 interaction through a monoclonal antibody that binds to either PD-1 or PD-L1, and in turn physically block their interaction, and allowing T cells to remain active [145]. Some anti-PD1 molecules used in cancer include nivolumab, pembrolizumab and cemiplimab. Several anti-PD-L1 molecules include atezolizumab, durvalumab and avelumab which are currently in advanced clinical trials [143, 146]. In bladder cancer, ICIs are mostly used in metastatic cases. As discussed previously, atezolizumab and pembrolizumab can be used as a first line of treatment under certain conditions. Although, only 30% of metastatic cases will end up responding to ICIs [147].

Resistance to ICIs in cancers is still not very well understood. There are two different pathways of resistance known so far to ICIs: primary or acquired resistance. Primary resistance occurs when the patient does not respond to the blockade from the start and will never respond. This can occur due to T cell exclusion, resistance to interferon signalling or a local immune suppressive environment [148]. Acquired resistance, on the other hand, occurs gradually. Patients initially respond to ICIs but develop a resistance once they have a secondary treatment phase. This occurs through multiple processes such as loss of T cell function or disrupted antigen presentation [148]. Recent studies have found other mechanisms by which resistance develops. One of these is through the upregulation of other inhibitory molecules such as TIM-3 and VISTA. TAMs were

also shown to clear the monoclonal antibodies that have bound to PD-1 on T cells. Finally, tumor mutation could also occur to reduce the efficiency of T cell killing [149, 150]. Research is still ongoing to find ways to counter these resistance mechanisms to improve the response rate of ICIs in cancer therapies.

5.2 DNAse I

NETs play an important role not only in cancer development. As discussed earlier, NETs were shown to promote tumor progressions through enhancing tumor metastasis through trapping circulating tumor cells and by degrading ECM, which allows tumor cells to extravasate and settle at a distant site [104, 105]. Tumor cells also promote the differentiation of TANs into a pro-tumorigenic phenotype, to promote their growth and survival. These NETs have also been shown to play a role in other diseases such as autoimmune diseases and infections [151]. A simple way to counter NET production is to degrade the DNA portion using DNAse I. DNAse I is an enzyme which breaks down DNA and therefore, in this context, degrades any NETs produced. By giving DNAse I, systemic degradation of NETs occurs. Currently, recombinant human DNAse is a standard of care for cystic fibrosis [152].

CHAPTER 2: Rationale

Radiation therapy (RT) is an appealing bladder-sparring treatment plan for muscle-invasive bladder cancer. As it was previously mentioned, RT is known to cause an increase in immune cell infiltration to the radiated site, and also systemically, through the abscopal effect. Combining RT with anti-PD-L1 therapies could also offer a better treatment response. In the hot tumor MB49 MIBC mouse model, our team was able to previously show that the combination of these two did improve overall survival and improve immune response, both *in situ* and through an abscopal effect [153]. More work on the MB49 model was done by our team and we were able to identify that neutrophils, through the formation of NETs, play a role in RT resistance. This is done by physically blocking $CD8^+$ T cell infiltration into the tumor core, which was rescued by administering DNAse I [154]. All this work being done on a hot tumor model. Whether it can be applied to a cold tumor model for MIBC (such as UPPL mouse model) requires further investigation.

The UPPL cell line is considered a cold tumor model and therefore, has a very low immune infiltration. Our team has recently started working with this cell line, given to us by Dr. Kim [65], and was able to demonstrate that it has a higher infiltration of neutrophils and a lower infiltration of $CD8^+$ T cells when compared to the MB49 model [155] (*Thesis*). Having more neutrophils may be indicative of higher NET production in the tumor and degrading these might prove even more beneficial than what was seen in our MB49 model. Combining RT and anti-PD-L1 may not be sufficient since high NET production may block effective T cell infiltration that is necessary for a good immunological anti-tumour response. By triggering the increase of immune cells through radiation therapy, degrading NET production and blocking the PD-L1 immunosuppressive cascade

in T cells, we may be able to transform the UPPL cold tumors into hot, immunologically active tumors and allow for a better response to treatment in this model.

CHAPTER 3: Hypothesis

We hypothesize that neutrophils through NET formation and expression of surface PD-L1 are associated with a radiation therapy resistance in a cold (luminal-like) tumor MIBC model. We believe the combination of RT and anti-PD-L1 may not be sufficient to improve the response to radiation therapy and thus, we want to accentuate this through the addition of DNAse I to the combination of treatments.

CHAPTER 4: Objectives

Our objectives can be separated into two distinct aims

1) Evaluate the effect of neutrophils and NETs in a luminal-like tumor model and efficiency of radiation therapy
2) Investigate the immunological changes within the tumor microenvironment caused by the presence of neutrophils and NETs, underlying the response to radiation therapy

CHAPTER 5: Materials and Methods

5.1 MIBC Luminal-like Cell Line and Cell Culture

The UPPL1540 (UPPL for short) syngeneic bladder cancer cell line was gifted by Dr. William Kim (University of North Carolina). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Wisent) supplemented with 10% fetal bovine serum (FBS, Wisent) and were incubated at 37 degrees Celsius, with 5% CO_2. When cells have reached 70-80% confluency, these were passaged. Detachment of cells was done using 0.25% trypsin (Wisent). Cells were passaged at least twice before injection in mice and for a maximum of ten times. When necessary, cells were frozen in a 5:4:1 ratio of DMEM with FBS, dimethyl sulfoxide (DMSO, ThermoFisher) and FBS. All cell counting was done using Vi-cell-XR cell viability analyzer (Beckman Coulter).

5.2 In Vivo Mouse Model

Male C57B/6 mice were acquired from Charles River Laboratories, Inc, and kept in the animal research facility at the Research Institute of McGill University Health Centers (RI-MUHC). The facility animal care committee (FACC) approved protocol follows the standard operating procedures (SOPs) put in place. 5 000 000 UPPL cells were injected subcutaneously in the right flank of the mice, and mice were monitored regularly for tumor growth. Once tumors were palpable and established (0.1-0.15 cm^3), mice were monitored every two days and respective treatments was started. Tumor length and width was measured with a caliper, and tumor volume was then estimated using an ellipsoidal formula (estimated tumor volume = $4/3*(3.14159)*(Length/2)*(Width/2)^2$). Primary endpoint was established at an approximate tumor volume of 1.5-2 cm^3. In extreme cases, primary endpoint was set at bad skin ulcerations. Midpoint tumor volumes were cut off at 21 days post start of treatment.

5.3 Radiation Therapy

Radiotherapy was given using X-RAD SmART Irradiator Pxi 225cx (Precision X-Ray). Once tumors reached 0.1-0.15 cm^3 and were established, 2 doses of 5Gy were given, 24 hours apart, based on our previous studies [153]. Fluoroscopic guidance was used to allow for targeted radiation of the tumor only.

5.4 Immune Checkpoint Inhibitor Treatment In Vivo

PD-1/PD-L1 blockade treatment was given through anti-mouse PD-L1 injections, given intraperitoneally to mice (*InVivoMAb,* clone 10F:9G2, BioCell). These injections were given a total of 4 times every other day, at a dose of 250ug, diluted in 200 ul of PBS. Treatment was started on the same day as radiation therapy for groups containing both treatments in combination.

5.5 DNAse I Treatment In Vivo

DNAse I treatment were given intramuscularly to mice (DNAse I, Bovine Pancreas, Biomatik). These injections were given every day for a duration of 14 days then every 3 days, until the tumor have reached endpoint. The dose of DNAse I given was 2.5mg/kg, given in 50ul of 0.9% NaCl solution. Intramuscular injections of DNAse I were given on the thighs, legs were alternated between each injection. Treatment was started the same day as radiation therapy for groups containing both treatments in combination.

5.6 Tissue Dissociation

Once tumors had reached endpoint or midpoint, tumors were excised and weighed. Tumors were separated into fragments for either immunofluorescence, flow cytometry analysis, RNA sequencing or DNA analysis. The fragment used for immunofluorescence were stored in 10% formalin for future paraffin embedding (FFPE). The fragment used for flow cytometry was kept in Roswell Park Memorial Institute 1640 media (RPMI, Wisent) with a supplementation of 10% FBS. Tumor fragments were cut into smaller pieces, before adding mouse tumor dissociation enzyme kit (Miltenyi Biotec). These were then dissociated using a gentleMACS dissociator (Miltenyi Biotec). Once the dissociation program has been completed, samples were passed through a 70-um cell strainer to obtain a single cell suspension. The cells were cleared of red blood cells through the treatment with ACK lysis buffer (Thermofisher). Cells were passed a second time through a 70um cell strainer to get rid of any clumps that may have been caused by the lysing buffer treatment. Cells were counted, and then extracellular flow cytometry staining was started and continued the next day.

5.7 Flow Cytometry Staining and Analysis.

Single cell suspension was then separated in 3 different immune panels to observe different types of immune cells. The first panel was used to identify myeloid immune cells. The second panel was used to identify different T cells. The third panel was used to assess the functionality of the different T cells through the production of cytokines. All samples were first stained with a viability dye (viability eFluor780, eBiosciences) and then blocked with an anti-CD16/CD32 used as an Fc Block (CD16/CD32 (Invitrogen)). Cell suspensions were then stained for extracellular markers and fixed for further intracellular markers. Cells were fixed using the

FOXP3/Transcription factor staining buffer set (Invitrogen, eBioscience). Staining for intracellular marker was then done the following day.

The first panel used for myeloid cells used the following antibodies: rat anti-mouse F4/80 – BUV393 (clone T45-2342; BD Bioscience), hamster anti-mouse CD11c – BUV737 (clone N418; BD Bioscience), rat anti-mouse Ly6G – BV421 (clone 1A8; BioLegend), rat anti-mouse PD-L1 – BV605 (clone 10F.9G2; BioLegend), rat anti-mouse CXCR4 – BV711 (clone L276F12; BioLegend), rat anti-mouse CD45 – FITC (clone 30-F11; BD Bioscience), rat anti-mouse MHCII – PerCP/Cy5.5 (clone M5/114.15.2; BioLegend), rat anti-mouse CXCR2 – PE (clone SA04E1; BioLegend), rat anti-mouse CD170 – PE/Cy7 (clone S17007L; BioLegend), rat anti-mouse iNOS – APC (clone CXNFT; Invitrogen) and rat anti-mouse CD11b – AF700 (clone M1/70; BioLegend).

The second panel for T cells used the following antibodies: hamster anti-mouse KLRG1 – BUV395 (clone 2F1; BD Bioscience), rat anti-mouse CD3 – BUV737 (clone 17A2; BD Bioscience), rat anti-mouse PD1 – BV421 (clone 29F.1A12; BioLegend), rat anti-mouse CD8 – BV510 (clone H35-17.2; BD Bioscience), rat anti-human/mouse RORyt – PE (clone AFKJS-9; Invitrogen eBioscience), rat anti-mouse/rat FoxP3 – FITC (clone FJK-16s; Invitrogen eBioscience), rat anti-mouse CD45 – PerCP/Cy5.5 (clone 30-F11; BD Bioscience), armenian hamster anti-human/mouse Helios – PE/Cy7 (clone 22F6; BioLegend), rat anti-mouse TIM3 – APC (clone B8.2C12; BioLegend) and rat anti-mouse CD4 – AF700 (clone GK1.5; Invitrogen eBioscience).

The third panel was first stimulated with a Cell Stimulation Cocktail Plus Protein Transport Inhibitors (500x) (Invitrogen, eBioscience) to stimulate cytokine production in the immune cells and inhibit the secretion of these in the extracellular environment. Cells were stimulated with the cocktail for 4 hours before being washed and proceeding to the staining steps. The third panel used

the following antibodies: rat anti-mouse CD45 – BUV395 (clone 30-F11; BD Bioscience), rat anti-mouse CD3 – BUV737 (clone 17A2; BD Bioscience), rat anti-mouse IL17A – V450 (clone TC11-18H10; BD Bioscience), rat anti-mouse CD8 – BV510 (clone H35-17.2; BD Bioscience), rat anti-human/mouse RORyt – PE (clone AFKJS-9; Invitrogen eBioscience), rat anti-mouse/rat FoxP3 – FITC (clone FJK-16s; Invitrogen eBioscience), mouse anti-human/mouse GranzymeB – PerCP/Cy5.5 (clone QA16A02; BioLegend), rat anti-mouse IFNγ – PE/Cy7 (clone XMG1.2; BD Bioscience), rat anti-mouse TNF – APC (clone MP6-XT22; BD Bioscience) and rat anti-mouse CD4 – AF700 (clone GK1.5; Invitrogen eBioscience).

Fluorescence minus one (FMO) controls were produced to acquire through flow cytometry to allow for better gating. The stained cells from all three panels were acquired using the BD LSRFortessa X-20 (BD Biosciences). All files obtained were analyzed using FlowJo 10.4 software.

5.8 Statistical Analysis

Statistical analysis was performed using GraphPad Prism v.6.0c. Survival curve statistics were done using the Mantel-Cox statistical test. 2-Way ANOVA's with Holm-Sidak correction for multiple comparisons were used for analysis of frequencies in immune infiltration. To compute the ratios, non-parametric Kruskal-Wallis test with Dunn's multiple comparisons was performed. All statistical analysis was considered significant with a p value <0.05.

CHAPTER 6: Results

6.1 Assessing tumor growth of the mice treated with different combination of RT, anti-PD-L1 and DNAse

Since the UPPL model has not been very well studied and its response to different treatment regimens is also unclear, we have set to use this model for the duration of our study. The UPPL mouse cell line allows the study of a luminal-like model which represents a cold tumor model, with low immune infiltration. Therefore, we injected mice on the right flank with 5 million UPPL cells, in a 200ul of phenol free media and growth and survival was monitored. Primary endpoint was set at 1.5-2 cm^3 for the endpoint mice, and 21 days for the midpoint mice. The midpoint was selected at 21 days since we hypothesized that the treated mice will survive over 40 days. Mice were randomized in the different treatment groups once their tumors had reached 0.1-0.15 cm^3 (Figure 2).

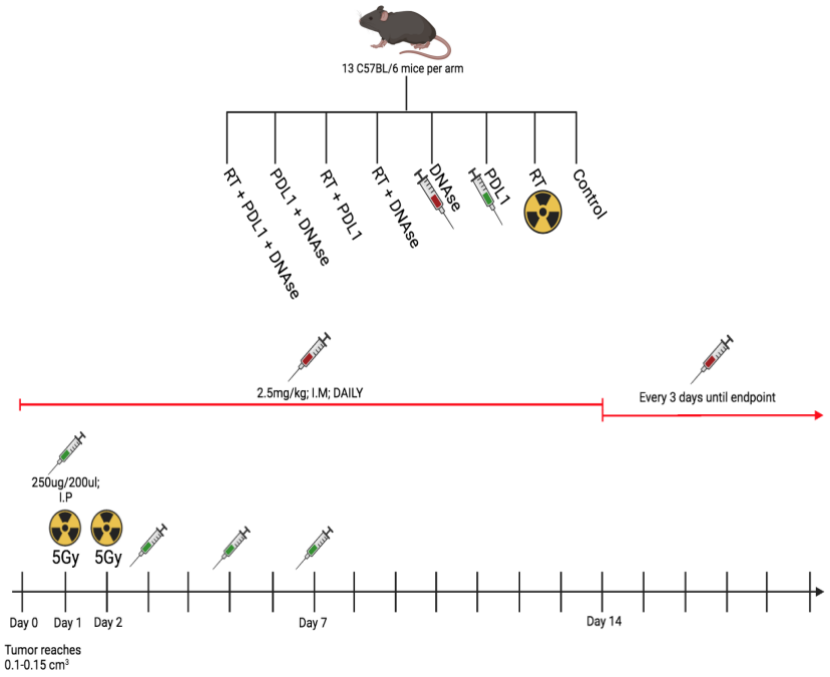


Figure 2: Treatment Plan of the* In Vivo *Study. Mice were randomized in the 8 different treatment groups identified in the figure. RT was given twice, with 24 hours between radiation sessions, 5 Gy each session. Anti-PD-L1 (green syringe) was given in 4 doses, every other day. DNAse I injections (red syringe) were daily for 14 days, after which, it was given every 3 days.

Tumor growth and survival of 8 mice was monitored until the primary endpoint of 1.5-2 cm^3 reached, after which these were plotted and analyzed. These are considered the endpoint mice. Survival plots were separated in groups with RT and groups without RT to facilitate analysis. Treatment with anti-PDL-L1 did not significantly increase survival compared to the control group. DNAse I treatment had a median survival of 31 days, which is significantly longer than the control or the anti-PD-L1 treatment alone. The combination of anti-PD-L1 and DNAse I performed similarly to the DNAse I treatment alone, showing the addition of anti-PD-L1 does not add any benefit in the context without RT (Figure 3). The addition of either anti-PD-L1, DNAse I or both to RT treatment did not significantly improve survival when compared to the group treated with RT alone (Figure 3). All RT groups survived longer than the Control groups (Figure 3).

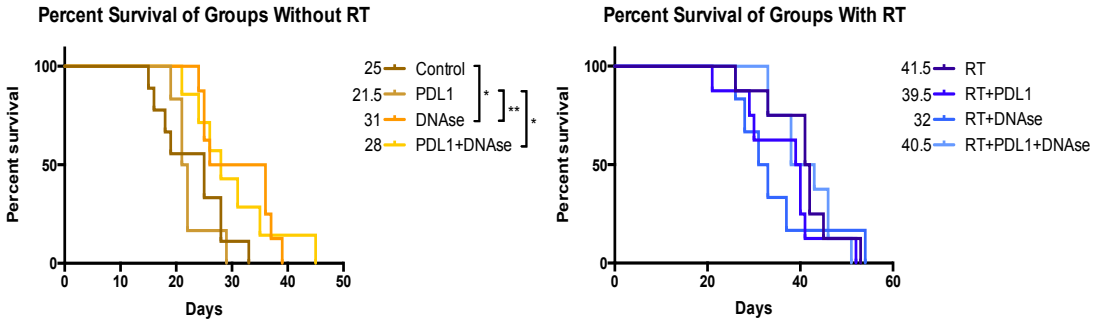


Figure 3: Survival Curves of the Mice. Survival curves were plotted with the 8 mice which were monitored. PDL1 represents treatment with monoclonal anti-PD-L1. Median survival is represented for each group and Log Rank (Mantel-Cox) test was conducted to determine significance, with $p < 0.05$ considered significant.

We've plotted the tumor growth of each individual mice in each treatment group (Figure 4). We distinguished that within each treatment group, two distinct population with different tumor growth kinetics. The control group was used as reference. All mice in this group reached endpoint before day 33. Therefore, we used day 33 as threshold to differentiate the mice into two groups. Within each treatment arm, some mice did not survive past day 33, whereas some mice had a slower growth kinetic and survived past day 33. We've classified these mice as non-responders and responders respectively (Figure 4). Each treatment arm had both non-responders and non-responder, with the exception of the triple combination group. The triple combination treated group only has responder mice. These differences within the group cause large heterogeneity in the results when pooling all the data per treatment group, which could cause masking of effects of the treatment in the responder mice.

We were able to show that day 33 is an accurate threshold to determine whether a mouse responds or not to the treatment. To do this, we've plotted both the mean tumor growth and survival curves of responder and non-responder (Figure 5). Tumor growth kinetics showed to be statistically different among the responder and non-responder. Survival curves showed a significantly larger survival median in the responder compared to the non-responder in each group (Figure 5). Therefore, day 33 is an adequate threshold to distinguish between responder and non-responders. Performing the analysis by segregating between responder and non-responders will allow us to determine what differences may be masked by the heterogeneity in each population.

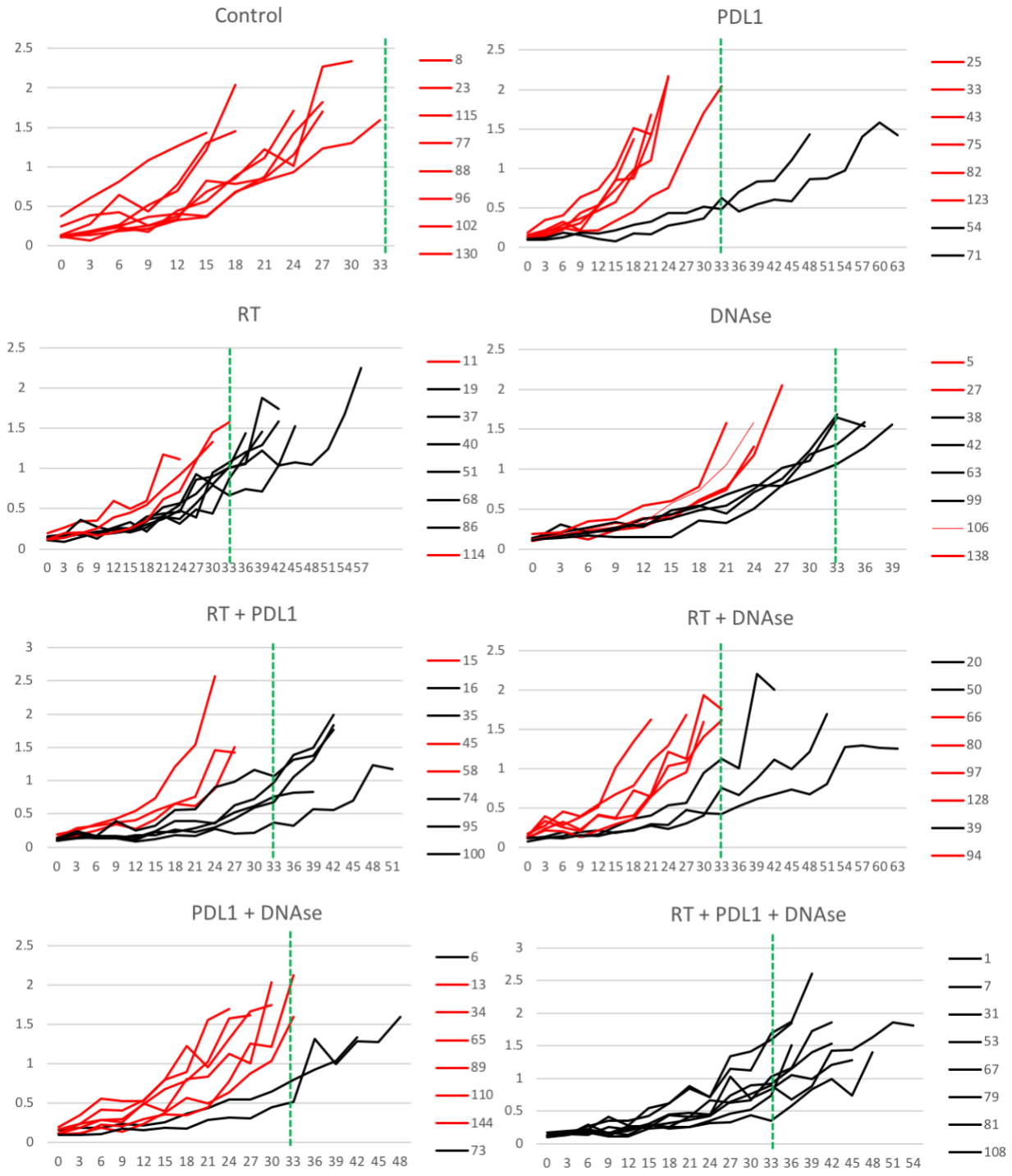


Figure 4: Tumor Growth of Individual Mice Until Endpoint is Reached. Each number represents a different mouse. The x axis represents the number of days while the y axis represents the estimated tumor volume calculated through the formula mentioned in the methods, using the length and the width of the tumor. The red lines in each graph represent the non-responders for each treatment group, while the black lines the responders. Green dotted lines represent the day 33 threshold to differentiated between the responder and non-responders.

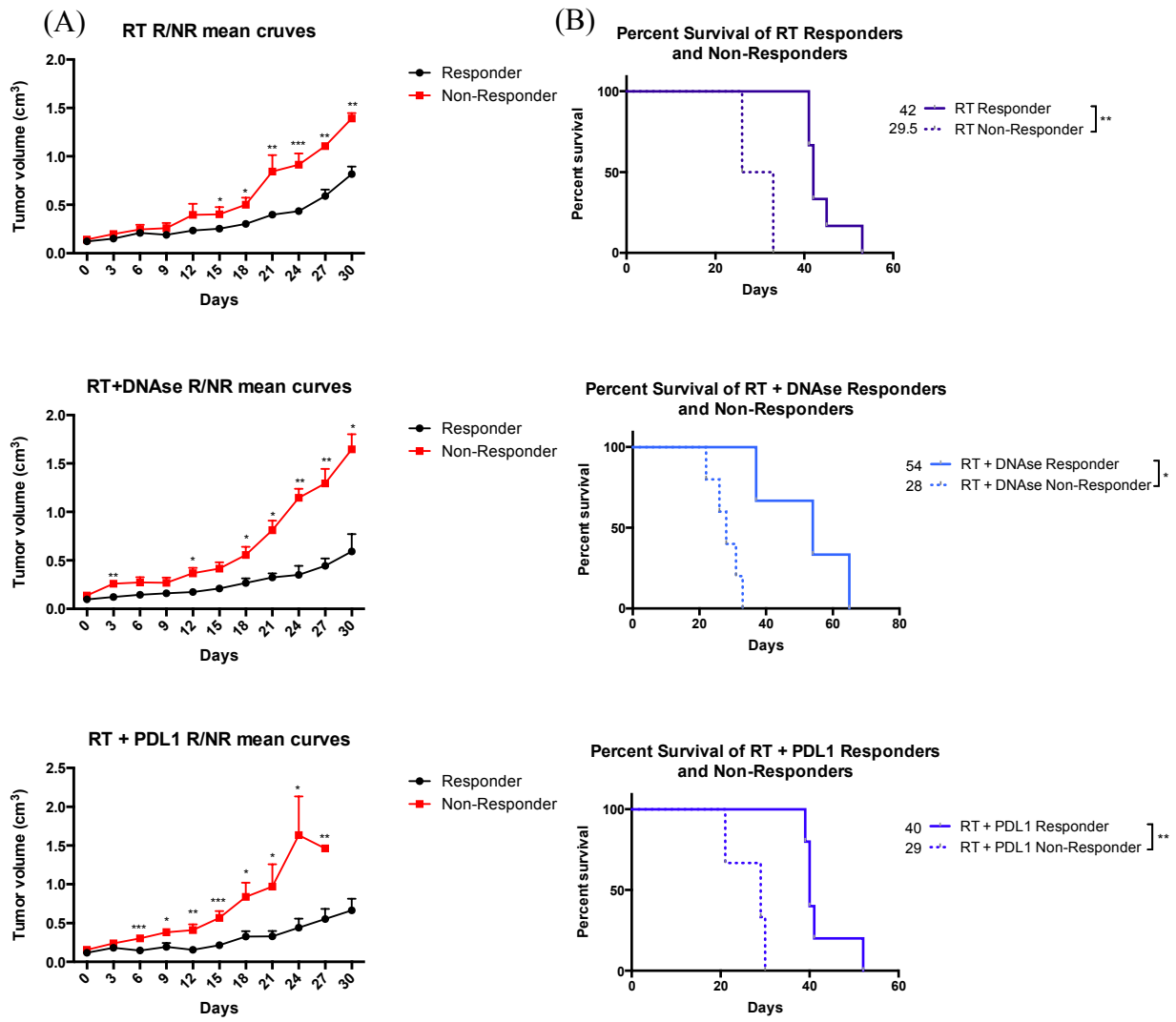


Figure 5: Mean Tumor Growth and Survival of Responder and Non-Responder. (A) Mean tumor growth of responders and non-responder in 3 treatment groups. Statistics were down using a multiple T test, with Holm-Sidak correction for multiple corrections. $p < 0.05$ was considered statistically significant. (B) Survival curves were plotted for the responder and non-responders in each of the 3 treatment groups. Median survival of each group is represented next to each group. Statistical analysis was done using Log Rank (Mantel-Cox) test. $p < 0.05$ was considered statistically significant.

6.2 Assessing Immune Infiltration of Responder and Non-Responder Mice in the Groups Receiving Radiation Therapy

To determine whether differences in response among the responder and non-responder within each of the four RT treated groups are due to differences in immune infiltration, flow cytometry analyses were done on tumor samples from the endpoint mice. Myeloid and T cell analyses were done with different gating strategy (Figure 6). Once final population are obtained, percentages of total $CD45^+$ cells (encompasses all of the immune cells) were obtained and graphed for responder and non-responders of the RT groups (Figure 7). To determine whether any differences could be observed among overall responders versus non-responders, we have grouped the data from three of the RT groups: RT alone group, RT and anti-PD-L1 group and RT and DNAse I. No statistical differences can be observed in the T cell compartment among responders and non-responders. In the myeloid compartment, only more neutrophils were observed among the non-responder group, but the differences did not reach statistical significance (Fig 7, $p=0.1819$). To understand why these disparities are seen at endpoint we sought to understand what is happening immunologically at an earlier timepoint, which are our midpoint mice, sacrificed 21 days after treatment. Of relevance, the responder and non-responder groups start segregating around day 21 (Figure 4). Therefore, events occurring earlier in the response could impact tumor progression across the different treatment arms.

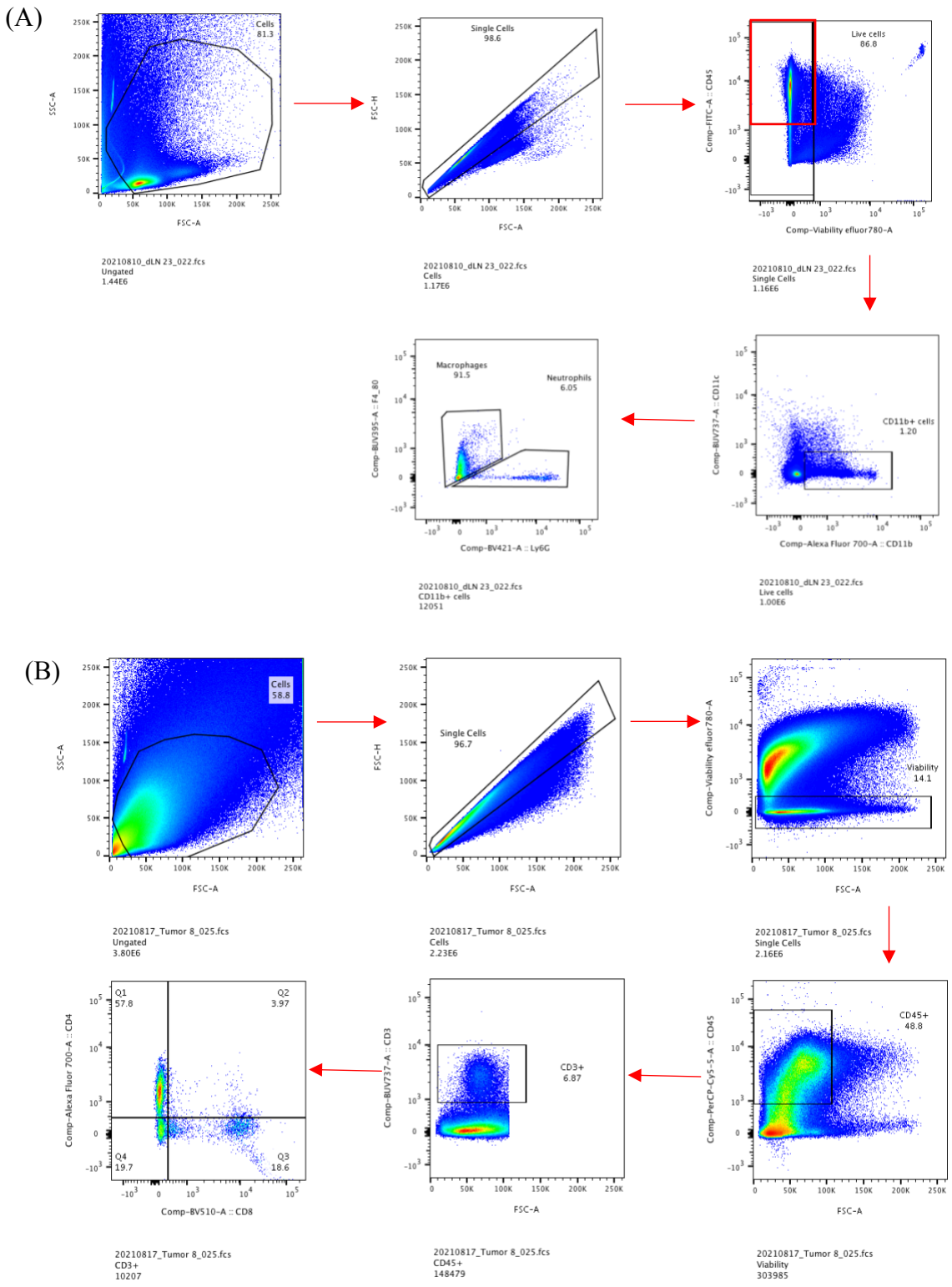


Figure 6: Flow Cytometry Gating Strategy for Immune Cells. All gating strategies are shown up until specific cells are attained. After which, each of the isolated immune populations was analyzed for specific markers for functionality of the cells. All flow cytometry analysis was done

using FlowJo v. 10.4. For both myeloid and T cell gating, lymphoid cells were first gated from FSC-H and FSC-A. Following which, doublet cells were excluded from the selected cells (A) Gating strategy for myeloid cells. Once all single cells were obtained, gating was done on all viable $CD45^+$ cells. Neutrophils were gated as $CD45^+CD11b^+Ly6G^+Ly6C^{low}F4/80^-$. Macrophages were gated as $CD45^+CD11b^+F4/80^+$. Dendritic cells were gated as $CD45^+CD11c^+MHCII^+$ (not shown). (B) Gating strategy for T cells. Once single cells were obtained, gating was done on viable cells, and then on $CD45^+$ cells. Cells were then gated for $CD3^+$ cells, with which we could get our populations of interest: $CD4^+$ and $CD8^+$ T cells.

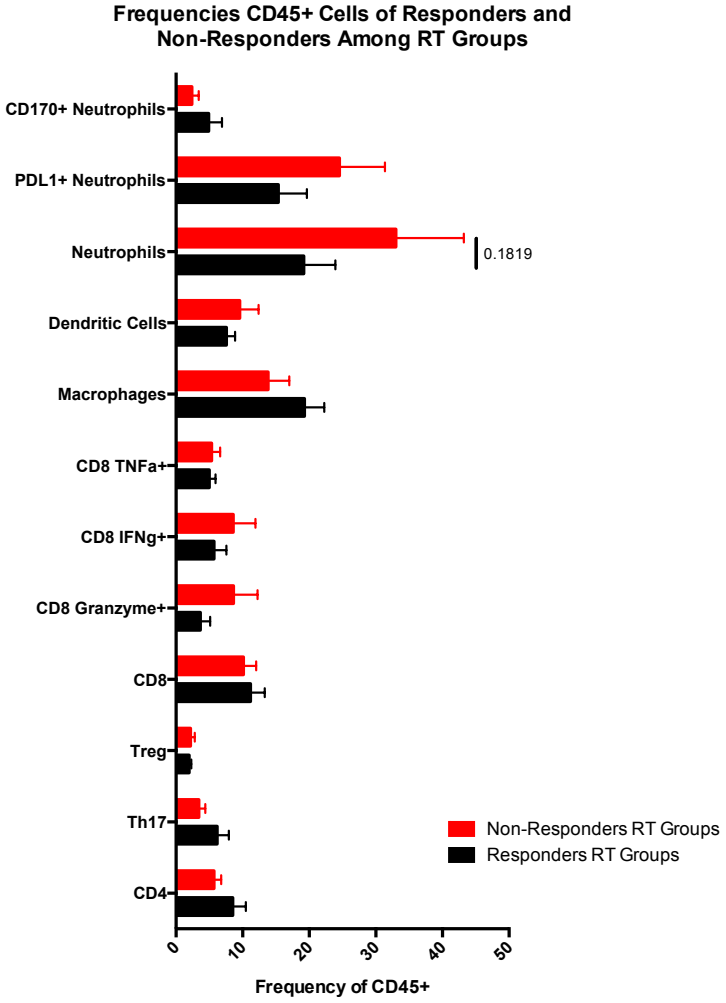


Figure 7: Tumor Immune Infiltration Among Responder and Non-Responder of the RT Groups. Percentages of each population from the total $CD45^+$ immune population. Percentages were gathered for each immune population. Markers of functionality were also added to some populations. Non-responders include all non-responders from the RT group, RT and anti-PD-L1 group and RT and DNAse I group. 2-way ANOVA was performed with multiple comparisons, with Holm-Sidak corrections. $p < 0.05$ is considered significant.

6.3 Assessing Immune Infiltration in the Tumors at an Earlier Timepoint in the Different Treatment Groups

Immune infiltration was assessed after 21 days post treatment initiation, to determine whether any immunological differences early on, could cause the delay in disease progression. The groups that had no RT treatment did not have significant variation in myeloid infiltration. However, the RT treated group had a significantly higher infiltration of neutrophils (mean of 35.6%) and more specifically PD-L1$^+$ neutrophils (mean 31.6%) and a trend pointing towards a higher CD170$^+$ neutrophils (mean 15.5%), when compared to the other groups treated with RT (Figure 8A). The other myeloid cells (macrophages and DCs), did not significantly change between any of the groups.

In terms of T cell infiltration, once again, the groups receiving no RT treatment did not vary in terms of their T cell infiltration. However, in the groups treated with RT, there are some differences and trends. Overall, RT alone had the lowest CD8$^+$ T cell infiltration (mean 12.59%), followed by the group receiving RT and DNAse I (mean 21.28%) and the group receiving RT and anti-PD-L1 (mean 27.08%). The group with the highest overall infiltration was the triple combination group (mean 34.18%). The group treated with RT, anti-PD-L1 and DNAse I had a higher infiltration of specifically cytotoxically active CD8$^+$ T cells (cells with Granzyme B, IFNγ or TNFα expression) as well. When comparing to RT alone, CD8$^+$ T cell infiltration was significantly lesser than in the triple combination treatment arm. The CD8$^+$ T cells had infiltrated less in the double combination groups and infiltrated the least in the group treated with only RT, showing an increase in infiltration as additional treatments are added to the mice (Figure 8B). However no significant different was reached when comparing the double combination to the triple combination groups. No differences in the CD4$^+$ T cell infiltration and the specific subsets.

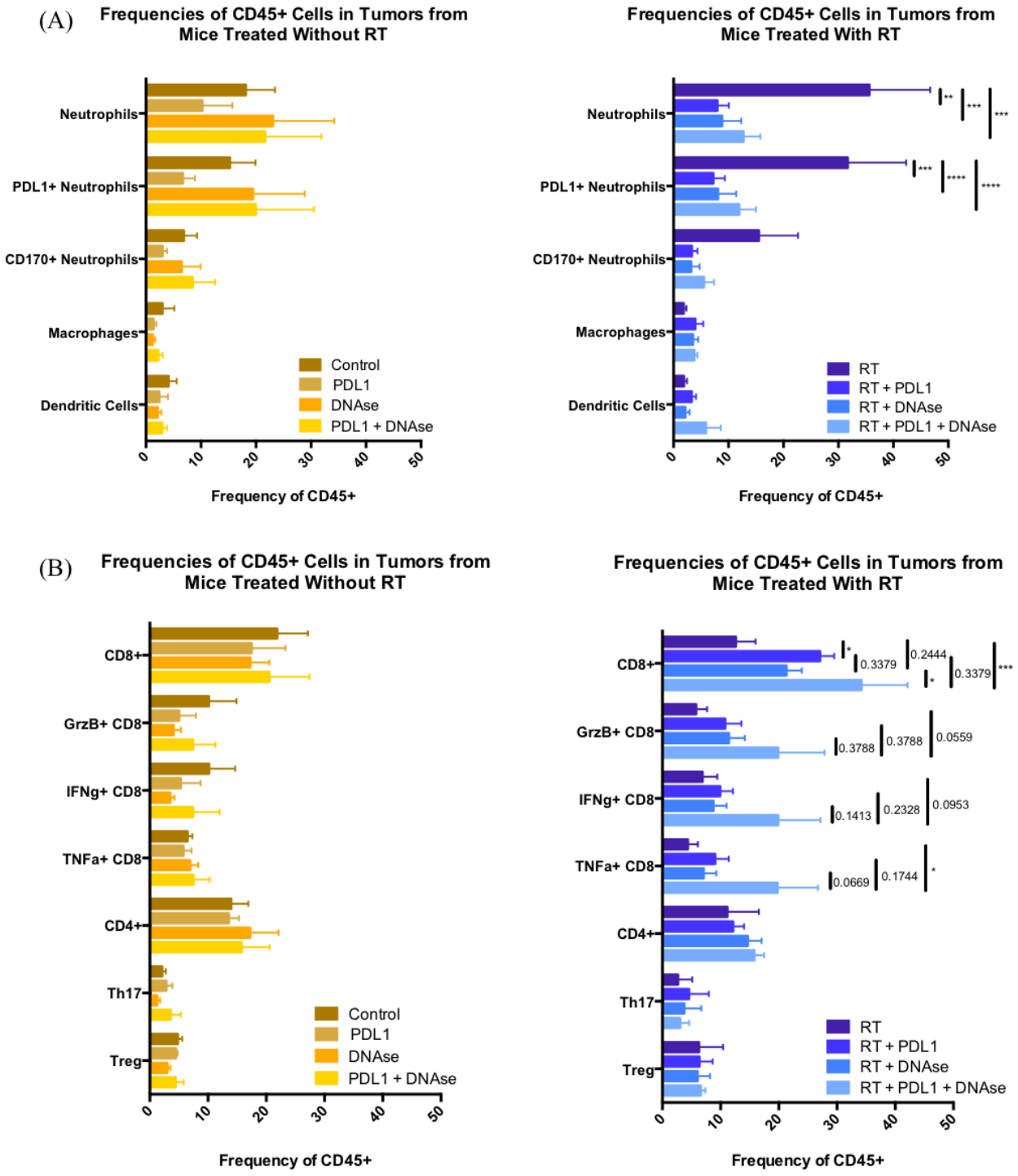


Figure 8: Tumor Immune Infiltration at Midpoint. Immune infiltration after 21 days post treatment initiation. Frequency of each subset of immune cell from the total $CD45^+$ cells is represented. (A) Myeloid tumor infiltration. Includes neutrophils and their pro-tumorigenic markers, macrophages and dendritic cells. (B) T cell tumor infiltration. Includes $CD4^+$ T cells and certain subsets (T_{H17} and T_{REG}), $CD8^+$ T cells and specific cytotoxic cytokine expression

associated with these. Statistical analysis was done using 2-way ANOVA with multiple comparisons, with Holm-Sidak correction. $p < 0.05$ was considered significant.

To summarize the immune infiltration at midpoint, especially in the data that shows variation among groups, neutrophil to $CD8^+$ T cell ratio were compiled from the frequencies of $CD45^+$ immune cells represented previously (Figure 8). The RT group had a much higher neutrophil to $CD8^+$ T cell ratio than any other of the treated groups (Figure 9A). The ratios were also done using PD-$L1^+$ neutrophils instead of overall neutrophils, to determine whether the ratio between pro-tumorigenic neutrophils and anti-tumorigenic $CD8^+$ T cells were also different among the groups. The group receiving RT also had a higher ratio compared to all of the other groups (Figure 9B).

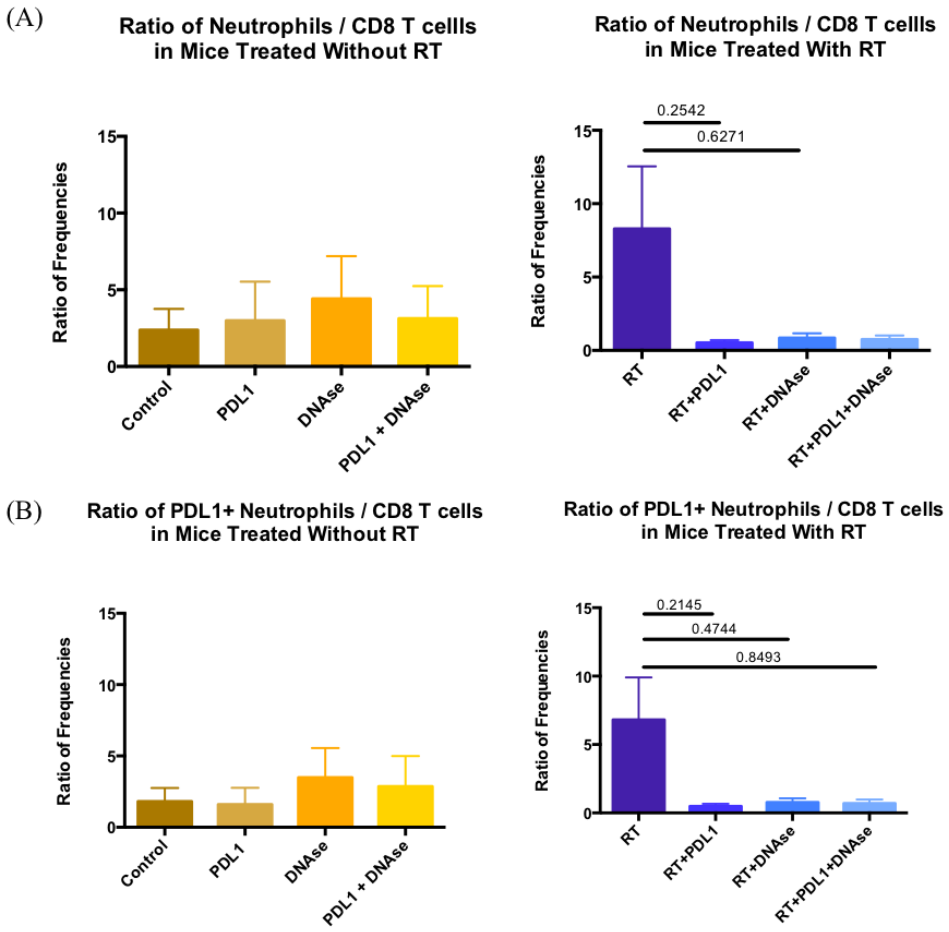


Figure 9: Neutrophil to CD8$^+$ T Cell Infiltration into the Tumor at Midpoint. Ratios were compiled using the previous infiltration frequencies. (A) Neutrophil to CD8$^+$ T cell infiltration in the tumor, for groups without RT and groups receiving RT. (B) PD-L1$^+$ neutrophils to CD8$^+$ T cell infiltration in the tumor for groups without RT and groups receiving RT. Statistical analysis was done using Kruskal-Wallis test with Dunn's multiple comparison test with $p < 0.05$ was considered significant.

6.4 Assessing Differences in Immune Infiltration in Each Groups Over Time

To determine whether these differences are sustained throughout the treatment, we compared the immune infiltrations in the control, RT and triple combination groups specifically over time. The RT and the triple combination group had the most variation in infiltration of neutrophils and CD8$^+$ T cells, and therefore, it is interesting to determine whether these variations are sustained over time. To do this, we have compared immune infiltration at midpoint with the immune infiltration of the endpoint mice in each of the three differentially treated groups.

In the myeloid compartment, we do see changes in the neutrophil distribution in both the RT and the triple combination group. At endpoint, there is a seemingly decrease in neutrophils in the RT group compared to the midpoint, whereas the opposite is seen in the triple combination group, who has a significantly large increase in neutrophil infiltration in the TME at endpoint (Figure 10A). Slight changes could also be observed in the macrophage compartment of certain groups.

In the T cell compartment, the triple combination had many changes in the immune tumor infiltration when comparing the endpoint to midpoint. There is a significantly large decrease in CD8$^+$ T cell infiltration and in cytotoxic CD8$^+$ T cell at endpoint in our triple combination group, which is not see in the other groups, who have a stable low infiltration. There was also a significant decrease in overall CD4$^+$ T cell could be observed at endpoint in the triple combination group (Figure 10B).

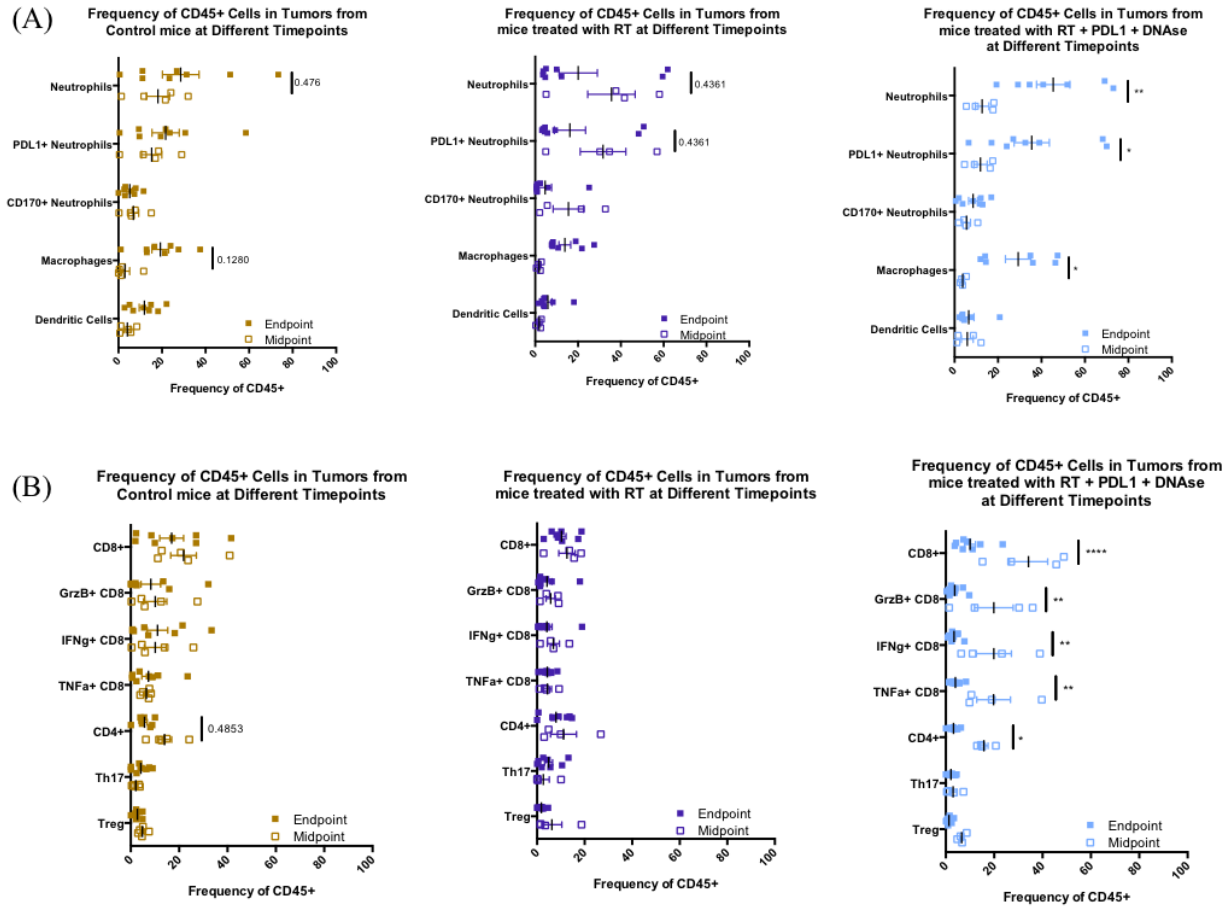


Figure 10: Variation in Immune Infiltration from Midpoint to Endpoint tumor samples. Midpoint frequencies were compared to endpoint frequencies which were computed using the same gating strategies from flow cytometry data. (A) Myeloid infiltration in the tumor in the control, RT and triple combination groups. (B) T cell infiltration in the tumor in the control, RT and triple combination groups. Statistical analysis was done using 2-way ANOVA with multiple comparisons, with Holm-Sidak correction. $p < 0.05$ was considered significant.

CHAPTER 7: Discussion

MIBC is a disease which has low survival and reduces greatly the quality of life of patients. The standard of treatment being radical cystectomy, there is a need to develop bladder-sparring strategies to maintain quality of life and improve patient care in the clinic. Currently, radiation therapy has been an interesting avenue to take for future patients, but many do not respond. We know that radiation causes an influx of immune cells in the radiated area, and therefore, understanding the underlying immune response is crucial to fully comprehend the radioresistance in patients.

So far, most of the pre-clinical work has been done using the MB49 cell. This cell line was used as a basal-like molecular model. Our team was able to previously show that this cell line has a high immune infiltration, thus, being a great hot tumor model [155]. With this model, it was shown that neutrophils infiltrate the TME after radiation, and through the formation of NETs, promote radioresistance. Degrading the NETs through the use of DNAse I or even by inhibiting their formation, our team was able to see an improvement in the response to RT, in this hot tumor model [129]. However, very little is known about the UPPL cell line, which grow as cold tumors with much higher neutrophil infiltration. Thus, the presence of more neutrophils and in turn, of more NETs might also promote radioresistance in this model as well. We seek to understand the underlying role of neutrophils in this model and following radiation therapy.

Our data shows that degradation of NETs through DNAse I treatment does improve survival when compared to control, and anti-PD-L1, strongly suggest that neutrophils and NETs do play a role in disease progression of MIBC in a luminal-like tumor model. Abrogating the NETs in an otherwise normal environment increases survival of these mice and therefore, shows the NETs play a pro-tumorigenic role in disease progression. However, treatments including RT do

not have a significant survival increase from one another, showing that the addition of anti-PD-L1 or DNAse I to RT treatment does not enhance RT efficacy nor improve the overall survival of the mice. However, a lot of heterogeneity is seen in each of the groups, as some mice respond better than others to the treatment, which could mask the effect of a better survival in these mice. When segregating the flow cytometry data into responder and non-responders and assessing the immune infiltration at endpoint into the TME, we were able to detect that neutrophils were the most important immune difference among the responder and non-responders, suggesting that these are one of the important players in response to radiation.

Early immunological events were looked at to understand why the mice start to segregate into these two groups. Flow cytometry data at midpoint showed that radiation alone, causes a high neutrophil infiltration and low $CD8^+$ T cell infiltration. The opposite was observed in our triple combination treatment which had very low neutrophil infiltration and high $CD8^+$ T cell infiltration. Functionally speaking, these neutrophils were pro-tumorigenic as they expressed PD-L1 and the $CD8^+$ T cell were cytotoxically active, as they express cytokine such as IFNγ, granzyme B and TNFα. In the clinic this ratio, also called the neutrophil-to-lymphocyte (NLR) ratio has been shown by our group to be a poor prognosis for patient, and patients with high NLR, seem to have responded poorly to RT [129, 156]. Therefore, early on in the treatment response, our triple combination treatment seems to alleviate the negative NLR ratio, allowing better infiltration of the active $CD8^+$ T cells, which promotes a slower initial tumor growth kinetic, hence why all of the mice in the triple combination group responded to the treatment, compared to the RT group alone or the double combination groups. However, this positive response is not sustained at endpoint in our triple combination group. A complete shift in the immune compartment is seen, where there is a significant increase of neutrophils and a significant decrease in $CD8^+$ T cells.

The anti-PD-L1 treatment did not seem to be effective in our luminal cold tumor model, without the addition of either RT and DNAse I. This could simply be due to the fact that the UPPL cell line being a cold tumor model, the effects of anti-PD-L1 could not be achieved. In fact, the anti-PD-L1 acts by blocking its interaction with PD-1 found on T cells which would promote an immunosuppressive environment. By blocking this interaction, we allow T cells to continuously remain active. However, as we're in a luminal-like model which is also a cold tumor model, the activated T cells cannot actually penetrate the core of the tumor and have their anti-tumorigenic effects. This is why the addition of RT and DNAse I, which trigger the immune response and allow the degradation of NETs allows anti-PD-L1 to have a stronger effect in the tumor. In fact, NETs were previously shown to physically block $CD8^+$ T cell infiltration and therefore, this phenomenon could be repeated in the cold tumor model [129]. To confirm this in the UPPL mode, immunofluorescence of tissues would be helpful. In short, RT causes and immune influx, DNAse I degrades any NETs that could physically block T cell infiltration and finally, anti-PD-L1 the does have an effect and keeps $CD8^+$ T cells active. We believe that to fully have its effect and improve response rate in RT treatment, anti-PD-L1 would need to be coupled to these other compounds in the model studied here.

The work done here also demonstrates how mice given the same treatment might not respond the same way, which is relevant to the clinic. We do see in the clinic how some patients will respond to RT, while others will not. Although this could be due to many environmental factors, we were able to show here that this could also be due to immunological factors, such as a higher infiltration of neutrophils. This could allow us to use neutrophil infiltration as a prognostic factor in the clinic but could also suggest a new avenue of treatment for MIBC. Targeting neutrophils and their formation of NETs could help improve the response to RT in the clinic. Our

triple combination treatment only had responders, showing that the combination of all three treatment options could be a viable treatment plan to improve the response to RT in the clinic. However, once treatment is switched to a less stringent DNAse I dosage, tumor growth restarts at a much higher pace then seen initially in this group. The shift in immunological changes could cause the increase in growth rate seen later on in this triple combination group. If we were able to sustain these initial immunological phenotype, more specifically high $CD8^+$ T cell infiltration and low neutrophils infiltration could be beneficial for patients in the clinic, as the tumor growth rate could be slowed down for a long period of time. Understanding the immunological shift in this population of mice could help understand ways to sustain a positive response to RT.

Many events could have explained this complete shift and the loss of an anti-tumorigenic immune environment seen in this experiment in our triple combination group. This could be caused by poor NET degradation over time, poorly timed DNAse I administration and finally, antibody production against DNAse I.

For instance, the treatment regimen could have been not stringent enough to degrade the NETs on a long-term basis. DNAse I must be administered every day for it to effectively degrade NETs and permanently remove these from the system. Neutrophils are the most short-lived cell in our immune system, reaching the target tissue after 6 to 10 hours in the circulation, and therefore must constantly be replenished by the bone marrow [157]. Since neutrophils are tightly regulated, follow a circadian rhythm and must be replenished as they have a short half-life, NETs are constantly being produced by the newly arriving neutrophils in the TME [157]. To ensure that the NETs produced by the newly arriving neutrophils are degraded, DNAse I must therefore, be given repeatedly. The UPPL tumor model can, on average take up to two months to reach the endpoint we had set, making it highly unpractical and to a certain extent, unethical, to give the mice daily

intramuscular injections of DNAse I for the duration of the 2 months. We therefore switched the regimen after 2 weeks, where we moved from daily injections to injections every three days for the duration of the experiment. Three days might not have been sufficient to maintain the phenotype we see in our midpoint mice, which could explain why at endpoint, our triple combination treatment loses the low neutrophil infiltration phenotype. Therefore, a more frequent regimens could have been more beneficial. To confirm whether this is the case in our study, immunofluorescence of the tissues at endpoint could prove beneficial. If NETs can be observed in the tissue at endpoint, we can clearly then assess that this regimen was not able to sustain NET degradation. In turn, this finding would allow us to confirm that once NETs are not degraded as efficiently, the tumor starts to progress, and a response is not sustained, proving our rationale is correct and that NETs do play an important role in this cold tumor model of MIBC.

This logic could also be applied to the other treatment given to the mice. As the kinetics of this tumor are very slow, and mice survive for 2 months following treatment initiation, giving multiple rounds of RT and anti-PD-L1 could have been more beneficial, as the effects could be lost after such a long period of time.

Another interesting possibility would be to alter the treatment regimen as to give DNAse I before RT doses. RT causes an influx in immune cells and one of the first cells to migrate to the TME are the neutrophils. By blocking NET production, we'd allow a more efficient infiltration of $CD8^+$ T cells in the TME early on, since NETs will not be able to form efficiently and durably. By giving DNAse I at least an hour prior to RT, any NETs that may be induced by RT will be immediately degraded. By giving RT before DNAse, some NETs can start being produced and impacting the TME before DNAse I could be administered and reach the target area and therefore, promoting a pro-tumorigenic microenvironment shortly at the beginning of the response.

A final possibility is the production of antibodies against DNAse I enzymes given systemically. B cells are a subset of immune cells that produce antibodies against specific antigens to target the danger at hand. B cell biology and production of antibodies is a process by which the B cell will undergo multiple rounds of antibody production in the germinal center, each round producing antibodies that have higher affinities to the detected danger or foreign object [158]. Therefore, the DNAse I used in our experiments, which is derived from bovine pancreas, could have been detected as a foreign object by the immune system, and an immune response could have been triggered against the enzyme. This would cause the DNAse I to be cleared out of the system much quicker than it would have at the beginning of the treatment. Therefore, reducing the amount of DNAse I given towards the end of the experiment, in synergy with the possibility of antibodies against the enzyme being produced, could have not been sufficient to block NET formation. NET could be degraded for a short period of time, but quickly start being produced and have their pro-tumorigenic effect in the TME. This presents a limitation to the setup of the experiment, as the slow kinetics of the UPPL tumor model does not allow us to administer DNAse I for a short period of time only.

The radiation doses used in this study also need to be optimized, as certain doses could potentially be more beneficial. Some pro-tumorigenic cells, such as T_{REG} cells are more resistant to radiation therapy compared to other immune cells [159]. This promotes the pro-tumorigenic and immunosuppressive environment in the TME, which allows cancer progression. By optimizing the dosages at which tumor cells are targeted and killed, while also preserving more of the pro-inflammatory and anti-tumorigenic immune cells in the TME, we may further enhance targeted radiation therapy in this study and eventually, in the clinic.

This experiment sets the stage for further research on immunological changes overall. Changes throughout time in our triple combination group have been observed not only in the neutrophils and $CD8^+$ T cells but also in other immune compartments. So far, the neutrophils and $CD8^+$ T cell changes in our triple combination group indicate that early on, there is an anti-tumorigenic environment, which switches to a pro-tumorigenic environment at the endpoint timepoint. An increase in macrophages towards the endpoint and a decrease in $CD4^+$ T cells are also seen, but the specific subsets of these have not been elucidated. The limitations of our flow cytometry panel do not allow us to determine whether the macrophages increasing over time are anti-tumorigenic (M1 macrophages) or pro-tumorigenic (M2 macrophages). This also applies to the $CD4^+$ T cells, which we did not have an extensive marker list to understand which subset of these is at play in the response observed. Understanding this would allow us to determine whether the changes in these other immune compartments also promote a shift towards a pro-tumorigenic environment in our triple combination group. Understanding this shift will bring us closer to finding a way to try and sustain the initial anti-tumorigenic response as long as possible. Another subset of cells that have recently been researched a lot in cancer studies are NK cells. These cells have been neglected in cancer progression for a while, but they are just as important for tumor clearance as $CD8^+$ T cells. Our panel had no marker for NK cells and therefore, their infiltration could not be assessed. Presence of NK cells in the TME could be beneficial, as they could aid $CD8^+$ T cells in tumor clearance.

CHAPTER 8: Conclusion

UPPL cell line model is a murine MIBC model, which simulates a luminal molecular subtype. It's also used as a cold tumor model, which has very low anti-tumorigenic immune infiltration, such as T cell infiltration. To determine whether we could improve radiation therapy response in this tumor model, we've tried to radiosensitize using combinations of anti-PD-L1 and DNAse I. Using both of these would allow to target T cells and increase their activity, while DNAse I would allow to degrade the NETs, which have been shown to play an important role in tumor progression in another of our murine MIBC mouse model. By modulating the immune system, we've sought out to understand the role of these neutrophils and NETs in this less commonly studied MIBC model.

By combining the different treatments, we've noticed that some mice respond to certain treatments while others do not. The main immune difference between the responder and non-responder was seen in an increase in neutrophils in mice who did not respond to RT or our double combination groups. However, our triple combination group, receiving a combination of RT, anti-PD-L1 and DNAse I only had responders. To understand why this group appeared to function this way, we observed immunological differences early in the response. We've established that this group had the highest cytotoxic $CD8^+$ T cell infiltration, coupled with a relatively low neutrophil infiltration, which could promote the response seen in all mice of this group. Nevertheless, this infiltration was not sustained over time, and better treatment regimens would be necessary to improve response and delay tumor growth even more.

Overall, the UPPL model has shown to be a good model to use in the study of MIBC, along with the widely used MB49 tumor model. Understanding radioresistance in both models, and the

differences among these models, could help predict outcome in the clinic and improve treatment of patients diagnosed with MIBC.

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