The role of HMGB1 in bladder cancer immune surveillance and radioresistance

BY

Mina Ayoub

Department of Medicine, Division of Experimental Surgery,

McGill University, Montréal

June 2018

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of M.Sc.

Acknowledgments

This research work was accomplished with the help and support of numerous people to whom I am truly indebted for making this such an incredible experience for myself.

I would like to express my deepest gratitude to my supervisor Dr. Wassim Kassouf and my Co-supervisor Dr. Ciriaco Piccirillo for providing me with the guidance and support I needed throughout my research project and for offering me an exemplary learning and research opportunity that I will remember for years to come.

I would like to extend my gratitude to Dr. Jose Joao Mansure who without his mentorship and advising, this project would not have been possible and I am truly grateful to him for all the knowledge I gained throughout these two amazing years.

A special thank for the chair of my research advisory committee Dr. Cristian O'Flaherty and the members of the committee Dr. Christopher Rudd and Dr. Woong-Kyung Suh for their interest in my research and for providing me with valuable input.

To my former and current lab members: Shurashri Shinde-Jadhav, Shraddha Solanki, Leonardo Lima Monteiro, Alexis Rompré-Brodeur, Liana Liu and Rodrigo Skowronski, I couldn't ask for a better team to work with and I am indeed grateful for all the help and encouragement you showed me.

I would like to thank also members of Dr. Piccirillo's laboratory: Fernando Alvarez, Roman Istomine, Tho-Alfakar Al-Aubodah, Khalid Bin Dhuban, Sabrina Bartolucci, Harry Yang, Qinhua Guo, Lei Li and Helen Mason for their continuous guidance. I indeed appreciate all the scientific knowledge and technical troubleshooting I learned from each one of you.

To my “masters support group” Surashri Shinde-Jadhav and Claire Wang, for all the fun time we had together, the laughs and jokes we shared and helped me overcoming tough times.

A special thanks to the urologic oncology research group at McGill University Health center - Research Institute for their valuable input and continuous support for my research. It’s been a privilege to work with all of you.

Last but certainly not least, to my sister Marina, my parents Nevein and Ayoub, I am extremely lucky to have you in my life and I am sincerely grateful for your support and encouragement throughout these two years even though we are miles apart.

Table of contents

Formatted: French (France)

Formatted: French (France)

Preface

Our research is focusing on better understanding the mechanisms of radioresistance and identifying novel therapeutic targets to improve radiation response of bladder cancer. In this context, we sought to study the effects of radiation on bladder cancer immune microenvironment and evaluate changes in the immune landscape post radiation. Furthermore, we investigated the role of a Damage Associated Molecular Pattern (DAMP) protein, HMGB1 in bladder cancer radioresistance through its function in modulating the tumor immune microenvironment.

HMGB1 has long been thought to be a nuclear protein that only exerts its functions within the cell boundaries. The main function of HMGB1 was identified as the reparation of DNA breaks by participating in the molecular mechanisms involved in repairing DNA damages. More research has led to the discovery of other intracellular functions for HMGB1 such as its role in regulating autophagy and its involvement in DNA transcription and replication.

However, a new era began after the discovery of DAMP proteins as danger signals released from cells in response to stressful events or cellular damage. Studies have then showed that HMGB1 itself is a member of the DAMP proteins family and is released from cells when they undergo necrotic cell death. Shortly after, studying the properties of this protein gained interest when research studies demonstrated its role in cancer progression and tumorigenesis.

Our group has previously investigated the effects of HMGB1 knockdown in several human bladder cancer cell lines on the radiation response of these cells both *in vitro* and *in vivo*. Results obtained from *in vitro* experiments as well as from *in vivo* work on nude mice suggested that HMGB1 knockdown improves radiation response and results in delayed tumor growth after

radiation by attenuating DNA damage repair and impairing autophagy. These findings confirmed that HMGB1 is involved in bladder cancer radioresistance through its intracellular functions.

In the current study, we investigated the role of HMGB1 as a DAMP protein in bladder cancer radioresistance given its extracellular properties in regulating many inflammatory and immune mechanisms. Using a series of *in vitro* experiments on MB49 murine bladder cancer cells, in addition to an *in vivo* syngeneic bladder cancer mouse model, we demonstrate a novel mechanism of radioresistance of bladder cancer through the immune modulatory effects of HMGB1. We have also evaluated changes in bladder cancer tumor immune landscape in response to radiation by comparing the levels of expression of a set of immune related genes between the different experimental groups.

Finally, I would like to declare that literature review, experiments and data analysis were all done by me under the supervision of my supervisor Dr. Wassim Kassouf and my co-supervisor Dr. Ciriaco Piccirillo and with the guidance of Dr. Jose Mansure. I would like also to mention that I don't have any conflicts of interest to disclose.

I, Mina Ayoub, have read, understood and abided by all norms and regulations of academic integrity of McGill University.

Abstract

Bladder cancer is the fifth most common cancer and the second among the urological cancers that affect Canadians according to the Canadian cancer society. Surgical removal of the bladder or radical cystectomy remains the gold standard treatment for bladder cancer. Less invasive treatment modalities for bladder cancer are available and have the advantage of sparing the bladder and maintaining the patients' quality of life after treatment compared to surgery. One treatment option that is currently of particular interest is radiotherapy. However, radioresistance of bladder cancer remains problematic and biomarkers for the prediction of the response of the tumor to radiation are still needed in order to better select patients that are more likely to benefit from this treatment option. In this context, HMGB1 is a highly suitable candidate to study the molecular and immunological mechanisms associated with bladder cancer radioresistance. It was demonstrated that HMGB1 is involved in bladder cancer radioresistance through its intracellular functions in promoting autophagy and DNA damage repair in cancer cells. Recently, it was discovered that HMGB1 can be passively released extracellularly from tumor cells upon exposure to chemotherapy or radiotherapy. Once outside, HMGB1 was found to regulate many inflammatory and immunological pathways and interact with a wide variety of immune cells. For example: HMGB1 can promote antigen presentation by dendritic cells within the tumor microenvironment. On the other hand, HMGB1 was shown to enhance the proliferation, survival and function of several immune suppressive cells such as MDSCs, TAMs and Tregs. Since the immune response induced by radiation therapy is dependent on the balance between the immune activation (antigen presenting cells, cytotoxic CD8 cells, CD4 T helper cells …etc.) and immune

suppressive (MDSCs, TAMs, Tregs …etc.) mechanisms, this project evaluated the role of HMGB1 in immune surveillance and its involvement in bladder cancer radioresistance.

We found that radiation induced the release of HMGB1 from bladder cancer cells. A significant radiosensitization effect was observed after the combination of radiotherapy with HMGB1 inhibition using Glycyrrhizin (GLZ) compared to radiation alone. Moreover, the combination of radiation and GLZ reduced the frequency of several tumor infiltrating immunosuppressive cells and shifted the immune balance within the tumor immune microenvironment towards more anti-tumor immune response. The evaluation of the tumor immune landscape showed significant changes in the expression of several immune related genes between the combination group and the radiation alone group.

Based on these results we concluded that extracellular HMGB1 is involved in bladder cancer radioresistance through its immune modulating functions. The inhibition of extracellular HMGB1 improves the response of bladder cancer to radiation possibly by shifting the immune balance within the tumor towards more anti-tumor immune responses. Targeting this pathway may provide a therapeutic approach to radiosensitize bladder tumors. Biomarkers such as HMGB1 and immune cells infiltration could be evaluated as potential predictive markers for radiation response.

Résumé

Le cancer de la vessie est le cinquième cancer le plus fréquent et le deuxième parmi les cancers urologiques qui touchent les Canadiens selon la Société canadienne du cancer. L'ablation chirurgicale de la vessie ou la cystectomie radicale reste le traitement de référence pour le cancer de la vessie. Des modalités de traitement moins invasives pour le traitement du cancer de la vessie sont disponibles et ont l'avantage d'épargner la vessie et de préserver la qualité de vie des patients après traitement par rapport à la chirurgie. Une option de traitement qui est actuellement d'un intérêt particulier est la radiothérapie. Cependant, la radiorésistance du cancer de la vessie reste problématique et des biomarqueurs pour la prédiction de la réponse de la tumeur à la radiothérapie sont toujours nécessaires afin de mieux sélectionner les patients les plus susceptibles de bénéficier de cette option de traitement. Dans ce contexte, HMGB1 est un candidat très approprié pour étudier les mécanismes moléculaires et immunologiques associés à la radiorésistance du cancer de la vessie. Il a été démontré que des taux plus élevés de HMGB1 sont associés à un mauvais pronostic du cancer de la vessie. Il a également été démontré que HMGB1 est impliqué dans la radiorésistance du cancer de la vessie grâce à ses fonctions intracellulaires dans la promotion de l'autophagie et la réparation des dommages de l'ADN dans les cellules cancéreuses. Récemment, il a été découvert que HMGB1 peut être libéré de manière passive extracellulaire à partir de cellules tumorales lors d'une exposition à un traitement par chimiothérapie ou radiothérapie. Une fois dehors, HMGB1 a été trouvé pour contrôler de nombreuses voies inflammatoires et immunologiques et interagir avec une grande variété de cellules immunitaires. HMGB1 peut favoriser la présentation d'antigène par les cellules dendritiques dans le microenvironnement de tumeur. En revanche, HMGB1 peut également

Formatted: French (France)

améliorer la prolifération, la survie et la fonction de plusieurs cellules immunosuppressives telles que les MDSCs, les TAMs et les Tregs. La réponse immunitaire induite par la radiothérapie dépend fortement de l'équilibre entre les mécanismes d'activation immunitaire (cellules présentatrices d'antigènes, cellules CD8 cytotoxiques, cellules auxiliaires CD4 ... etc.) Et immunosuppresseurs (MDSCs, TAMs, Tregs ... etc.), Ce projet étudie le rôle du HMGB1 dans la surveillance immunitaire et son implication dans la radiorésistance du cancer de la vessie.

Nous avons constaté que le rayonnement induit la libération de HMGB1 des cellules cancéreuses de la vessie. Un effet significatif de radiosensibilisation a été observé après la combinaison de la radiothérapie avec l'inhibition de HMGB1 en utilisant la glycyrrhizine (GLZ) par rapport au rayonnement seul. De plus, la combinaison de rayonnement et de GLZ a réduit la fréquence de plusieurs immunosuppresseurs infiltrant la tumeur et déplacé l'équilibre immunitaire au sein du microenvironnement immunitaire tumoral vers une réponse immunitaire plus anti-tumorale. L'évaluation du paysage immunitaire de la tumeur a montré des changements significatifs dans l'expression de plusieurs gènes liés au système immunitaire entre le groupe de combinaison et le groupe traité avec le rayonnement seul.

Sur la base de ces résultats, nous avons conclu que HMGB1 extracellulaire est impliqué dans la radiorésistance du cancer de la vessie grâce à ses fonctions immunomodulatrices. Cibler cette voie peut fournir une approche thérapeutique pour améliorer la réponse au rayonnement du cancer de la vessie. Des marqueurs tels que HMGB1 et l'infiltration de cellules immunitaires pourraient être évalués en tant que biomarqueurs prédictifs potentiels pour la réponse au rayonnement.

List of abbreviations

DAMPs: Damage associated molecular patterns

HMGB1: High mobility group box-1

MDSCs: Myeloid derived suppressor cells

TAMs: Tumor associated macrophages

Tregs: Regulatory T cells

NAT2: *N*-acetyltransferase-2

GSTM1: Glutathione S-transferase mu1

FGFR3: Fibroblast growth factor receptor 3

HPV: Human papilloma virus

CT scan: Computerized Tomography

RBCs: Red blood cells

HPF: High power field

MRI: Magnetic resonance imaging

TURBT: Transurethral resection of bladder tumor

BCG: Bacille Calmette Guerin

DNA: deoxyribonucleic acid

MHC-1: Major histocompatibility complex-1

CTLA-4: Cytotoxic T-lymphocyte–associated antigen-4

PD-1: Programmed death-1

PD-L1: Programmed death ligand-1

STAT3: Signal transducer and activator of transcription-3

HMGA: High mobility group AT-hooks

HMGN: High mobility group nucleosome binding

RAGE: Receptor for advanced glycation end-products

Tim-3: T cell immunoglobulin mucin-3

Formatted: French (France)

TLRs: Toll like receptors

LPS: Lipopolysaccharides

GLZ: Glycyrrhizin

PDX: Patient derived xenograft

EP: Ethyl Pyruvate

HMG-coA: 3-hydroxy-3-methyl-glutaryl-coenzyme A

VCAM: vascular cell adhesion molecule-1

Formatted: Spanish (Spain, Traditional Sort)

TNF: Tumor necrosis factor

APO-E: Apolipoprotein E

HSP: Heat shock protein

ARG1: Arginase-1

TGF: Tumor growth factor

CSF1: Colony stimulating factor

IFN: Interferon

Foxp3: forkhead box P3

NK cells: Natural killer cells

RIPA: Radioimmunoprecipitation assay

HRP: horseradish peroxidase

TBST: Tris-buffered saline-Tween 20

ELISA: Enzyme Linked Immunosorbent Assay

FACC: Facility Animal Care Committees

RPMI medium: Roswell Park Memorial Institute medium

ACK: Ammonium-Chloride-Potassium

XRT: Irradiation

CTRL: Control

qRT-PCR: quantitative real-time polymerase chain reaction

IDO: Indoleamine 2,3- dioxygenase

ROS: Reactive oxygen species

CHAPTER I: (INTRODUCTION)

Section 1: BLADDER CANCER

1.1 Epidemiology

Bladder cancer is the 5th most common cancer in Canada. Around 9000 new cases are diagnosed each year according to Bladder Cancer Canada with increased incidence in the last few decades. In 2017, it was estimated that 2400 Canadians will die from bladder cancer [1]. The risk of bladder cancer is increasing with age, peaking at the 8th decade of life and a median age of diagnosis 70 years of age. Worldwide, Bladder cancer ranks 9th most common cancer and 13th most common cause of death, with 357,000 new cases recorded in 2002 [2].

Bladder cancer is more common in men than in women (4th most common cancer in men and 12th most common cancer in women), this is possibly attributed to the higher prevalence of smoking and exposure to toxins among men. In terms of racial differences in bladder cancer prevalence, white men tend to have 3 times higher risk of developing bladder cancer than African American males; however, African American males have higher mortality rates from bladder cancer [2].

1.2 Etiology

Although not yet fully understood, a wide variety of genetic and environmental factors contributes to the development of bladder cancer. Genetic abnormalities have been proposed as risk factors for bladder cancer, specifically *NAT2* slow acetylation and *GSTM1* null genotype increase the risk of bladder cancer possibly by increasing susceptibility to external carcinogens

such as tobacco [3]. *TP53* as well as *FGFR3* mutations have been also reported as potential genetic abnormalities associated with bladder cancer [4].

In addition to genetic abnormalities, exposure to certain environmental toxins, specially smoking, increases the risk of developing bladder cancer. Another important risk factor for bladder cancer is chronic inflammation and bladder infections; 14% to 16% relative risk of developing bladder cancer with any history of urinary tract infection is reported [5].

1.3 Clinical presentation, symptoms and signs

The most common presenting symptom for bladder cancer is blood in the urine (known as hematuria) which occurs in 80% of cases and usually occurs in the absence of any pain. Other presenting complaints include pain in different anatomic locations depending on tumor stage and invasiveness, voiding symptoms including frequency, urgency and incontinence. Constitutional symptoms such as low-grade fever, weight loss, fatigue and anorexia can occur as well.

Although physical examination may not reveal anything, some signs can be present in advanced cases which usually include a pelvic mass, inguinal lymph nodes enlargement and induration of prostate gland on digital rectal examination.

1.4 Diagnosis and pathology

The gold standard for the diagnosis of bladder is cystoscopy, where a camera is introduced into the bladder through the urethra in order to visualize the lumen of the bladder and detect any structural abnormalities. A biopsy could be obtained during the procedure in order to obtain a pathological evidence of the tumor in addition to urine and bladder irrigation cytology. Bladder tumors can be present in various shapes and can take several forms within the bladder including a sessile lesion, nodule or solid mass. The extent of the tumor in terms of muscle invasion is

usually also very important in order to evaluate the tumor grade and stage. Other investigations for bladder cancer may include imaging with CT or MRI scans, intravenous pyelograms and ultrasound. Evaluation of the presence or absence of metastasis is also performed using imaging modalities and pet scans.

Urothelial cell carcinoma is the most common histologic type of bladder cancer with 90% of bladder tumors are of urothelial origin, 5% are squamous cell carcinoma and 2% are adenocarcinomas. Bladder cancer is often then further subdivided into non-muscle invasive bladder cancer and muscle invasive bladder cancer where the muscle layer of the bladder is invaded by the tumor.

1.5 Management and treatment options

1.5.1 Management of non-muscle invasive bladder cancer

a. Transurethral resection of bladder tumor (TURBT):

In general, patients with non-muscle invasive bladder cancer are offered transurethral resection of bladder tumor (TURBT) where the tumor is resected using a cystoscope. A fluorescent microscope is used in some cases in order to improve the detection of lesions and facilitate the visualization of abnormal areas. Patients with high risk non-muscle invasive bladder cancer may undergo a repeat for the procedure in order to ensure the complete removal of the tumor.

b. Intravesical therapy:

By definition, Intravesical therapy involves the instillation of therapeutic agents directly to the bladder epithelium usually two-four weeks after the initial resection of the tumor with several follow up treatments that could last for 2-3 years after surgery. Agents introduced are usually either chemotherapeutic agents with cytotoxic effects or BCG, a type of immune therapy

composed of a live attenuated *mycobacterium bovis* capable of inducing an immune response against remaining tumor cells.

1.5.2 Management of muscle invasive bladder cancer

a. Radical cystectomy:

Radical cystectomy involves the surgical removal of the bladder and the adjacent organs as well as regional lymph nodes with a subsequent urinary diversion. It is considered the gold standard treatment for patients suffering from muscle invasive bladder cancer. Treatment outcomes greatly depends on several factors including tumor stage and grade, local and distant invasion and the quality of the surgical procedure.

Although effective, radical cystectomy is usually associated with high rates of morbidity after surgery. A negative impact on general as well as disease specific quality of life of bladder cancer patients was shown to be associated with radical cystectomy [6]. Partial cystectomy can be considered for selected patients that have limited disease and lesions that involve certain anatomical sites within the bladder.

b. Systemic Chemotherapy:

Neoadjuvant chemotherapy given before radical cystectomy for patients with muscle invasive bladder cancer has been shown to improve clinical outcomes [7]. Moreover, adjuvant chemotherapy after radical cystectomy was shown to be associated with better overall survival rates and disease specific survival rates compared to cystectomy alone and it is now recommended for high risk patients [8]. Agents used are usually a combination of Gemcitabine and Cisplatin or Methotrexate, Vinblastine, Doxorubicin and Cisplatin.

In addition, concurrent treatment with chemotherapy and radiotherapy along with a maximally performed TURBT is a treatment modality offered for patients who refuse surgery and or who are not surgical candidates. This approach is usually referred to as trimodal therapy or TMT. Studies comparing outcomes of TMT versus radical cystectomy for patients with muscle invasive bladder cancer are showing promising results for TMT in appropriately selected patients [9].

c. Radiation therapy:

Radiation is usually used in combination with chemotherapy and a maximally performed TURBT in patients who do not receive radical cystectomy. In general, external beam radiation is the approach used in the management of bladder cancer and a total dose of radiation of 50 GY is usually delivered in 20 fractions of 2.5 GY each.

Several studies evaluated the efficacy of radiotherapy as an alternative treatment to radical cystectomy for muscle invasive bladder cancer patients who are not surgical candidates. Results showed a 5 years overall survival rate of around 40% with the preservation of a functional bladder [10]. A prospective study evaluated the treatment outcomes and quality of life for muscle invasive bladder cancer patients treated with TURBT and concurrent chemoradiation and showed satisfactory quality of life and good treatment outcomes [11].

Given the previously mentioned advantages, radiation therapy is considered a promising alternative treatment option for patients with bladder cancer. However, the estimated local residual/recurrence rate after radiation requiring salvage cystectomy is 25-30% of patients and half will develop metastasis [12]. Thus, more research is needed in order to improve efficacy of radiation therapy.

d. Immunotherapy (immune checkpoints inhibitors):

Cancer immunotherapy has become a standard treatment in the management of many cancer types. It involves the stimulation of the immune system against tumor cells and comprises many mechanisms such as: cytokine therapy for the stimulation of immune cells e.g. IL-2 and IFN-α, cancer vaccines, adoptive cell transfer and more recently immune checkpoints blockade [13]. Combination treatments of several immunotherapeutic agents or between immunotherapeutic drugs and other conventional treatments are increasingly used in cancer management.

Besides BCG vaccine that is currently used in the management of non-muscle invasive bladder cancer, immune checkpoints inhibitors have been recently incorporated into the management of bladder cancer as novel immunotherapies. These drugs target mainly inhibitory receptors present on the surface of immune cells or their ligands on tumor cells thus reducing immune evasion of the tumor and further enhance anti-tumor immune responses [14].

Atezolizumab, a monoclonal antibody against PD-L1 was the first immune checkpoint inhibitor drug to be approved as second line treatment for muscle invasive bladder cancer patients who progress during their course of management after surgery as well as metastatic patients. Anti-PD-1 drugs such as Pembrolizumab and Nivolumab also showed promising results in advanced stages of bladder cancer [14]. Clinical trials are currently conducted in order to determine the efficacy of combination therapies of immune checkpoints inhibitors and other standard treatments in bladder cancer including radiotherapy and chemotherapy [15].

1.5.3 Management of metastatic bladder cancer

The first line of treatment for patients with metastatic bladder cancer is a Cisplatin-based chemotherapy. However, 50% of metastatic bladder cancer patients are poor candidates for

systemic chemotherapy and require a second line treatment. Immunotherapy with immune checkpoints inhibitors (e.g. PD-1 inhibitors and PD-L1 inhibitors) are the second line treatment for metastatic bladder cancer patients and are mainly used after failure of the first line chemotherapy treatment.

Section 2: Radiation therapy and immune response

2.1 Introduction

Radiotherapy is an important non-invasive treatment approach that is widely used in the management of numerous cancer types including bladder cancer. Recent advances in understanding radiation properties and the biology of cancer led to significant improvements in the ways radiation is delivered, increasing the efficacy of radiation and limiting the undesired adverse effects associated with radiation use. Worldwide, it is estimated that around 40% of cancer patients will require radiation treatment during their course of management and 60% are treated with curative intent. With its high efficacy and relatively lower cost, radiotherapy is considered one of the most cost effective cancer therapies [16].

Radiation is a type of energy that can be used in clinical settings for both diagnostic and therapeutic aims. When delivered in low doses, radiation can provide images for body structures as used in X-ray imaging. Higher doses of radiation are used to destroy abnormal cells such as cancer cells by targeting DNA present in their nuclei, creating chromosomal breaks leading to slowing of the rate of cellular proliferation and potentially complete eradication of these abnormal cells. The type of radiation used for these applications is the external beam photon based radiation therapy.

Generally, radiation targets highly dividing cells –as in the case of cancer cells- however, normal cells are also affected by radiation, and that leads to various adverse effects that can be associated with radiotherapy, which range from simple inflammation and burns to the development of secondary tumors and malignancies [17].

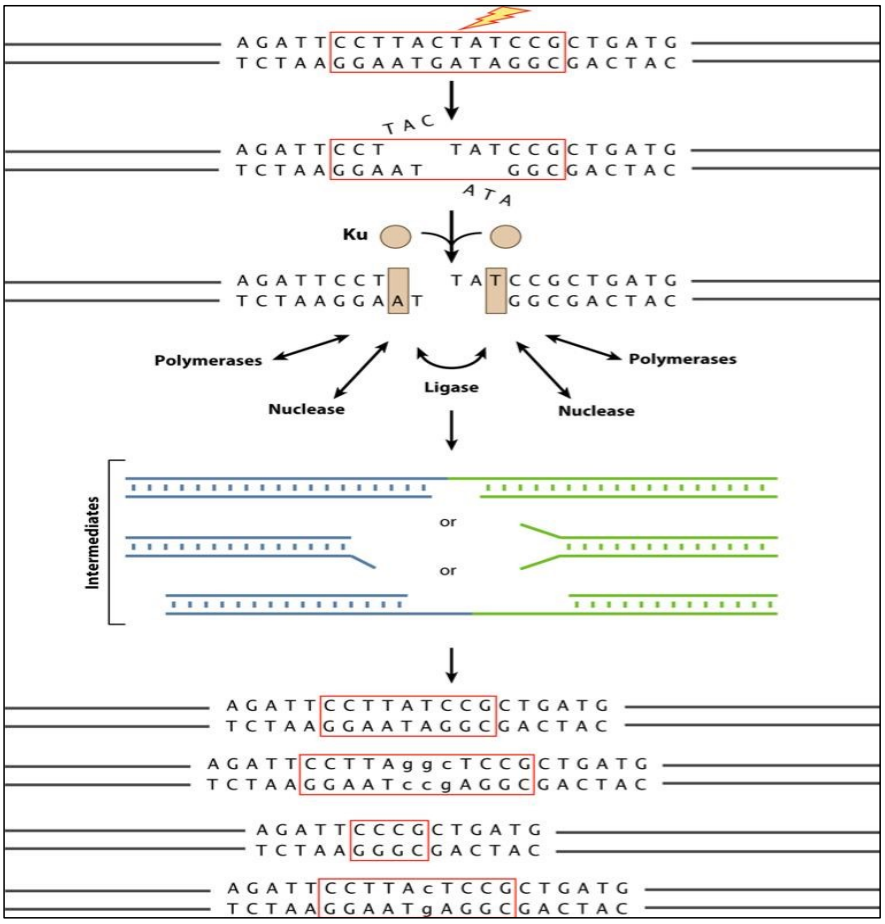
2.2 DNA damage and DNA damage repair

The main effect of ionizing radiation is creating irreversible DNA defects that prevent the cell from further dividing and leading eventually to cell death. DNA damage results from the emitted radiation photons and electrons passing through the chromatin structure at high speed creating breaks in the covalent bonds between molecules along their track and leading to single or double strands DNA breaks [18]. The magnitude of the DNA breaks is directly proportional not only to the increase in radiation dose, but also with the structural configuration of the chromatin, where the damage is maximal when the chromatin is highly compacted [19].

Another element that is involved in radiation induced DNA damage is the creation of reactive oxygen species (ROS) within the milieu in response to the lysis of water molecules [20]. The availability of oxygen within the tumor microenvironment might have a great impact on the response of the tissue to radiation. The lack of oxygen within the tissues may increase their resistance to radiation damage. This is particularly important in the context of cancer since tumors are usually associated with hypoxia and a high consumption of oxygen, which in turn might play a role in the resistance of these tumors to radiation [21]. Moreover, it has been described that tumor hypoxia could be a marker for poor prognosis in patients treated with radiotherapy [22].

On the other hand, cellular mechanisms regulating DNA damage repair do exist in almost all the cells of the body including malignant cells. In fact, most of the DNA breaks are usually repaired within hours, with around 90% of the breaks repaired within the first 24 hours post radiation [18]. It is then anticipated that tumor cells that possess a robust DNA damage repair machinery are more radioresistant and are able to survive radiotherapy [23].

Two main pathways are involved in regulating DNA damage repair within the cells: the non-homologous end joining pathway (NHEJ pathway) and the homologous recombination pathway (HR pathway) [24]. During the NHEJ pathway, a protein called Ku is first recruited to the site of the DNA double strand break which in turn helps recruit a number of DNA enzymes (such as nucleases, ligases and polymerases) to form a complex and bridge both ends of the break together [25]. Despite the importance of this pathway in repairing DNA breaks and preventing cell death, small sequence errors may erupt as a result of the rapid process of joining the ends of the breaks. These errors may themselves drive the cell into undergoing apoptosis or even developing mutations and malignant transformation (Figure 1) [26].



(Figure 1) DNA repair via non-homologous end joining (NHEJ) pathway [26]

On the other hand, DNA repair via HR pathway involves complex of proteins that guide the single stranded DNA filament resulted from the break to invade a similar DNA sequence and then DNA polymerases start to fill in the gaps to repair the damage [27]. In contrast to the NHEJ pathway, chances of DNA mutations and sequence errors during the HR pathway are lower since the later represents a more complex -but delayed- mechanism of DNA repair [28].

HMGB1 has been shown to help in joining the ends of DNA breaks during the non-homologous end joining pathway [29]. Moreover, HMGB1 was found to help guiding DNA protein enzymes towards DNA break ends and facilitating the recognition of damaged sites, which thus enhances DNA repair efficiency [30]. This adds to the rational for studying the role of HMGB1 in radioresistance of tumors.

2.3 Radiation and cellular responses

Mechanisms of cell damage caused by radiation are numerous and contribute to its highly effective therapeutic outcomes. In general, cell death is the main effect resulting from radiation exposure. However, several cellular responses can result from targeting rapidly dividing cells with radiation. These responses include the following:

2.3.1 Apoptosis: Apoptosis or programmed cell death is one of the main biological consequences of radiation. Radiation causes cell shrinkage, chromatin condensation within the nucleus with DNA breaks but the cell membrane usually remains intact [31]. This process of creating DNA breaks activates DNA repair machinery within the cells in order to compensate for the damage induced by radiation using pathways of DNA damage repair. And since the level of expression of genes and proteins involved in this process can differ from one cell type to the other, the sensitivity of cells to radiation induced apoptosis varies [32].

2.3.2 Mitotic catastrophe: Mitotic catastrophe is another major mechanism of cell death caused by radiation. It occurs as the result of failure of cell cycle checkpoints and the formation of giant cells due to an abnormal segregation of chromosomes during an aberrant mitosis. Most of the effects observed by radiation in solid tumors are believed to be the result of mitotic catastrophe in solid tumors cells [33].

2.3.3 Necrosis: Necrosis is a type of cell death which involves the disintegration of cellular organelles and breakage of cellular membrane [34]. In contrast to apoptosis, necrotic cell death occurs in response to relatively higher doses of radiation affecting cell membrane integrity. Uncontrolled release of cellular contents occurs during necrotic cell death and results in inflammation and immune reaction.

2.3.4 Autophagy: Radiation induced autophagy is considered a survival maintaining cellular mechanism rather than a cell death mechanism, and it is associated with radioresistance [35].

Autophagy occurs when autophagosomes containing segregated cellular organelles are formed by tumor cells exposed to radiation. These autophagosomes then undergo lysosomal degradation, this process is usually used by cancer cells to survive stressful environment by recycling cellular components [36].

2.3.5 Immunogenic cell death: In a study in 1953, tumor regression of distant non irradiated tumors was observed after local radiation of a primary tumor [37]. Later, the mechanism behind this finding was attributed to a systemic immune response that was induced by radiating the primary tumor [38].

Radiation induced immunogenic cell death is characterized by the translocation of certain antigens within the targeted cells towards the cell membrane and the extracellular release of

other proteins into the microenvironment, which combined activate antigen presenting cells and stimulate an immune response against tumor cells [39]. Among the secreted proteins in response to radiation are Calreticulin, Adenosine Triphosphate (ATP), Heat Shock Protein 70 (HSP70), Heat Shock Protein 90 (HSP90), and HMGB1; these secreted factors are usually referred to as danger signals or DAMP proteins. The release of DAMP proteins is considered a hallmark for immunogenic cells death for their well-established role in stimulating the immune system [40].

2.4 Molecular mechanisms of radioresistance

Radiation induced DNA breaks stimulate DNA repair mechanisms in cancer cells in order to circumvent the damage created. In this context, DNA repair aberrations represent one of the molecular mechanisms that leads to radioresistance of cancer cells and attenuate their response to radiation therapy. However, other intrinsic and extrinsic mechanisms are also involved in protecting cancer cells from radiation effects.

2.4.1 Intrinsic mechanisms of radioresistance: Several molecular mechanisms occur within cancer cells can promote cell survival and protect the cells against the deleterious effects of radiation. Cell cycle checkpoints regulate the process of cellular division by mediating cell cycle arrest in response to any dysfunction that could occur during the process. While cell cycle arrest provides time for cells to repair any damage that could have happened during the process of DNA replication and cell division, cancer cells may take advantage of this mechanism to overcome damage created by radiation. The selective inhibition of G2 cell cycle checkpoint by a small molecule inhibitor has been found to increase radiosensitivity of esophageal cancer cells [41].

Another intrinsic radioresistance mechanism is autophagy or cell recycling [42]. Cancer cells experiencing stressful events such as radiation may undergo autophagy in order to maintain their survival and avoid cell death. A recent study showed that targeting autophagy pathways by Chloroquine may enhance radiation response of bladder cancer cells [43].

2.4.2 Extrinsic mechanisms of radioresistance: In contrast to the intrinsic mechanisms exerted by cancer cells to resist radiation effects, the tumor microenvironment may also play a role in promoting cancer cell survival and radioresistance. A prominent example is the hypoxic environment in some solid tumors where less ROS are generated in response to radiation and oxygen radicals are regularly scavenged by thiol molecules released from cancer cells [44].

In addition to hypoxic conditions, the tumor immune microenvironment might provide protection for tumor cells from radiation by creating an immunosuppressive environment that helps the tumor to grow and avoid being recognized by circulating immune cells [45]. It is then speculated that there is cross talk between the microenvironment and the tumor cells to circumvent the effects of radiation. HMGB1 is believed to be an important mediator of this intercellular communication.

2.5 Radiation and tumor immune response

The immune system is indeed able to recognize tumorigenesis and in turn react accordingly. In addition, it was found that most of the conventional anti-cancer therapies may have immunogenic effects and could play a role in re-shaping an already existing immune response within the tumor [46]. Radiotherapy is not an exception, in fact it is one of the most immune modulating anti-cancer therapy known to date [47] [48].

Whereas it is now well established that the activation of antigen presenting cells (APCs) such as dendritic cells (DCs) is a critical step in the induction of an immune reaction in response to radiation exposure. Several mechanisms have been proposed to explain how this immune response is initiated. One of the proposed mechanisms is the activation of interferon pathways by the damaged DNA taken up by antigen presenting cells and sensed in the cytosol with the subsequent activation of stimulator of interferon genes (STING) that results in the release of interferons [49]. Another mechanism is the ability to induce immunogenic cell death and the release of DAMPs which in turn lead to the activation of antigen presenting cells and the induction of an adaptive immune response [50].

Radiation also has been found to enhance the infiltration of effector immune cells within the tumor and to promote several anti-tumor immune mechanisms that help in attenuating tumor progression. Numerous studies on several cancer types reported an increase in the frequency of cytotoxic CD8 T lymphocytes in response to radiation therapy [51]. Effector CD4 T lymphocytes were shown also to be recruited towards the tumor site after radiation [52]. In addition, radiation was responsible for the upregulation of certain cancer specific antigens as well as the antigen presentation molecule MHC-I on tumor cells [53]. These observations highlight the activation of adaptive immune responses by radiation therapy.

In contrast, radiation induced immune responses not only involve the activation of the adaptive immune system but also innate immunity. Studies have shown that radiation can promote the recruitment of myeloid cells within the tumor microenvironment resulting in repolarization of these cells and their differentiation into a wide variety of phenotypes [54]. Moreover, the depletion of macrophages was shown to improve radiation response by impairing tumor angiogenesis mediated by macrophages derived cytokines [55].

On the other hand, radiation was shown to promote tumor immune tolerance and mechanisms of tumor immune escape as well. The recruitment of several immune suppressor cells e.g. regulatory T Cells (Tregs), Myeloid Derived Suppressor Cells (MDSCs) and Tumor Associated Macrophages (TAMs) towards the tumor microenvironment was found to be enhanced by radiation in addition to an increase in the release of many immunosuppressive cytokines such as TGF-β and IL-10 [56]. Moreover, radiation was found to induce functional and phenotypic changes in the recruited immunosuppressive cells. In a recent study, radiation increased the frequency of tumor infiltrating Tregs and altered the phenotype of these cells towards a more suppressive phenotype as shown by the increase in the expression of CTLA-4, Helios and 4-1BB [57]. Similarly, radiation has been shown to upregulate STAT3 signaling pathways in MDSCs [58].

The expression of several immune checkpoint receptors and their ligands was also shown to be modulated by radiation. CTLA-4 expression on Tregs was upregulated by radiation in mouse models [57]. In addition, radiation resulted in increased expression of PD-L1 in bladder cancer [59]. The observed increase in expression of immune checkpoints in tumors after radiation has directed the research to develop combinatory therapeutic strategies between radiation and immune checkpoint inhibitors with the aim of maximally stimulating anti-tumor immune responses induced by radiation and limiting the pro-tumor immune escape mechanisms that develop within the tumor immune microenvironment; these combination therapies are showing positive results in many tumor types [60].

2.6 Radiation and DAMPs

DAMPs are a group of proteins that are either actively or passively released from cells under stressful environment including radiation exposure. Currently, the role of DAMPs in cancer including the role of HMGB1 is still under investigation, however studies have shown that the release of DAMPs is associated with immunogenic cell death of tumor cells, where DAMP proteins are orchestrating the crosstalk between the tumor microenvironment and the immune system [61].

The release of DAMP proteins in response to radiotherapy in several cancer types is associated with an anti-tumor response that leads to tumor regression by stimulating effector immune cells recruitment and cytokine production in order to eliminate tumor cells [62]. On the other hand, several studies have shown a role for DAMPs in maintaining an immunosuppressive environment within the tumor and promoting tumor immune escape [63]. For example, ATP released from dying tumor cells was found to enhance the proliferation of Tregs [64] and attenuate DCs functions [65] [66] whereas HMGB1 is reported to enhance the proliferation of function of several immunosuppressive cells including MDSCs, Tregs and TAMs [67] [68]. A better understanding of the exact role of this group of proteins is essential for identifying mechanisms of radioresistance in cancer cells.

Section 3: HIGH MOBILITY GROUP BOX-1 (HMGB1)

3.1 Introduction

HMGB1 is a highly conserved ubiquitous nuclear protein that is present in almost all cell types of the body. It belongs to a family of proteins called high mobility group proteins that share a similar amino acid sequences and functions to a certain extent [69]. This family of proteins has been isolated and characterized in 1973 from calf thymus nuclei [70]. It was found that they play essential roles in the maintenance of DNA integrity and the enhancement of transcription, replication and recombination processes within the nucleus by acting as DNA chaperones [71]. High mobility group proteins consist of 3 main sub-families: HMGA, HMGB and HMGN which are similar in certain aspects of their function, however they differ in their DNA binding motifs and preferred binding substrates [72]

The sub-family HMGB includes three members HMGB1, 2 and 3 that possess a similar structure by having special DNA binding regions called HMG boxes followed by an acidic tail. They bind to DNA minor grooves and result in bending of the DNA backbone in order to facilitate DNA repair. They also bind with high affinity to distorted DNA fragments [73].

3.2 HMGB1 structure

HMGB1 is a more abundant nonhistone nuclear protein in cells compared to HMGB2 or HMGB3. It is composed of two DNA binding motifs box-A and box-B with one nuclear localization signal located at each box (amino acids 28-44 in box-A and amino acids 179-185 in box-B). The boxes are attached to an acidic tail at the C-terminal of the protein.

HMGB1 has also three cysteine groups at the 23, 45 and 106 positions that can be either reduced or oxidized or could form a disulfide bond between the C23 and C45 which in turn can change the redox state of the protein [74]. This change in the redox state of HMGB1 may increase the affinity of the protein to one receptor over the others [75].

3.3 HMGB1 redox state and receptors

HMGB1 in its extracellular form serves as a ligand for several receptors including toll like receptors (TLRs) 2, 4 and 9, receptor for advanced glycation end-products (RAGE) and T cell immunoglobulin and mucin (TIM-3) among others. Studies have shown that the redox state of extracellular HMGB1 dictates the affinity of the protein to its receptors and in turn its biological functions [76]. Furthermore, distinct redox states of HMGB1 was reported to have clinical significance and to be associated with different diseases [77] [78].

The full reduction of the three cysteine groups leads to a reduced protein that has a greater affinity to RAGE, as the formation of a disulfide bond between C23 and C45 (disulfide HMGB1) increases the affinity of the protein to TLR4 [79]. In the oxidized form, the pro-inflammatory characteristics and cytokine-like effects of HMGB1 are attenuated [80].

3.4 HMGB1 functions

As mentioned earlier, HMGB1 can be present as a chromatin bound protein within the nucleus, in the cytosol or it can also be released extracellularly either actively or passively. The functions of the protein can be divided into intracellular and extracellular functions:

3.4.1 *Intracellular functions*: With its ability to bind the DNA, HMGB1 inside the nucleus maintains the architecture of the chromatin and helps in increasing its accessibility.

HMGB1 also contributes to DNA repair after DNA damage by chemotherapeutic agents, oxidative stresses as well as radiation. It directly binds to DNA and allows a variety of DNA repair mechanisms such as in the NHEJ pathway. HMGB1 also participates in gene transcription and gene recombination as well as in maintaining telomere integrity [81].It was also observed that HMGB1 can regulate the process of cell autophagy by shuttling from the nucleus to the cytosol and binding to Beclin1 which plays a key role in initiating autophagy in the cell [81].

3.4.2 Extracellular functions: HMGB1 can also be released extracellularly either actively or passively. Active release of HMGB1 usually happens after the protein is post translationally acetylated and then translocated from the nucleus to the cytosol. After this step, HMGB1 is actively released outside the cell either by exocytosis or in secretory lysosomes [82]. On the other hand, passive release of HMGB1 happens in the context of necrotic cell death, either in the fully reduced form, oxidized form or the disulfide form [82].

Extracellularly released HMGB1 is believed to regulate many inflammatory and immunological mechanisms including cytokine secretion, promotion of adhesion and migration of immune cells as well as their proliferation. In addition, it was found that extracellular HMGB1 is associated with cancer progression and treatment resistance [83]. More studies are currently evaluating different strategies for HMGB1 inhibition as potential cancer therapies [84].

3.5 HMGB1 inhibitors and their therapeutic effects

Inhibitors of HMGB1 were identified and used either clinically or for research purposes for many years in order to reverse functions exerted by this protein. It is also important to

mention that although they might share some similarities, different HMGB1 inhibitors have different mechanisms of inhibition and their use depend mainly on the specific function or site of action of HMGB1 that needs to be blocked [85].

3.5.1 *Recombinant box B*: this synthetic inhibitor competes with the full-length protein for RAGE binding site without causing the activation of the downstream signals. This strategy is usually used in order to inhibit the pro-inflammatory and cytokine production ability induced by the binding of box B of HMGB1 to RAGE [86]. Interestingly, the recombinant box B was used in several research models of diseases both *in vitro* and *in vivo* such as sepsis [87] and LPS induced lung injury [88] with promising results.

3.5.2 *Ethyl Pyruvate (EP)*: Another potent HMGB1 inhibitor that is commonly used is EP. This HMGB1 inhibitor blocks the release of HMGB1 by inhibiting the translocation of the protein from the nucleus to the cytoplasm [89]. EP was also tested for its potential anti-cancer effects in gallbladder cancer where it was found to reduce the proliferation of gall bladder cancer cells and induce cell cycle arrest [90]. Furthermore, EP impaired the release of HMGB1 from malignant mesothelioma cells and subsequently resulted in inhibition of tumor growth *in vivo* [91].

3.5.3 *Atorvastatin and simvastatin*: Two HMG-CoA reductase inhibitor molecules, Atorvastatin and Simvastatin are mainly used for the management of dyslipidemia. However, it was reported that both drugs might serve as inhibitors for HMGB1. In one study, the administration of Atorvastatin immediately after middle cerebral artery occlusion resulted in a reduction in brain infarct size and dramatically improved clinical outcomes. This was associated with a reduction in the expression of HMGB1

and its receptors RAGE and TLR-4 [92]. On the other hand, HMGB1 was found to induce changes in human umbilical vein endothelial cells by increasing the expression VCAM-1 and RAGE, both playing pivotal roles in promoting the attraction of macrophages and atherosclerosis. Pretreatment of human umbilical vein endothelial cells with Simvastatin inhibited the changes induced by HMGB1 and resulted in decreased levels of expression of VCAM-1 and RAGE [93].

3.5.4 *Methotrexate*: In addition to its use as a chemotherapeutic drug, Methotrexate possesses some anti-inflammatory effects. A group of researchers showed that Methotrexate can interact with HMGB1 through two independent binding sites and that this interaction prevents the binding of HMGB1 to RAGE; however, it did not prevent the binding of HMGB1 to DNA. Furthermore, Methotrexate blocked the HMGB1/RAGE induced TNF-α release and the mitogenic activity of murine RAW 264.7 cells [94].

3.5.5 *Glycyrrhizin (GLZ)*: A natural compound extracted from licorice plant (Glycyrrhiza glabra). It is one of the most commonly used compounds in the literature to reverse functions exerted by HMGB1. GLZ is described to directly bind to both boxes of HMGB1 preventing the binding of the protein to its receptors; however it only mildly interferes with the binding of intranuclear HMGB1 to DNA [95]. In addition, GLZ inhibits the release of HMGB1 from cells causing reduced levels of HMGB1 detected in the sera of animals treated with GLZ [96]. Recently, it was shown that GLZ suppresses the HMGB1 mediated invasion and migration of lung cancer cells both *in vitro* and in a PDX mouse model, possibly by reducing the activity of JAK/STAT

pathway upstream of HMGB1 [97]. GLZ was also successfully tested both *in vitro* and in animal models in glioblastomas and colon cancer [98].

Given the previously mentioned properties, GLZ is considered a highly effective and selective HMGB1 inhibitor. In our study, we used GLZ to inhibit the active release of HMGB1 within the tumor microenvironment and more importantly, to block the interaction between the passively released HMGB1 from dying tumor cells in response to radiation and its receptors, TLR4 and RAGE, without interfering with the intracellular functions of HMGB1.

3.6 HMGB1 and cancer

Since its discovery in 1973, HMGB1 has been studied extensively for its potential role in many different types of diseases including cardiovascular diseases [99], sepsis [100], diabetes [101] and cancer [102]. HMGB1 has been shown to be involved in several mechanisms of cancer progression and treatment resistance [81]. Below is a brief literature review on the role of HMGB1 in some of the cancer hallmarks:

i. Resisting cell death: Cancer cells have to resist death signals in order to maintain their survival and proliferation. In general, it has been shown that HMGB1 regulates the process of apoptotic cell death as well as necrosis [103]. For example, it has been demonstrated that the knockdown of HMGB1 in LNCAP prostate cancer cells attenuates cellular growth due to increase in caspase-3 dependent apoptosis [104]. HMGB1 is then promoting cell survival and resistance to anti-tumor therapies.

ii. Cellular proliferation: Extracellular HMGB1 has been shown to promote pancreatic cancer cells proliferation through the HMGB1/RAGE pathway [105]. Recently, released HMGB1 from irradiated cancer cells was shown to promote the proliferation and survival of living cells after radiation and was associated with poor clinical outcome in colorectal cancer [106].

iii. Invasion and metastasis: Several studies highlighted the role of HMGB1 in tumor metastasis. In breast cancer, the downregulation of HMGB1 resulted in attenuating the migration and invasion of breast cancer cells possibly through targeting the expression of the nuclear protein SMARCC1 [110]. Similarly, the knockdown of HMGB1 in HCCLM3 hepatocellular carcinoma cell line reduced their invasive and migratory properties [111]. Moreover, HMGB1 released from neutrophil extracellular traps (NETs) was found to promote liver metastasis after surgical stress by binding to TLR9 [112].

iv. Angiogenesis: In a study to identify some of the tumor specific angiogenesis markers, HMGB1 stood out as one of the angiogenesis markers of tumors [113]. Binding of HMGB1 to RAGE and TLR4 has been demonstrated to promote angiogenesis in several tumor types [114].

v. Avoiding immune destruction: An important feature in cancer development is the ability to escape being recognized by patrolling immune cells. Several mechanisms have been proposed that help the tumor evade the immune system. The role of HMGB1 in promoting tumor immune escape will be discussed further in the following section.

Section 4: HMGB1 AND CANCER IMMUNOLOGY

4.1 Introduction

The first documented cases of cancer management using an immune/infection based treatment was during the nineteenth century by Doctor William B. Colley, a New York surgeon who used a heat killed mixture of *B. prodigious* and *S. pyogenes* bacteria injection in patients with inoperable sarcomas and achieved excellent results. In 1959, a husband and wife team, Ruth and John Graham, published the first ever clinical trial of tumor vaccines using tumor lysate in patients suffering from a variety of gynecological malignancies [115]. These historic advances were followed by the emergence of the field of cancer immunology. As our understanding of both the immune system and cancer biology increased, the implementation of an immune based management strategy for tumors stands now at the same level as standard cancer therapies such as surgery, radiation, and chemotherapy.

Despite these advances, response rates as well as overall survival rates for immunotherapies remain only in the minority of patients. The lack of useful predictive markers to evaluate treatment outcomes and the development of resistance to some immunotherapies, in addition to the higher cost of these immunotherapeutic agents compared to conventional treatments, represent challenges that require further research and more studies [116].

The basic concept behind cancer immunotherapy is to induce an anti-tumor immune response against tumor cells. In general, these therapies are boosting the patient's own immune system to recognize and eliminate abnormal cells that adopted a malignant genotype/phenotype or earlier during the premalignant transformations of the cells. However, one of the hallmarks of cancer cells is evading the immune system and maintaining a pro-tumor immune microenvironment that

helps these cells proliferate and eventually invade new tissues and organs [117]. Thus, the balance within the tumor microenvironment between anti-tumor immune responses and pro-tumor immune mechanisms is critical in determining the fate of these cancer cells. Factors released from tumor cells could play a key role in altering the immune balance within the tumor microenvironment in order to promote cancer progression.

4.2 Cancer immunology, immune surveillance and tumor immunoediting

More than 5 decades ago, tumor immunoediting and immune surveillance theories were proposed in order to describe the relationship between the immune system and malignant cells [118]. Studies have shown that carcinogen induced tumor development is hindered in immunocompetent mice compared to immunodeficient mice, which suggest that the immune system plays a critical role in tumorigenesis [119]. Moreover, the immune system was found to regulate not only the induction of *de novo* tumors, but also the immunogenicity of these tumors and their susceptibility to be recognized by the circulating immune cells [119]. Thus, tumor development and progression are markedly controlled by the immune status of the host.

Three phases were described to explain the mechanisms of immune surveillance and tumor immunoediting: elimination, equilibrium, and escape. These three phases represent a dynamic process that occur when normal cells acquire a malignant behavior whether being due to the exposure to a carcinogen or a genetic mutation. The resulting outcome of this process will then determine the fate of these newly transformed tumor cells [120].

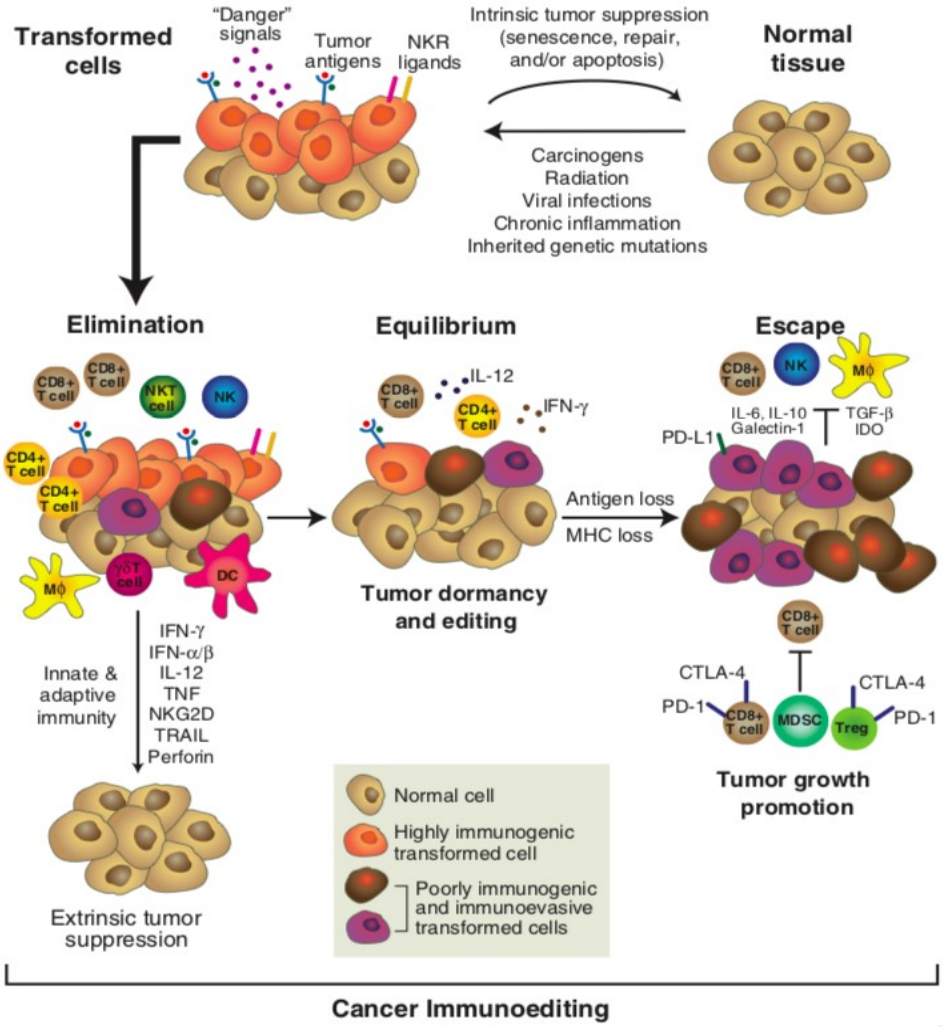
The elimination phase occurs when normal cells turn into tumor cells and “alarm signals” are sent to help localize the newly transformed cells. These alarm signals are still yet to be defined; however, they are believed to include tumor antigens, inflammatory cytokines, and DAMP

proteins released from dying tumor cells. The activation of the immune system occurs then as a result of these alarm signals by the release of interferons that enhances the cross presentation of tumor antigens and stimulates adaptive and innate immune cells [121].

A state of equilibrium can then be reached between the elimination of highly immunogenic tumor cells and the preferential proliferation of poorly immunogenic cells. The end result is a balance between anti-tumor immune responses and pro-tumor immune evasion that could last for years. Any change in this balance due to alterations in the immune status of the host can lead to tumor progression and in turn a dramatic change in clinical outcomes [121].

During the escape phase, poorly immunogenic tumor cells proliferate in a way that surpasses the ability of the immune system to control their growth [121]. Tumor cells acquire mechanisms to resist being recognized and eliminated by immune cells. These escape mechanisms include: the loss of antigenicity by attenuating antigen processing and presentation, loss of immunogenicity by the expression of immune checkpoints receptors and the acquisition of an immunosuppressive microenvironment by promoting the recruitment of immunosuppressive cells [122].

Thus, the role of the immune system in the detection, recognition and eradication of tumor cells is a continuous process that creates a balance between anti-tumor and pro-tumor factors. The ultimate goal of immune modulating cancer therapies is to shift the immune balance towards more anti-tumor immune responses by stimulating effector immune cells and impairing tumor immune escape mechanisms.



(Figure 2) cancer immune surveillance theory and tumor immunoediting [119]

4.3 CD4 and CD8 T cells

The role of HMGB1 in cancer is well established, and its implication in cancer cells proliferation, angiogenesis and metastasis is relatively well studied. However, the role of HMGB1 in the modulation of the tumor immune microenvironment is yet to be fully unraveled. Since HMGB1 is known to regulate many inflammatory and immune mechanisms [123], research studies investigated its role in the modulation of the tumor immune microenvironment. These studies demonstrated that HMGB1 could possibly be at the heart of a crosstalk between cancer cells and tumor infiltrating immune cells. More interestingly, recent studies showed that HMGB1 could be playing a dual role in inducing an anti-tumor immune response while promoting pro-tumor immune escape mechanisms. Thus, the exact role of HMGB1 in tumor immune surveillance is yet to be fully understood [124].

The effect of HMGB1 on T cells was evaluated in several studies and important observations were reported. It was found that extracellular HMGB1 secreted actively by mature dendritic cells is required for the proliferation and survival of naïve CD4 T cells and induces the polarization of these cells towards a Th1 phenotype as identified by IFN-γ expression. Interestingly, the effect of HMGB1 on CD4 cells was abrogated after the use of a RAGE blocking antibody [125]. Another study investigated the effect of recombinant HMGB1 on CD8 and CD4 T cells proliferation and found a stimulating effect for HMGB1 on the proliferation of CD4 and CD8 cells activated with low doses of anti-CD3 antibody [126]. In autoimmune diseases, levels of HMGB1 was found to be significantly increased in the serum of SLE and rheumatoid arthritis patients and positively correlated with disease progression. HMGB1 was found to play a role in SLE pathogenesis by promoting DNA methylation of CD4 cells [127].

In cancer, a positive correlation was suggested by many studies between HMGB1 expression and the frequency of tumor infiltrating T lymphocytes in several types of cancer [128-131]. However, few publications studied the exact effect of HMGB1 on tumor infiltrating lymphocytes proliferation and function whether *in vitro* or *in vivo*. The role of HMGB1 in activation and proliferation of tumor-specific T cells was studied in a murine prostate cancer model where the inhibition of HMGB1 by a neutralizing antibody resulted in a significantly reduced numbers of CD4 and CD8 cells within the tumors. Moreover, the development of invasive carcinoma in adult mice was hindered in the absence of tumor specific T cells –but not B cells- supporting evidence that tumor specific T cells promote the development of invasive prostate cancer in mice after puberty [132]; a claim although still controversial, was supported by a recent study that showed similar results [133].

HMGB1 serves as a ligand for several receptors expressed by CD4 and CD8 cells including TLRs, RAGE and Tim-3 and many of these receptors were shown to play an important in the tumor immune surveillance. Further research on HMGB1 effects on tumor infiltrating CD4 and CD8 cells is needed [134] [135] [136].

4.4 Tregs

An important characteristic of the immune system is the ability to induce immune tolerance in response to excessive immune activation. Failure of regulating the immune system over-stimulation is associated with the development of autoimmune diseases, where effector immune cells react to the host's own antigens leading the destruction of many tissues and organs [137].

Several mechanisms are involved in mediating immune tolerance in physiologic conditions. These mechanisms include the secretion of certain immunosuppressive cytokines e.g. IL-10, IL-

6, IL-4 and TGF- β and the expression of immune checkpoints receptors. In addition, some immune cells are specialized in inhibiting the immune response within a certain microenvironment. These cells include: regulatory dendritic cells, myeloid derived suppressor cells (MDSCs) in addition to regulatory T cells (Tregs) [138].

Phenotypic identification of Tregs in humans requires the expression of CD4, IL-2 receptor (CD25), the transcription factor FOXP3, and the lack of expression of CD127. However, in mice, CD4 and FOXP3 expression are generally used and are sufficient to identify Tregs, unlike in humans where other cell populations than Tregs could express FOXP3 including effector T cells [139]. The transcription factor FOXP3 plays an important role in the differentiation of T cells into Tregs and in orchestrating their regulatory functions. The loss of expression of FOXP3 results in an autoimmune condition named Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome that is characterized by a wide variety of autoimmune pathologies that affect mainly boys at early age [140]. FOXP3 in Tregs induces the expression of immunosuppressive cytokines and receptors while inhibiting the expression of pro-inflammatory cytokines thus promoting the immune regulatory characteristics of Tregs.

Tregs exert their suppressive functions by different mechanisms: the secretion of inhibitory cytokines (such as IL-10, IL-35 and TGF- β), direct contact cytolysis of effector cells, cytokine deprivation, and metabolic disruption by depriving effector cells from IL-2 in addition to targeting DCs to turn them into regulatory DCs with immunosuppressive abilities [141]. Given their ability to suppress immune responses through a wide variety of mechanisms, Tregs are generally considered one of the most important immunosuppressive cell populations.

In this context, recent studies have shown that Tregs might experience functional plasticity by losing the transcription factor FOXP3 and acquiring some pro-inflammatory characteristics [142]. Moreover, single cell analysis of human Tregs revealed a population of FOXP3+ Tregs that lack suppressive function and failed to suppress effector cells cytokine production [143]. Although not yet fully understood, studies have suggested a role for inflammatory cytokines and cellular derived soluble factors in mediating Tregs functional instability in addition to the inflammatory microenvironment where Tregs are residing [144].

While Tregs dysfunction might lead to many undesirable effects including IPEX syndrome and a number of autoimmune diseases, Tregs suppressive capacity was found to promote tumorigenesis and to contribute to tumor immune evasion. Currently, Tregs are considered key players in promoting tumor immune escape through their role in suppressing anti-tumor immune cells proliferation and function [145] [146]. Moreover, higher numbers of tumor infiltrating Tregs or higher Tregs:CD8 ratio within tumors were associated with poor prognosis in most cancer types [147].

Growing evidence show that HMGB1 may be involved in the modulation of Tregs function and cytokine production; however, it is still controversial whether HMGB1 is potentiating or impeding Tregs suppressive capacity. In a rat model of thermal injury, the inhibition of HMGB1 using ethyl pyruvate or the blockade of RAGE markedly decreased the expression of CTLA-4 and Foxp3 in Tregs and resulted in decreased IL-10 production suggesting a stimulatory effect of HMGB1 on Tregs via RAGE [148]. On the other hand, several reports found that the expression of Foxp3 and the production of IL-10 by Tregs was markedly impaired following treatment with HMGB1 [149].

In the context of cancer, a group of researchers performed phenotypic characterization of Tregs and CD4 effector T cells in order to determine the levels of expression of TLR4 and RAGE as known receptors for HMGB1. Interestingly the levels of RAGE receptor on the surface of Tregs was significantly higher on Tregs compared to effector cells while the levels of TLR4 remained comparable in both subsets. In the same study, HMGB1 derived from the supernatant of necrotic cancer cells was found to enhance the migration, survival as well as IL-10 production by Tregs; This effect was abolished by blockade of RAGE. Moreover, proliferation and IFN-γ secretion by effector T cells was hindered by HMGB1 [68]. In another study, knockdown of HMGB1 in lung cancer cells resulted in reduced absolute numbers -but not frequencies- of Tregs in spleens or lymph nodes of tumor bearing mice and enhanced IL-10 secretion by Tregs [150]. These studies concluded that HMGB1 signaling promotes Tregs suppressive functions and represents a potential therapeutic target for cancer management.

On the other hand, several studies showed contradicting results suggesting an inhibitory effect of HMGB1 on Tregs. In a recent study, HMGB1 treatment of Tregs weakened their ability to inhibit effector CD4 cells proliferation and that effect was abolished when TLR4 antibodies were used, whereas LPS treated Tregs showed stronger suppressive capacity compared to untreated controls [151], suggesting that HMGB1 is impairing the immunosuppressive function of Tregs.

These seemingly contradictory results could possibly be explained by the fact that HMGB1 could be simply binding to two different receptors on the surface of Tregs leading to opposite outcomes, as it seems that the stimulatory effect of HMGB1 on Tregs is mediated by RAGE whereas the inhibitory effects of HMGB1 on Tregs results from the binding of HMGB1 to TLR4. Thus, it is very important to study the microenvironment where HMGB1 is generated

whether *in vitro* or *in vivo*, as it could play an important role in changing the redox state of the protein which in turn determines to which receptor it will bind to [79].

4.5 MDSCs

MDSCs are subtypes of immature myeloid cells originating from bone marrow hematopoietic cells. They exhibit immunosuppressive characteristics through their ability to secrete immunosuppressive cytokines (TGF-β, IL-10, ARG1 etc.) and induce T cell proliferation arrest [152]. Tumors may recruit MDSCs in order to maintain an immunosuppressive environment and inhibit anti-tumor immune responses. Certain transcription factors and signaling pathways are required for MDSCs proliferation and function. STAT family of transcription factors have been shown to regulate MDSCs expansion and activation [152]. Studies have demonstrated that the inhibition of STAT signaling promotes anti-tumor immune cells activation and results in tumor growth inhibition [153]. Attenuating the proliferation and function of tumor infiltrating MDSCs by targeting these signaling pathways may represent a novel therapeutic approach for cancer patients.

It has been suggested that the main drive for MDSCs recruitment is the inflammatory microenvironment within the tumors that promote MDSCs' trafficking and suppressive functions leading to tumor immune evasion and progression [154]. In addition, a number of chemokines and inflammatory cytokines were found to be associated with MDSCs recruitment. CXCR2 signaling pathway within MDSCs is stimulated by the binding of this receptor to a number of chemokines such as CXCL1 and CXCL5 mediating MDSCs migration and accumulation in tumor sites [155]. Cytokines such as IL-1β and IL-8 are also associated with MDSCs recruitment towards tumors [156].

In contrast to the controversy around the exact effects of HMGB1 on Tregs, the role of HMGB1 in MDSCs recruitment, differentiation, and function is relatively well established [154]. In an animal model of colorectal cancer peritoneal metastasis, abdominal surgical trauma resulted in the elevation of levels of HMGB1 in the peritoneal cavity of the mice. Then, in order to determine whether the release of HMGB1 is associated with MDSCs recruitment, recombinant HMGB1 was injected into the peritoneal cavities of the animals, where it promoted the recruitment of MDSCs. In the same study, presence of MDSCs were found to be associated with higher metastatic burden. Interestingly, the elimination of MDSCs with Gemcitabine or the inhibition of HMGB1 by a neutralizing antibody markedly decreased peritoneal metastasis after surgery [157]. In another study, the use of anti-HMGB1 antibodies also significantly decreased the frequency of MDSCs and TAMs within the tumors as well as the spleens of tumor bearing mice in a renal cell carcinoma model and resulted in tumor regression; whereas the inhibition of HMGB1 did not change the percentages of T or B cells in the spleens [67].

HMGB1 was found to facilitate the differentiation of bone marrow cells into functional MDSCs and enhance their survival through autophagy [158] [67], the thing that highlights again the potential role of tumor derived HMGB1 in modulating the tumor immune microenvironment [159]. In addition, HMGB1 was shown to enhance MDSCs' suppressive function and IL-10 secretion, suggesting a functional regulation by HMGB1 on MDSCs in addition to its survival and differentiation effects mentioned earlier [160].

4.6 TAMs

TAMs consist of two main functionally distinguishable cell types: M1 and M2, where M1 exhibit anti-tumor responses by secreting IL-1, TNF-α and CXCL10 while M2 cells are involved in immune suppression and pro-tumor immune response by secreting IL-10, TGF-β and CCL22

[161].

Although still debatable, the origin of TAMs has been widely accepted as being from differentiated monocytes from bone marrow hematopoietic stem cells [162]. It is also now widely accepted that CSF1 is the major regulator of monocytes differentiation into TAMs [163]. However, TAMs differentiation, proliferation and suppressive function within the tumor microenvironment are also regulated by other tumor derived factors including HMGB1. In one study, TAMs co-cultured with recombinant HMGB1 significantly promoted lymphatic endothelial cells proliferation, migration and tube formation in an ovarian cancer model [164]. In melanoma, HMGB1 knockdown *in vivo* resulted in reduced tumor growth possibly by decreasing the numbers of TAMs. Moreover, phenotypic characterization of these TAMs showed higher expression of markers associated with M2 phenotype compared to M1 markers in the HMGB1 knockdown group. Furthermore, HMGB1 induced the secretion of IL-10, a potent immunosuppressive cytokine, by TAMs in a RAGE dependent manner [108]. Another study showed that HMGB1 enhanced M2 functional ability to promote tumor invasiveness and angiogenesis but not M1 functions [165].

Taken together, these results suggest that HMGB1 is promoting the recruitment and the immunosuppressive functions of TAMs, favoring the accumulation of pro-tumor M2 macrophages through RAGE. However, it is worth mentioning that some studies on autoimmune and inflammatory diseases suggested a role for HMGB1 in skewing monocytes differentiation towards M1 phenotype rather than M2, mainly through binding to TLR4 [166] [167] [168]. Moreover, HMGB1 deficient murine embryonic fibroblasts with Kras mutation tumors induced an M2-like panel of cytokines, whereas wild type tumors induced an M1-like panel of cytokines suggesting that HMGB1 is responsible for polarization of macrophages into an M1 phenotype

[169]. This could be also attributed to the type of environment where HMGB1 is released and the target receptor that may favor one pathway over the other.

4.7 Dendritic cells

Dendritic cells are a group of prominent antigen presenting cells; their function is to uptake, process, and then present antigens to effector T cells in order to induce an immune response. In cancer, dendritic cells play a major role in inducing an anti-tumor immune response. A number of studies showed that higher numbers of tumor infiltrating dendritic cells correlated with good prognosis in different cancer types [170]. However, recently it was discovered that subsets of dendritic cells can play a role in tumor immune tolerance and promote a number of pro-tumor immune cells such as Tregs and MDSCs [171] [172]. In this context, a study sought to investigate the modulation of dendritic cells phenotype and function upon the exposure to tumor derived soluble factors in an *in vitro* cervical cancer model and showed that dendritic cells co-cultured in a trans-well plate with squamous cell carcinoma cells exhibited a tolerogenic like phenotype, a reduced ability of secreting IFN-α, and induced the differentiation of naïve CD4 cells into Foxp3+ Tregs when exposed to soluble factors derived from cultured cancer cells. In the same study, HMGB1 was identified as a major tumor derived factor involved in the modulation of dendritic cells [173]. Another recent study showed that dendritic cells mediated Tregs activation, is induced by tumor derived HMGB1 through the release of thymic stromal lymphoprotein from tumor cells [174].

More recently, a role for HMGB1 in modulating antigen presentation and processing by dendritic cells post cancer treatment was described. It was shown that HMGB1 released from chemotherapy or radiotherapy treated cancer cells binds to TLR4 and promotes antigen presentation and processing by dendritic cells *in vivo*[50]. This mechanism could explain the

immune changes that occur in the tumor microenvironment following treatment with radiation or chemotherapy.

4.8 Natural Killer cells

Natural killer (NK) cells are key players in innate anti-tumor immune responses [175]. Anti-tumor immune effects of NK cells are carried out by a variety of different mechanisms including cytokine-mediated targeting of tumor cells e.g. granzymes and TNF-α, in addition to the activation of anti-tumor immune T lymphocytes [176]. Similar to tumor infiltrating T cells, NK cells recruitment and activity within the tumor immune microenvironment is dependent on a number of immunological signals governing NK cells stimulation or inhibition [177].

HMGB1 was found to directly modulate NK cells survival and function. NK cells cultured with recombinant HMGB1 showed upregulation of proteins involved in cell survival, proliferation and increased the motility of the cells compared to cells cultured in the absence of HMGB1 [178]. HMGB1 deficient tumor cells failed to recruit NK cells towards the tumor area whereas wild type tumors induced the recruitment of NK cells [169]. Moreover, Monocytes derived HMGB1 enhanced the ability of NK cells to release IFN-γ when coupled with other pro-inflammatory cytokines such as IL-1, IL-2 and IL-12 through RAGE [179].

Taken together, there seem to be a role for HMGB1 in NK cells recruitment, proliferation and function mainly through RAGE receptor on NK cells. NK cells do express other receptors for HMGB1 such as Tim-3; however, the effect of binding of HMGB1 to these receptors has not been yet elucidated [180].

4.9 Neutrophils

Despite a compelling evidence for the infiltration of neutrophils in numerous tumor types, the role of tumor infiltrating neutrophils in cancer is still not fully understood [181]. Whereas numerous studies showed pro-tumor role of tumor associated neutrophils [182], other studies reported a number of anti-tumor effects for neutrophils within the tumor microenvironment including the activation of tumor infiltrating T lymphocytes and inducing cancer cells apoptosis [183] [184].

HMGB1 seems to be an important factor for neutrophils recruitment towards tumors site. In a prostate cancer murine model, Cabozantinib treatment resulted in immunogenic cell death by unleashing an anti-tumor innate immune response. Cabozantinib also induced CXCL12 and HMGB1 extracellular release from tumor cells which in turn formed a heterocomplex to bind to CXCR4 and increase neutrophils recruitment [185].

Another important mechanism for HMGB1 mediated neutrophils modulation, is neutrophils extracellular traps (NETs) where recent reports showed an important role for HMGB1 in the process of netosis [186]. NETs are associated with increased risk of tumor progression and metastasis [112]. Moreover, HMGB1 is an important constituent of NETs and might have a role in NETs mediated tumor progression.

4.10 Immune checkpoints

Recent advances in the field of immune-oncology revealed a new tumor promoting mechanism that involves tumor cells escape being recognized by the immune system using a number of inhibitory signals induced by the engagement of certain receptors named immune checkpoints (e.g. PD-1, CTLA-4, Tim-3, LAG-3 etc.) and the blockade of these checkpoints offered a new

therapeutic approach for cancer patients [187].

Tim-3 is one of the immune checkpoint receptors that is expressed on a variety of different acquired and innate immune cells such as T-helper 1 cells, Tregs, NK cells, naïve dendritic cells, monocytes and macrophages [188]. HMGB1 serves as a ligand for Tim-3; however, little is known about the effects of HMGB1 binding to Tim-3 in the context of cancer. It was shown that HMGB1 binding to Tim-3 expressed on tumor infiltrating dendritic cells suppresses the processing of nucleic acids derived from dying tumor cells and thus impedes anti-tumor immune responses [189]. The role of HMGB1 interaction with Tim-3 expressed on other immune cells is yet to be studied.

4.11 Conclusions

Since the emergence of tumor immunoediting theory and the discovery of mechanisms of tumor immune surveillance, the focus of recent research studies was to understand the complex network of molecular signaling and cellular interactions between tumor cells and the immune system. In this context, tumor derived factors are believed to mediate the cross talk between cancer cells and the immune system [195].

Given the overwhelming evidence of the immune modulatory effects of HMGB1 [190], it could serve as a potential target for further research in order to identify its effects on the different constituents of the tumor immune microenvironment. Moreover, HMGB1 is known to be released from cancer cells upon exposure to anti-cancer drugs such as chemotherapy and radiotherapy where it induces immunogenic cell death [40]. This makes HMGB1 a perfect candidate to study the immunological mechanisms associated with the resistance to many anti-cancer therapies.

However, many aspects are still not fully understood about the interaction between HMGB1 and many tumor infiltrating immune cells. For example, HMGB1 is known to serve as a ligand for several receptors on immune cells (Tim-3 on T cells and NK cells, RAGE, TLRs etc.). However, the effects of HMGB1 binding to these receptors on the immune balance within the tumor microenvironment are still not known. In addition, the driving factors for HMGB1 to preferentially bind to one receptor over the other are not yet fully understood. Given these reasons, further studies are required in order to better understand the effects exerted by HMGB1 on the tumor immune microenvironment.

Since the tumor immune microenvironment is a dynamic milieu that develop through the interaction of a wide variety of immune and non-immune cells, it is crucial to study the modulation of a single factor, for example HMGB1, on all different components of the microenvironment rather than its effect on a single population of cells. This will allow a better understanding of the biological processes that occur within the tumor.

In conclusion, HMGB1 represents a promising protein candidate to study its involvement in the resistance to many anti-cancer and immunotherapeutic drugs. The numerous immunological functions exerted by HMGB1 affects almost all components of the tumor immune microenvironment and could serve as potential targets for modulation in order to promote anti-tumor immune responses and attenuate pro-tumor immune escape mechanisms.

CHAPTER II: (RATIONAL)

Bladder cancer ranks fifth among most common cancers in Canada. Twenty five percent of bladder cancer patients in Canada are estimated to suffer muscle invasive disease. Mortality rates for muscle invasive bladder cancer are as high as 40% in the first five years. Currently, the gold standard treatment for muscle invasive bladder cancer is radical cystectomy where the bladder is surgically removed along with the adjacent structures and a urinary diversion is created [1]. However, Patients undergoing radical cystectomy usually experience high morbidity related to abnormal urinary and sexual functions after surgery which certainly have negative impacts on the quality of life of patients after treatment [196].

Radiation therapy can serve as a non-invasive alternative to radical cystectomy with improved quality of life and less morbidity rates post treatment. And although radiotherapy can induce tumor regression and long-term response in the majority of bladder cancer patients, 25-30% of patients will still experience local recurrence and even distant metastasis after treatment which will require salvage cystectomy [197]. For these reasons, a better understanding of the mechanisms involved in radioresistance and the identification of biomarkers to predict radiation response are needed.

Molecular mechanisms of cancer cells radiation resistance involve both intrinsic and extrinsic pathways. Enhanced DNA damage repair machinery helps tumor cells circumvent damage induced by radiation in the form of DNA breaks and maintain their survival post treatment. Likewise, tumor cells are able to take advantage of the surrounding environment to resist the anti-tumor effects of radiation by promoting pro-tumor immune escape mechanisms and scavenging ROS generated by radiation. In this context, HMGB1 was found to be involved in

promoting many of these pathways [69] [56], which makes it a prime candidate protein to study molecular and immune mechanisms of radiation resistance.

Despite the fact that radiation is able to induce an anti-tumor immune response that might be synergizing the cytotoxic effects of radiation, yet recent studies have shown that radiation may enhance the development of several pro-tumor immune mechanisms that involve the infiltration of certain immunosuppressive cells within the tumor microenvironment and that may play a role in radioresistance and tumor recurrence [56].

DAMP proteins released within the tumor microenvironment in response to radiation are known to mediate the crosstalk between tumor cells and the different tumor infiltrating immune cell subsets. In this context, HMGB1 is of particular interest as one of the DAMP proteins released from tumor cells in response to radiation and for its many previously mentioned immunological functions. Moreover, we have previously identified a role for HMGB1 in bladder cancer radioresistance through its intracellular role in DNA damage repair and enhancing autophagy [198]; however the question of whether the immune modulating effects of HMGB1 also play a role in bladder cancer radioresistance remains to be answered. In the current study, we sought to investigate the role of HMGB1 in bladder cancer radioresistance through its immunological functions.

Our findings suggest that extracellular HMGB1 is involved in radioresistance of bladder cancer. The inhibition of HMGB1 using GLZ does improve radiation response of tumors possibly by the attenuation of recruitment of MDSCs and TAMs and shifting the tumor immune microenvironment towards more anti-tumor immune response. Targeting HMGB1 could serve as a novel therapeutic approach to overcome radiation resistance in bladder cancer.

CHAPTER III: (HYPOTHESIS)

Given its immune modulating effects, we hypothesize that extracellular HMGB1 is involved in radioresistance of bladder cancer by promoting pro-tumor immune mechanisms. The proliferation and function of several major immunosuppressive cells within the tumor microenvironment are enhanced by the release of extracellular HMGB1 from irradiated tumor cells. The modulation of HMGB1 within the tumor microenvironment will result in activating anti-tumor immune mechanisms and inhibiting pro-tumor immune escape.

We hypothesize also that radiation will have a major impact on the tumor immune landscape. Radiation induced changes in the level of expression of certain immune genes within the tumor microenvironment will include the upregulation of both anti-tumor and pro-tumor immune pathways. HMGB1 inhibition could result in a shift in the immune balance within the tumor microenvironment towards more anti-tumor immune response.

The clinical implications of this project include the identification of a novel mechanism of bladder cancer radioresistance and the demonstration of alterations in the tumor immune microenvironment that could attenuate the response of bladder cancer to radiation. The inhibition of HMGB1 and targeting the tumor immune microenvironment may provide a therapeutic approach for bladder cancer radiosensitization. In addition, HMGB1 and immune cell infiltration could be evaluated as useful predictive markers for radiation response.

CHAPTER IV: (AIMS)

1. Assessing HMGB1 expression and its release from MB49 murine bladder cancer cell line.
2. Establishing an *in vivo* syngeneic bladder cancer animal model to study the immunological effects of radiation induced HMGB1.
3. Investigating the role of extracellular HMGB1 in bladder cancer radioresistance.
4. Evaluating the tumor immune microenvironment post radiation.
5. Studying the immunomodulatory effects of HMGB1 inhibition in the context of radiation and correlating these effects with tumor response to radiation.

CHAPTER V: (MATERIALS & METHODS)

CELL LINE AND CELL CULTURE

Murine bladder cancer cells MB49 were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Wisent) supplemented with 10% fetal bovine serum (FBS, Wisent). Cells were routinely passaged when 70-80% confluent. Cells used for both *in-vitro* and *in-vivo* experiments were at least at 2nd passage and not more than 5th passage.

IN-VITRO IRRADIATION OF MB49 CELLS

MB49 cells were plated at a density of 500,000 cells in a 100 mm2 plate and irradiated using Faxitron machine with different doses of radiation (2GY, 4GY, 6GY). Non-irradiated cells were kept in the same conditions as the irradiated cells except for the delivery of a radiation dose. After 24 hours post radiation, the conditioned media was collected from the cells for ELISA as well as the intracellular cellular fraction for western blot.

PROTEIN EXTRACTION AND WESTERN BLOT

Adherent cells were scrapped from the plate in a sterile environment and treated with RIPA buffer. BCA protein kit was used for protein quantification and 20 ug of protein was loaded on the gel after being mixed with 5x laemmli buffer and heated for 7 mins at 96 degrees. Following protein separation, proteins on the gel were transferred to a nitrocellulose membrane then the membrane was blocked using 5% milk in TBS for 1 hour at room temperature. Primary antibody against HMGB1 was diluted at a concentration of 1:25000 in TBS and incubated with the membrane at 4 C overnight then washed. HRP conjugated secondary antibody was added at a concentration of 1:2000 in 5% milk in TBST for 1 hour at room temperature. Membranes were

then imaged using the chemidoc machine. Western blot results analysis and protein quantification were done using the image lab software (Bio-rad). Normalization of data was done using stain free gel approach from (Bio-rad) and is performed based on the total proteins measured directly from the membrane [199].

ELISA

Conditioned media from irradiated cells as well as control was collected 24 hours post radiation. Extracellular HMGB1 levels in the conditioned media was quantified using an ELISA kit according to the manufacturer's instructions.

IMMUNOFLUORESCENCE

Sections from formalin fixed paraffin embedded (FFPE) tissues were deparaffinized using Xylene for 10 mins, then rehydrated using serial dilutions of ethanol in distilled H_2O (100%, 95% and 70%). Antigen retrieval was done using Tris-EDTA buffer (PH 9) in a pressure cooker and slides where removed at boiling temperature and washed twice using distilled H_2O. Blocking was performed using goat serum for 1 hour then slides were permeabilized using 0.025 triton X-100 for 10 mins and rabbit anti-mouse monoclonal primary antibodies against HMGB1 (abcam, 1:250) was added and slides were incubated with the primary antibody at 4°C overnight. Alexa-fluor 568 anti-rabbit secondary antibody (Thermofisher) was added at a concentration of 1:200 for 1 hour at room temperature. Slides were then incubated with DAPI (Thermofisher) for 5 mins and mounted with gold-antifade (Thermofisher) then visualized under the confocal microscope. Zeiss software was used to evaluate the intensities of fluorescence between images. At least 4 field views images were taken for each slide in order to compare fluorescence intensities between groups.

IN-VIVO BLADDER CANCER SYNGENEIC MOUSE MODEL AND RADIATION

C57BL/6 mice were purchased from Charles River Laboratories, Inc., and kept at the McGill University Health Center-Research Institute animal facility. Ethical approval for the protocol was obtained and standards of the FACC at McGill University were followed for all in-vivo animal experiments. Only male mice between 6-8 weeks of age were used for experiments and 500,000 MB49 murine bladder cancer cells were injected subcutaneously in the right flanks of the animals. Tumors were allowed to grow to a maximum volume of 1.5 cm^3 and they were regularly monitored at least 3 times/week. Tumor measurements were performed using a digital caliper and palpable tumors were irradiated using the X-RAD smart irradiator machine (Precision X-Ray, Inc.)

GLYCYRRHIZIN IN-VIVO TREATMENT

GLZ was purchased from Sigma-Aldrich and was dissolved in warm RPMI. In-vivo administration was done by intraperitoneal injection of 50 mg/Kg per mouse at 1 hour before radiation delivery then 4 hours after and then once daily until the end point.

TUMOR DISSOCIATION

Mice were sacrificed at the respective time points and tumors were collected into tubes filled with RPMI medium and then chopped into small pieces. Tumor Dissociation Kit (Miltenyi Biotec Inc.) was used for tumor digestion as indicated in protocol and tumors were dissociated using the GentleMACS Dissociator (Miltenyi Biotec Inc.). Single cell suspensions obtained from tumors were strained against 70 um cell strainers and then treated with ACK lysis buffer (Thermofisher) in order to eliminate red blood cells and cells were counted using an automated counting machine.

FLOW CYTOMETRY AND CELL ACQUISITION

In order to obtain the best results, a minimum of 40 million tumor cells were magnetically labeled using CD4 T cell isolation kit (Miltenyi Biotec Inc.) in order to separate CD4 T cells from other cell subsets. A minimum of 5 million CD4 positive cells were stained with CD4-APC and then fixed and permeabilized using the Foxp3 staining kit (Thermofisher) and stained intracellularly with Foxp3-FITC. CD4 negative cells were stimulated using PMA-Ionomycin and then stained for surface markers CD8-BV650, Gr-1-APC-Cy7, CD11b-APC, CD206-BV421, F4/80-BUV395 and fixed then permeabilized using the Foxp3 staining kit and stained intracellularly for IFN-γ-PE. At least 1 million events were recorded and FMOs were used for gating.

RNA EXTRACTION AND RT-PCR ARRAY

Tumors established in C56B/6 mice by the injection of MB49 cell line subcutaneously into the animals were collected 1 weeks after radiation and 25 mg tumor tissues were kept in RNA-later solution (Thermofisher scientific) overnight at -20 degrees then RNA was extracted from these tissues using miRNeasy Mini Kit (Qiagen). Four mice from each group were pooled together to make a pool of samples for each group. After the elimination of genomic DNA from the samples, 500 ng/ul of RNA was used for each sample in order to construct the cDNA using RT^2 First Strand Kit (Qiagen). PCR array 96 well plates were purchased from Qiagen (RT^2 Profiler™ PCR Array Mouse Cancer Inflammation & Immunity Crosstalk). Analysis and normalization were done using software analysis provided by Qiagen. A set of housekeeping genes already included in the array were used to normalize results. Statistical significance was set to a threshold of more than 2 fold change in expression between groups.

STATISCAL ANALYSIS

The statistical analysis was done using student's T test. Graph pad prism software was used to create the graphs and calculate P-values. A P-value of <0.05 was considered statistical significant.

CHAPTER VI: (RESULTS)

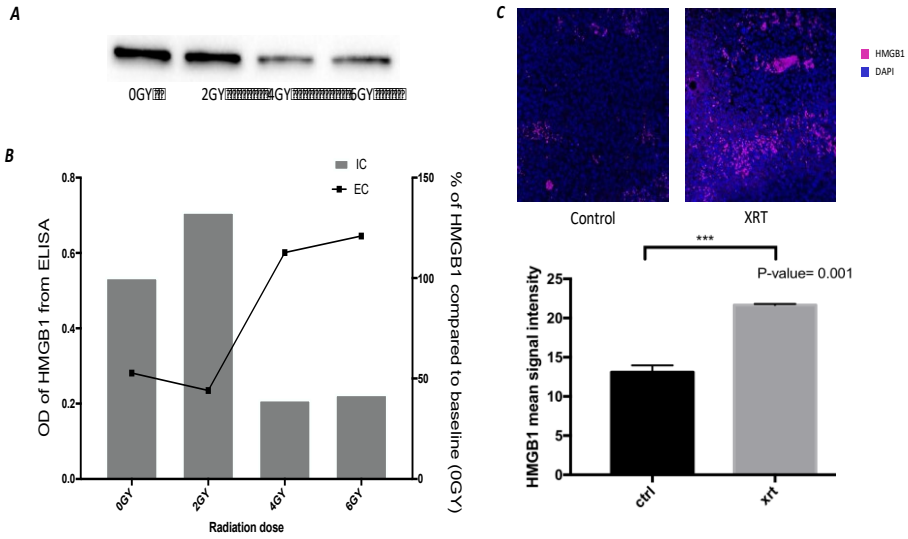
1. The expression and release of HMGB1 from MB49 bladder cancer tumors *in vitro* and *in vivo*

HMGB1 was observed to be associated with bladder cancer progression [200], invasion [201] and chemotherapy resistance [202]. Moreover, our group have shown a role for HMGB1 in bladder cancer radioresistance through its intracellular function in DNA damage repair and autophagy [198]. Radiation has been shown to promote the extracellular release of HMGB1 from cells both *in vitro* and *in vivo* [203] [50]. In order to study the role of extracellular HMGB1 in bladder cancer radioresistance, we first validated MB49 cell line for the expression and release of HMGB1 both *in vitro* and *in vivo.*

In vitro, we found that MB49 cells express HMGB1 at baseline (0 GY) as well as after *in vitro* radiation of the cells with increasing doses of radiation (2, 4 and 6 GY). The protein expression at baseline and at lower dose of radiation (2GY) was higher than the expression at 4GY or 6GY (Figure 3A). Furthermore, HMGB1 was detected in the conditioned media of cultured MB49 cells at 24 hours post radiation and its levels considerably increased with higher irradiation doses as shown in (Figure 3B). These results are consistent with previous studies [204] [205]. We speculated that MB49 cells do express HMGB1 at baseline and that irradiation of these cells lead to the release of HMGB1 into the conditioned media.

Next, in order to evaluate the expression of HMGB1 after radiation *in vivo,* MB49 bladder cancer tumors grown in C57BL/6 mice were collected and stained for HMGB1 by immunofluorescence in FFPE slides. We then compared the expression of HMGB1 in the

irradiated group vs. the control group. In contrast to our *in vitro* observations, the levels of expression of HMGB1 in the irradiated tumors were significantly elevated compared to the non-irradiated tumors as indicated by the mean fluorescence intensity of HMGB1 staining (P-value= 0.001) (Figure 3C). These findings suggest that *in vivo* irradiation of MB49 bladder cancer tumors results in higher expression of HMGB1.



(Figure 3) HMGB1 expression and release from MB49 bladder cancer model *in-vitro* and *in-vivo*

A) Western blot for intracellular expression of HMGB1 in MB49 cells at baseline and at different doses of radiation. B) Western blot quantification bar graphs showing percentages of intracellular (IC) HMGB1 compared to non-irradiated cells, the line is showing extracellular (EC) levels of HMGB1 in the conditioned media of cultured MB49 upon exposure to different doses of radiation at 24 hours post radiation. C) *Ex-vivo* Immunofluorescence staining of HMGB1 showing higher levels of expression in the irradiated tumors compared to control. *** P< 0.001 (N= 3 mice per group)

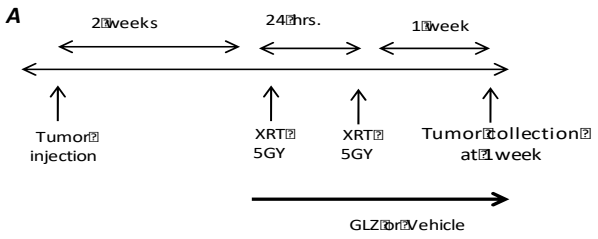
2. Extracellular HMGB1 inhibition with GLZ improves bladder cancer response to radiation *in vivo*

We then tested the hypothesis that HMGB1 is involved in bladder cancer radioresistance through its extracellular functions. Several compounds were shown to inhibit the release of HMGB1 or to bind directly to extracellular HMGB1 inhibiting its extracellular functions [85]. Glycyrrhizin (GLZ) among the other HMGB1 inhibitors has the advantage of inhibiting both the extracellular release and the interaction between HMGB1 and its receptors by specifically binding to both boxes of HMGB1 preventing it from performing its extracellular functions [95] [84]. This strategy for HMGB1 inhibition ensures near complete elimination of actively and passively released HMGB1 within the tumor microenvironment without the interference with its intracellular functions.

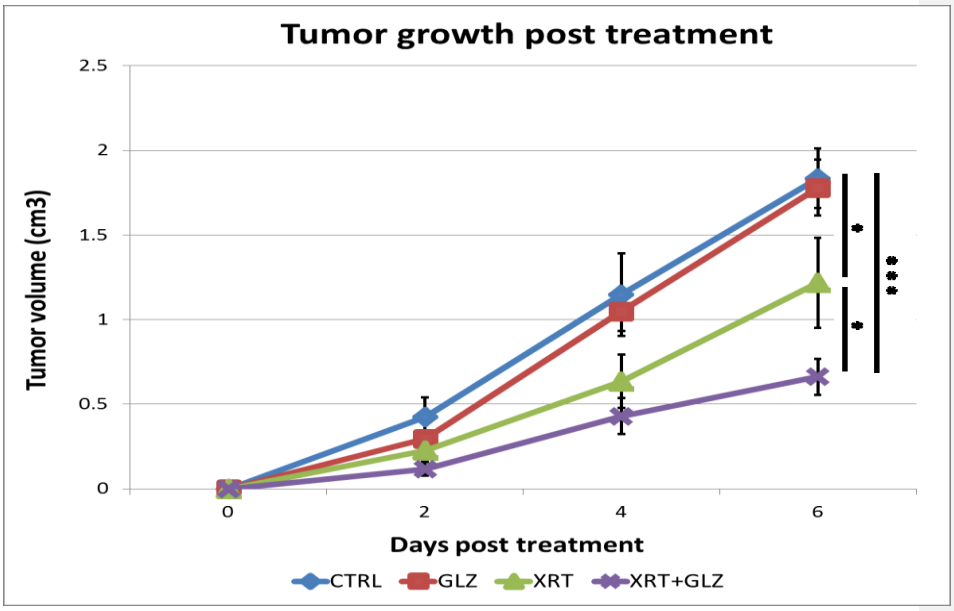
Tumors were induced and mice were randomized into four groups control (CTRL); glycyrrhizin alone (GLZ); radiation alone (XRT); and radiation + glycyrrhizin (XRT+GLZ). The timeline of the experiment as well as the treatment schedule are shown in (Figure 4A). Tumor growth was monitored by serial caliper measurements and mice were sacrificed 1 week post treatment.

We noticed a similar growth pattern in the non-irradiated groups regardless of GLZ treatment (1.83 ±0.17 cm^3 in CTRL and 1.78 ±0.16 cm^3 in GLZ). However, a significant improvement in the radiation response of the tumors was observed in the combination group compared to radiation alone as indicated by tumor volumes (0.66 ±0.1 cm^3 and 1.21 ±0.26 cm^3 respectively, *P-value*= 0.04) as shown in (Figure 4B).

These results demonstrate that extracellular HMGB1 does play a role in bladder cancer radioresistance and that the combination of radiation and HMGB1 inhibition leads to an improved radiation response of the tumor at 1 week post radiation.



B



(Figure 4) Extracellular HMGB1 inhibition results in improved radiation response of bladder cancer *in-vivo*

A) *In-vivo* experiment timeline showing treatment schedule and tumor collection end points.

B) Tumor kinetics graph showing tumor growth rate for each of the four groups (n >= 4 mice), N=3. * P< 0.05, *** P< 0.001

3) Extracellular HMGB1 is mediating bladder cancer radioresistance possibly through its immunological effects in promoting pro-tumor immunosuppressive cells

Given our previous observations, we sought to study the mechanism by which extracellular HMGB1 is mediating radioresistance of bladder cancer. We hypothesized that extracellular HMGB1 is mediating radioresistance of the tumor through its immunological functions.

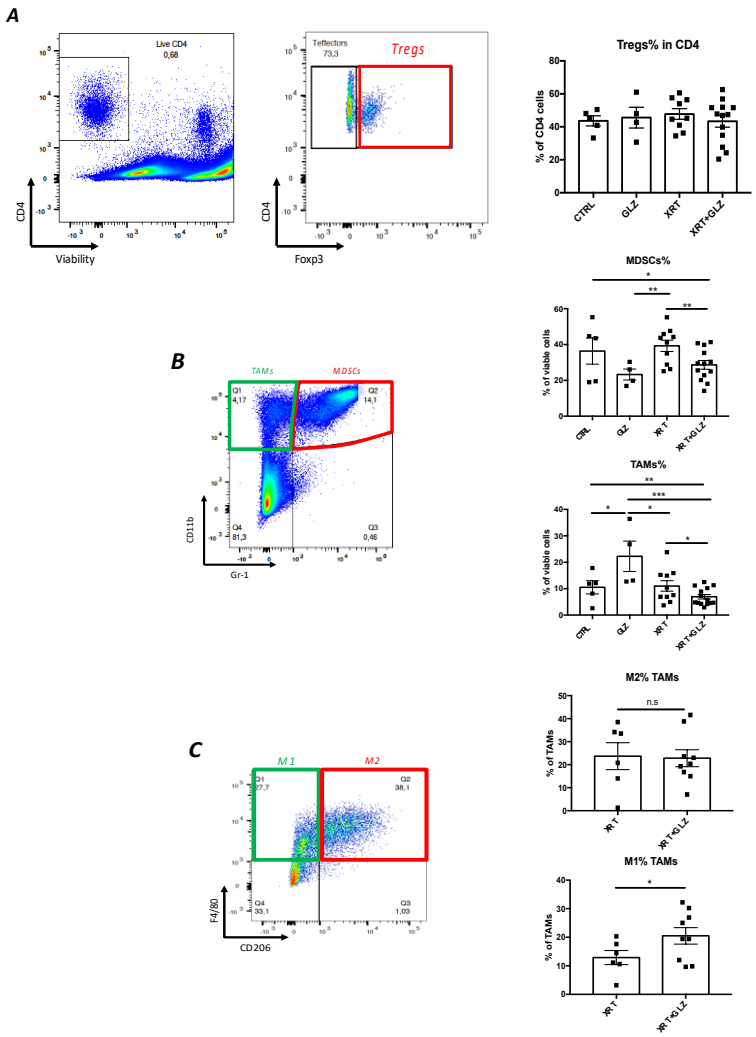
Several reports demonstrated a role for DAMP proteins released in response to radiation in mediating a pro-tumor immune microenvironment and promoting tumor progression [61]. In addition, HMGB1 was shown to promote the recruitment, proliferation and function of some immunosuppressive cells including Tregs [150] [68], MDSCs [67] [160] and TAMs [108] [164]. We evaluated whether the combination of radiation and HMGB1 inhibition would result in decreased frequencies of these cells within the tumors.

Tumors were collected at 1 week post radiation and processed into single cell suspensions. cells were then stained for flow cytometric analysis. Our results showed no significant differences in the percentage of Tregs in CD4 cells across the four groups (Figure 5A). Interestingly, the percentages of MDSCs and TAMs were significantly decreased in the combination group compared to radiation alone (*P-values*= 0.0221 and 0.0244 respectively) (Figure 5B).

It was reported that tumor infiltrating macrophages can be further subdivided into two distinct populations with different functions. M1 macrophages exhibit anti-tumor characteristics, while M2 macrophages adopt an immunosuppressive phenotype and promote tumor progression [206]. We were interested in looking at the frequencies of M1 and M2 TAMs in response to radiation and the combination of radiation and HMGB1 inhibition. We found a significant increase in the frequency of anti-tumor M1 TAMs in the combination group compared to radiation alone (*P-*

values= 0.04) while no significant difference was observed in the frequency of M2 TAMs (Figure 5C).

Taken together, we demonstrate that the combination of radiation and HMGB1 inhibition results in attenuation of immunosuppressive mechanisms within the tumor immune microenvironment by decreasing the frequency of pro-tumor immune cells.



(Figure 5) The combination of radiation and GLZ results in decreased frequency of tumor infiltrating MDSCs and TAMs at 1 week post radiation

A) Gating strategy and quantification bar graphs of Tregs percentages in CD4 cells, Tregs were identified as CD4+ Foxp3+. B) Gating strategy and quantification bar graphs of MDSCs and

TAMs percentages of live cells, MDSCs were identified as CD11b+ Gr-1+, TAMs were identified as CD11b+ Gr-1-. C) Gating strategy and quantification bar graphs of M1 and M2 TAMs, M1 and M2 were gated from TAMs cells and identified as F4/80+ CD206- or F4/80+ CD206+ respectively. * $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$

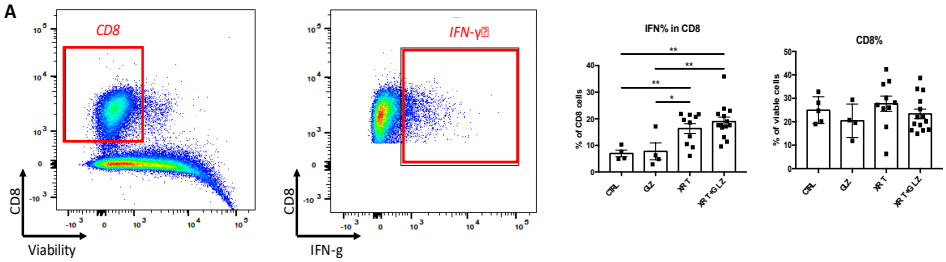
4) The effects of combining radiation with HMGB1 inhibition on the balance between pro-tumor and anti-tumor immune responses

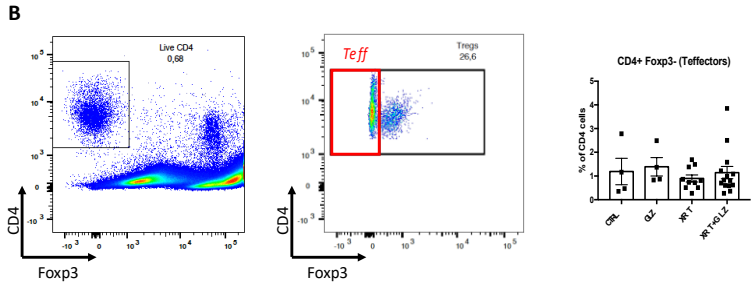
Given our previous findings regarding the changes in the frequency of certain immunosuppressive cells within the tumor microenvironment when combining radiation and HMGB1 inhibition, we then decided to look at the anti-tumor immune cell subsets.

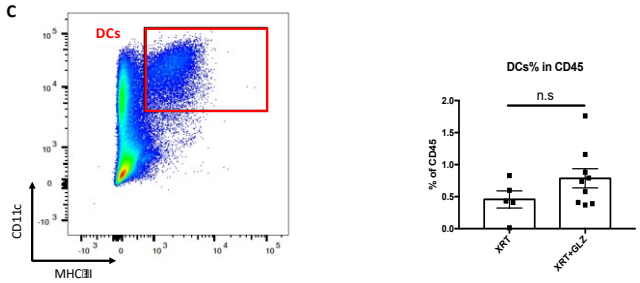
As shown in (Figure 6A & 6B), No significant differences were observed in the percentage of Foxp3-CD4+ effector T cells and CD8+ cells whereas the frequency of IFNγ+CD8+ cells was only significantly elevated in the irradiated groups compared to the non-irradiated groups indicating a role for radiation in enhancing anti-tumor immune responses via increasing the frequency of IFN-γ secreting CD8 cells.

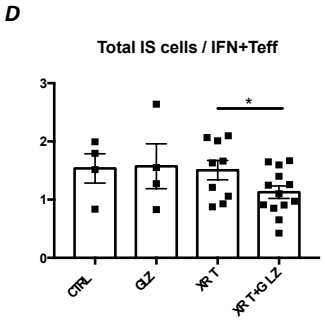
It has been reported also that radiation induced HMGB1 have a role in priming dendritic cells and enhancing their ability to present tumor antigens and in turn activating T cells [50]. We decided to test whether the inhibition of HMGB1 will result in decreased frequencies of tumor infiltrating dendritic cells. We compared the percentages of tumor infiltrating dendritic cells between XRT and XRT+GLZ groups. Despite observing a trend towards increased percentages of dendritic cells in the combination group the change didn't reach the significance level (*P-values*= 0.085), (Figure 6C).

We then calculated the ratio of immunosuppressive cells to immune effector cells as an indicator of the tumor immune balance between pro-tumor immune responses and anti-tumor immune mechanisms. As expected, the combination group had a significantly lower ratio compared to all the three other groups (*P-values*= 0.029) suggesting a shift in the tumor immune balance towards more anti-tumor response (Figure 6D).


A
CD8
Viability
IFN-γ
IFN-g
IFN% in CD8
% of CD8 cells
CD8%
% of viable cells
CTRL
GLZ
XRT
XRT+GLZ


B
Live CD4
0,68
CD4
Foxp3
Teff
Tregs
26,6
CD4+ Foxp3- (Teffectors)
% of CD4 cells


C
DCs
CD11c
MHCII
DCs% in CD45
n.s
% of CD45
XRT
XRT+GLZ



(Figure 6) Radiation plus GLZ shift the tumor immune microenvironment towards more anti-tumor response

A) Gating strategy and quantification bar graphs for the percentages of CD8 in live cells and IFN+CD8+ cells. B) Gating strategy and quantification bar graphs for the percentages of CD4+ Foxp3- cells effector T cells in CD4+ cells. C) Gating strategy and quantification bar graphs for the percentages of dendritic cells in CD45+ cells, dendritic cells were identified as MHCII+ CD11c+ from CD45+ cells. D) Quantification bar graph showing the ratio of the total immunosuppressive (IS) cells (Tregs + MDSCs + TAMs) to total anti-tumor immune cells (CD4 effector cells and IFN+CD8+ cells)

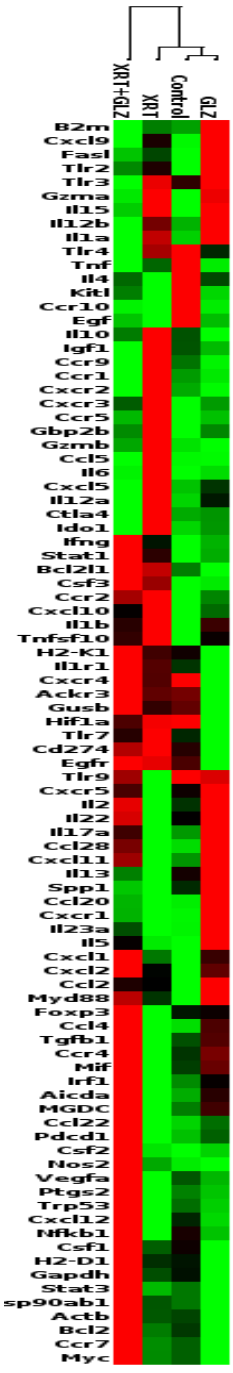
5. Differential gene expression associated with the combination of radiation and HMGB1 inhibition

With the aim to identify changes in the immune landscape of the tumors after the combination of radiation and inhibition of HMGB1, we performed a qRT-PCR micro-array from *in vivo* tumor tissues, looking at a set of genes involved in cancer immunology.

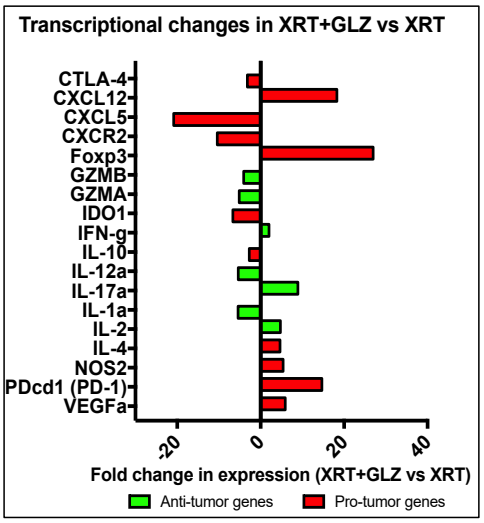
As anticipated, we observed several changes in the level of expression of certain genes across the four groups suggesting a change in the immune landscape of these tumors (Figure 7A). Among the genes that were significantly downregulated in the combination group compared to radiation alone are genes encoding for the chemokine CXCL5 and its receptor CXCR2 -both known for being involved in MDSCs and TAMs recruitment [207]- (change in gene expression of -20.58 and -10.39 folds respectively). Moreover, other pro-tumor immune genes including IDO, CTLA-4 and IL-10 were also downregulated in the combination group (Figure 7B).

These findings might suggest that the decrease in tumor infiltrating MDSCs and TAMs in the combination group is possibly due the downregulation of genes associated with their recruitment towards the tumor microenvironment.

A



B



C

	Genes	Immune effect	XRT+GLZ
Upregulation	IL-17a	Anti-tumor	8.93
	IL-2	Anti-tumor	4.77
	IFN-g	Anti-tumor	2.01
Downregulation	GZMA	Anti-tumor	-4.06
	GZMB	Anti-tumor	-5.14
	IL-12a	Anti-tumor	-5.36
	IL-1a	Anti-tumor	-5.39
Upregulation	FOXP3	Pro-tumor	26.98
	CXCL12	Pro-tumor	18.26
	PDcd1	Pro-tumor	14.67
	VEGFa	Pro-tumor	5.92
	NOS2	Pro-tumor	5.38
	IL-4	Pro-tumor	4.63
Downregulation	IL-10	Pro-tumor	-2.73
	CTLA-4	Pro-tumor	-3.17
	IDO1	Pro-tumor	-6.65
	CXCR2	Pro-tumor	-10.39
	CXCL5	Pro-tumor	-20.58

(Figure 7) Changes in gene expression levels of certain immune related genes within the tumor immune microenvironment

A) Heat map showing the differential gene expression of a set of immune related genes between groups. B) Graph showing differential gene expression between XRT group and XRT+GLZ (fold change in gene expression ≥ 2 folds), red bars representing pro-tumor genes and green bars representing anti-tumor genes. Fold changes below zero indicating a downregulation of expression in the combination group compared to radiation group. C) Table showing genes that were significantly up/downregulated in XRT+GLZ compared to XRT and their role in the tumor microenvironment.

CHAPTER VII: (DISCUSSION)

Radiation is considered a non-invasive alternative to radical cystectomy for the management of muscle invasive bladder cancer patients [9]. However, around 25-30% of muscle invasive bladder cancer patients experience local recurrence after radiation treatment [12]. Therefore, mechanisms of radioresistance in bladder cancer are still yet to be further investigated in order to achieve better outcomes.

It has been shown that the modulation of either the local or systemic immune environment might dictate the response of the tumor to radiation therapy. Depletion of certain immunosuppressive cells was found to improve radiosensitivity of many tumors [208] [209]. More recently, combination of radiation and immune checkpoints inhibitors showed promising results in clinical studies [60] [210].

Many reports demonstrated that HMGB1 is promoting radioresistance of several cancer types including esophageal cancer [211] and breast cancer [212]. In addition, we have previously shown that HMGB1 is involved in bladder cancer radioresistance by promoting DNA damage repair and autophagy of cancer cells [198]. In this study, we demonstrate a role for extracellular HMGB1 in bladder cancer radioresistance possibly mediated by its immune modulatory effects within the tumor microenvironment.

We first evaluated HMGB1 expression and extracellular release in MB49 cells *in vitro* with different doses of radiation ranging from (0GY to 6GY). The expression of HMGB1 initially increased at 2GY then sharply decreased at higher irradiation doses of 4GY and 6GY compared to the non-irradiated cells. Interestingly, extracellular HMGB1 levels measured in the

conditioned media from the irradiated cells at 24 hours post radiation, showed a sharp increase at 4GY and 6GY compared to lower doses of radiation or the non-irradiated cells. This finding suggested that radiation caused HMGB1 release likely due to necrosis of the cells and passive secretion of HMGB1 into the surrounding medium. *In vivo* staining of HMGB1 in MB49 tumors confirmed this finding when higher levels of HMGB1 were observed in irradiated tumors compared to the control tumors.

We then investigated the effect of extracellular HMGB1 inhibition using GLZ, on the radiation response of bladder cancer tumors in a syngeneic mouse model. GLZ is a known inhibitor for HMGB1 release that also selectively binds to HMGB1 [95] [84]. We show that the combination of radiation in addition to extracellular HMGB1 inhibition with GLZ results in a significant improvement in radiation response compared to radiation alone. Whereas we cannot affirm the absence of any off-target effects of GLZ in addition to extracellular HMGB1 inhibition, our results showed that *in vivo* administration of GLZ in the absence of radiation resulted in a similar tumor growth as un-treated control tumors.

The significant radiosensitization effect observed when tumors were treated with radiation in addition to HMGB1 inhibition demonstrates that HMGB1 is promoting radioresistance of bladder cancer not only through its intrinsic function within the cancer cells as we showed in our previous study [198], but also through its extracellular effects on the tumor microenvironment. This radioresistance mechanism in bladder cancer is novel and highlight the crucial role of tumor derived factors in dictating treatment outcomes.

Several reports have demonstrated that HMGB1 exerts many immunological functions that involve promoting the proliferation and function of MDSCs [67], TAMs [108] and Tregs [68]. In

order to test the hypothesis that extracellular HMGB1 is mediating bladder cancer radioresistance through its immune modulatory effects, we looked at the frequency of different anti-tumor as well as pro-tumor immune cell subsets within the tumor microenvironment including Tregs, MDSCs and TAMs. Compared to radiation alone, GLZ plus radiation resulted in a significant decrease in the frequency of tumor infiltrating MDSCs and TAMs but not Tregs. The ratio of pro-tumor immune cells to anti-tumor immune cells was significantly lower in the combination group compared to the other groups suggesting a shift in the tumor immune microenvironment towards anti-tumor immune response.

These results suggested major changes in the immune profile within the tumor microenvironment mediated by radiation and the inhibition of radiation induced extracellular HMGB1. We decided then to study the alterations in the immune landscape within the tumors at the transcriptional level by determining the changes in the level of expression of several immune related genes among the different experimental groups. Again, qRT-PCR results showed a similar gene expression profiles between the control group and the GLZ treated group. Interestingly, significant downregulation of several immunosuppressive genes was observed in the combination group compared to the radiation group.

Another interesting finding that was observed is the downregulation of the chemokine CXCL5 and its receptor CXCR2 in the combination group compared to the radiation alone group. CXLC5 is a known chemoattractant for MDSCs and TAMs and was found to be associated with their accumulation within the tumor microenvironment [207] [156]. While it was not clear whether the decreased frequency of MDSCs and TAMs in the combination group was the result of a decreased proliferative capacity of already existing cells or due to impaired recruitment of new cells, the downregulation of CXCL5 and its receptor in the combination group does suggest

an attenuated ability of the tumor to recruit MDSCs and TAMs cells towards its local microenvironment. Further research is needed in order to determine the exact mechanism by which HMGB1 is downregulating the expression of this chemokine.

From a clinical perspective, radioresistance is not the only challenge for wider use of radiotherapy in the management of muscle invasive bladder cancer. Other challenges include the lack of selection criteria that help in counseling patients that are more likely to benefit from radiation and the absence of a reliable predictive marker for treatment outcomes. Studying radiation induced changes in the tumor microenvironment might be useful in tackling some of these challenges by helping identify potential candidate factors that could serve as biomarkers for treatment response.

HMGB1 was evaluated in many studies as a biomarker for the detection of several diseases including cancer [213]. Moreover, current knowledge on HMGB1 release from dying cancer cells in response to anti-cancer therapies pointed to its potential use as a predictive marker for treatment response. One study has demonstrated that HMGB1 could serve as a predictive marker for response to oncolytic virus immunotherapy in cancer patients [214]. Another study showed that HMGB1 could be used for the early evaluation of response to radioembolization treatment in colorectal cancer [215]. Whereas radiation is known to induce HMGB1 release from cancer cells, the potential role of HMGB1 as a predictive marker for radiation response has not yet been evaluated. A possible positive correlation between higher serum levels of HMGB1 after radiation and treatment response could be hypothesized based on our observations in this current study as well as findings from previous studies. Furthermore, the infiltration of certain immunosuppressive immune cells within the tumor such as MDSCs, TAMs and Tregs after radiation may as well help in estimating the response to treatment. Though, determining the time

gap between radiation therapy initiation and biomarker level evaluation remains subject to further research.

Our findings pave the way for more future studies on the role of extracellular HMGB1 in cancer immunology and radiation resistance. Follow up studies may identify the exact mechanisms by which HMGB1 is affecting the proliferation and function of certain immune cells, this is essential in order to understand mechanisms of therapy resistance. In addition, several chemokines and receptors identified in this study such as CXCL5 and CXCR2 may also serve as future therapeutic targets for immunotherapy.

In summary, our study provides an evidence for the role of radiation in modulating the tumor microenvironment and altering the immune landscape of the tumor in bladder cancer. Moreover, we identify a novel mechanism of radioresistance in bladder cancer mediated by the immunological functions of extracellular HMGB1. Therapeutic targeting of this pathway may provide a new radiosensitization approach for bladder tumors and may result in improved outcomes for patients after radiation. Further studies are needed to validate the use of biomarkers such as serum levels of HMGB1 and tumor infiltrating immune cells as predictive markers for radiation response.

CHAPTER VIII: (CONCLUSIONS)

In conclusion, Radiation induced extracellular release of the DAMP protein HMGB1 both *in-vitro* and *in-vivo* in a bladder cancer model. Radiation induced extracellular HMGB1 is involved in radioresistance of bladder cancer as indicated by the radiosensitization effect observed after the combination of radiation in addition to the inhibition of extracellular HMGB1 using GLZ.

The improved radiation response observed in the XRT+GLZ group is possibly due to a decrease in the frequencies of tumor infiltrating MDSCs and TAMs that results in a shift in the immune balance within the tumor microenvironment towards anti-tumor immune responses.

The combinatory therapeutic approach of radiotherapy in addition to HMGB1 inhibition also results in alteration of the tumor immune landscape by downregulating the expression of several pro-tumor immunosuppressive genes.

REFERENCES

1. society, C.c. *Bladder cancer statistics*. 2018.
2. Wein, A.J., Louis R. Kavoussi, Meredith F. Campbell, and Patrick C. Walsh, *Campbell-Walsh urology*. 2012.
3. García-Closas, M., et al., *NAT2 slow acetylation and GSTM1 null genotypes increase bladder cancer risk: results from the Spanish Bladder Cancer Study and meta-analyses.* Lancet, 2005. **366**(9486): p. 649-659.
4. van Rhijn, B.W., et al., *FGFR3 and P53 characterize alternative genetic pathways in the pathogenesis of urothelial cell carcinoma.* (0008-5472 (Print)).
5. Abol-Enein, H., *Infection: is it a cause of bladder cancer?* (0300-8886 (Print)).
6. Tyson, M.D., 2nd and D.A. Barocas, *Quality of Life After Radical Cystectomy.* (1558-318X (Electronic)).
7. *Neoadjuvant chemotherapy in invasive bladder cancer: a systematic review and meta-analysis.* (0140-6736 (Print)).
8. Leow, J.J., et al., *Adjuvant chemotherapy for invasive bladder cancer: a 2013 updated systematic review and meta-analysis of randomized trials.* (1873-7560 (Electronic)).
9. Giacalone, N.J., et al., *Long-term Outcomes After Bladder-preserving Tri-modality Therapy for Patients with Muscle-invasive Bladder Cancer: An Updated Analysis of the Massachusetts General Hospital Experience.* (1873-7560 (Electronic)).
10. Zhang, S., et al., *Radiotherapy in muscle-invasive bladder cancer: the latest research progress and clinical application.* American Journal of Cancer Research, 2015. **5**(2): p. 854-868.
11. Lagrange, J.L., et al., *Quality of life assessment after concurrent chemoradiation for invasive bladder cancer: results of a multicenter prospective study (GETUG 97-015).* (1879-355X (Electronic)).
12. James, N.D., et al., *Radiotherapy with or without chemotherapy in muscle-invasive bladder cancer.* (1533-4406 (Electronic)).
13. Disis, M.L., *Mechanism of action of immunotherapy.* (1532-8708 (Electronic)).
14. Massari, F., et al., *Immune checkpoint inhibitors for metastatic bladder cancer.* Cancer Treatment Reviews, 2018. **64**: p. 11-20.
15. Gupta, S., et al., *Systemic Immunotherapy for Urothelial Cancer: Current Trends and Future Directions.* Cancers, 2017. **9**(2): p. 15.
16. Ringborg, U., et al., *The Swedish Council on Technology Assessment in Health Care (SBU) Systematic Overview of Radiotherapy for Cancer including a Prospective Survey of Radiotherapy Practice in Sweden 2001--Summary and Conclusions.* Acta Oncologica, 2003. **42**(5-6): p. 357-365.
17. Barnett, G.C., et al., *Normal tissue reactions to radiotherapy: towards tailoring treatment dose by genotype.* Nature reviews. Cancer, 2009. **9**(2): p. 134-142.
18. Lomax, M.E., L.K. Folkes, and P. O'Neill, *Biological Consequences of Radiation-induced DNA Damage: Relevance to Radiotherapy.* Clinical Oncology, 2013. **25**(10): p. 578-585.
19. Magnander, K., et al., *Clustered DNA damage in irradiated human diploid fibroblasts: influence of chromatin organization.* (1938-5404 (Electronic)).
20. Gray Lh Fau - Conger, A.D., et al., *The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy.* (0007-1285 (Print)).
21. Thomlinson Rh Fau - Gray, L.H. and L.H. Gray, *The histological structure of some human lung cancers and the possible implications for radiotherapy.* (0007-0920 (Print)).

22. Nordsmark, M., et al., *Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study.* Radiotherapy and Oncology, 2005. **77**(1): p. 18-24.
23. Mladenov, E., et al., *DNA Double-Strand Break Repair as Determinant of Cellular Radiosensitivity to Killing and Target in Radiation Therapy.* Frontiers in Oncology, 2013. **3**: p. 113.
24. Borrego-Soto, G., R. Ortiz-López, and A. Rojas-Martínez, *Ionizing radiation-induced DNA injury and damage detection in patients with breast cancer.* Genetics and Molecular Biology, 2015. **38**(4): p. 420-432.
25. Davis, A.J. and D.J. Chen, *DNA double strand break repair via non-homologous end-joining.* Translational cancer research, 2013. **2**(3): p. 130-143.
26. Lieber, M.R., *The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End Joining Pathway.* Annual review of biochemistry, 2010. **79**: p. 181-211.
27. Li, X. and W.-D. Heyer, *Homologous recombination in DNA repair and DNA damage tolerance.* Cell research, 2008. **18**(1): p. 99-113.
28. Wright, W.D., S.S. Shah, and W.D. Heyer, *Homologous recombination and the repair of DNA double-strand breaks.* (1083-351X (Electronic)).
29. Stros, M., T.M. Cherny D Fau - Jovin, and T.M. Jovin, *HMG1 protein stimulates DNA end joining by promoting association of DNA molecules via their ends.* (0014-2956 (Print)).
30. Yumoto, Y., et al., *High mobility group proteins 1 and 2 can function as DNA-binding regulatory components for DNA-dependent protein kinase in vitro.* (0021-924X (Print)).
31. Eriksson, D. and T. Stigbrand, *Radiation-induced cell death mechanisms.* (1423-0380 (Electronic)).
32. Verheij, M. and H. Bartelink, *Radiation-induced apoptosis.* Cell and Tissue Research, 2000. **301**(1): p. 133-142.
33. Vakifahmetoglu, H., B. Olsson M Fau - Zhivotovsky, and B. Zhivotovsky, *Death through a tragedy: mitotic catastrophe.* (1350-9047 (Print)).
34. Baskar, R., et al., *Cancer and Radiation Therapy: Current Advances and Future Directions.* International Journal of Medical Sciences, 2012. **9**(3): p. 193-199.
35. Chaachouay, H., et al., *Autophagy contributes to resistance of tumor cells to ionizing radiation.* (1879-0887 (Electronic)).
36. Mathew, R., V. Karantza-Wadsworth, and E. White, *Role of autophagy in cancer.* Nature reviews. Cancer, 2007. **7**(12): p. 961-967.
37. Mole, R.H., *Whole body irradiation; radiobiology or medicine?* (0007-1285 (Print)).
38. Brix, N., et al., *Abscopal, immunological effects of radiotherapy: Narrowing the gap between clinical and preclinical experiences.* (1600-065X (Electronic)).
39. Golden, E.B., et al., *The convergence of radiation and immunogenic cell death signaling pathways.* Frontiers in Oncology, 2012. **2**: p. 88.
40. Tesniere, A., et al., *Molecular characteristics of immunogenic cancer cell death.* (1350-9047 (Print)).
41. Chen, G.-Z., et al., *The mechanisms of radioresistance in esophageal squamous cell carcinoma and current strategies in radiosensitivity.* Journal of Thoracic Disease, 2017. **9**(3): p. 849-859.
42. Glick, D., S. Barth, and K.F. Macleod, *Autophagy: cellular and molecular mechanisms.* The Journal of pathology, 2010. **221**(1): p. 3-12.
43. Wang, F., et al., *Chloroquine Enhances the Radiosensitivity of Bladder Cancer Cells by Inhibiting Autophagy and Activating Apoptosis.* Cellular Physiology and Biochemistry, 2018. **45**(1): p. 54-66.
44. Huber, S., et al., *Ionizing radiation, ion transports, and radioresistance of cancer cells.* Frontiers in Physiology, 2013. **4**: p. 212.

45. Shiao, S.L. and L.M. Coussens, *The Tumor-Immune Microenvironment and Response to Radiation Therapy.* Journal of Mammary Gland Biology and Neoplasia, 2010. **15**(4): p. 411-421.
46. Zitvogel, L., et al., *Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance.* (1097-4180 (Electronic)).
47. Formenti, S.C. and S. Demaria, *Systemic effects of local radiotherapy.* The lancet oncology, 2009. **10**(7): p. 718-726.
48. Galluzzi, L., O. Kepp, and G. Kroemer, *Immunogenic cell death in radiation therapy.* Oncoimmunology, 2013. **2**(10): p. e26536.
49. Burnette, B., et al., *The Efficacy of Radiotherapy Relies Upon Induction of Type I Interferon-Dependent Innate and Adaptive Immunity.* Cancer research, 2011. **71**(7): p. 2488-2496.
50. Apetoh, L., et al., *The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy.* (0105-2896 (Print)).
51. Filatenkov, A., et al., *Ablative Tumor Radiation Can Change the Tumor Immune Cell Microenvironment to Induce Durable Complete Remissions.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2015. **21**(16): p. 3727-3739.
52. Qinfeng, S., et al., *In situ observation of the effects of local irradiation on cytotoxic and regulatory T lymphocytes in cervical cancer tissue.* (1938-5404 (Electronic)).
53. Sharma, A., et al., *Radiotherapy of human sarcoma promotes an intratumoral immune effector signature.* (1078-0432 (Print)).
54. Vatner, R.E. and S.C. Formenti, *Myeloid-derived cells in tumors: effects of radiation.* (1532-9461 (Electronic)).
55. Meng, Y., et al., *Blockade of tumor necrosis factor alpha signaling in tumor-associated macrophages as a radiosensitizing strategy.* (1538-7445 (Electronic)).
56. Barker, H.E., et al., *The Tumour Microenvironment after Radiotherapy: Mechanisms of Resistance and Recurrence.* Nature reviews. Cancer, 2015. **15**(7): p. 409-425.
57. Muroyama, Y., et al., *Stereotactic Radiotherapy Increases Functionally Suppressive Regulatory T Cells in the Tumor Microenvironment.* Cancer immunology research, 2017. **5**(11): p. 992-1004.
58. Sampath, S., et al., *Combined modality radiation therapy promotes tolerogenic myeloid cell populations and STAT3-related gene expression in head and neck cancer patients.* Oncotarget, 2018. **9**(13): p. 11279-11290.
59. Wu, C.-T., et al., *The role of PD-L1 in the radiation response and clinical outcome for bladder cancer.* Scientific Reports, 2016. **6**: p. 19740.
60. Ko, E.C. and S.C. Formenti, *Radiotherapy and checkpoint inhibitors: a winning new combination?* Therapeutic Advances in Medical Oncology, 2018. **10**: p. 1758835918768240.
61. Krysko, O., et al., *Many faces of DAMPs in cancer therapy.* (2041-4889 (Electronic)).
62. Krysko, D.V., et al., *Immunogenic cell death and DAMPs in cancer therapy.* (1474-1768 (Electronic)).
63. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, Inflammation, and Cancer.* Cell, 2010. **140**(6): p. 883-899.
64. Trabanelli, S., et al., *Extracellular ATP Exerts Opposite Effects on Activated and Regulatory CD4⁺ T Cells via Purinergic P2 Receptor Activation.* The Journal of Immunology, 2012. **189**(3): p. 1303.
65. la Sala, A., et al., *Extracellular ATP Induces a Distorted Maturation of Dendritic Cells and Inhibits Their Capacity to Initiate Th1 Responses.* The Journal of Immunology, 2001. **166**(3): p. 1611.
66. la Sala, A., et al., *Dendritic cells exposed to extracellular adenosine triphosphate acquire the migratory properties of mature cells and show a reduced capacity to attract type 1 T lymphocytes.* Blood, 2002. **99**(5): p. 1715.

67. Li, J., et al., *HMGB1 promotes myeloid-derived suppressor cells and renal cell carcinoma immune escape.* Oncotarget, 2017. **8**(38): p. 63290-63298.
68. Wild, C.A., et al., *HMGB1 conveys immunosuppressive characteristics on regulatory and conventional T cells.* (1460-2377 (Electronic)).
69. Stros, M., *HMGB proteins: interactions with DNA and chromatin.* Biochim Biophys Acta, 2010. **1799**(1-2): p. 101-13.
70. Goodwin, G.H., C. Sanders, and E.W. Johns, *A new group of chromatin-associated proteins with a high content of acidic and basic amino acids.* Eur J Biochem, 1973. **38**(1): p. 14-9.
71. Hock, R., et al., *HMG chromosomal proteins in development and disease.* Trends in Cell Biology, 2007. **17**(2): p. 72-79.
72. Reeves, R., *HMG Nuclear Proteins: Linking Chromatin Structure to Cellular Phenotype.* Biochimica et biophysica acta, 2010. **1799**(1-2): p. 3.
73. Pil, P.M. and S.J. Lippard, *Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin.* (0036-8075 (Print)).
74. Yang, H., et al., *The many faces of HMGB1: molecular structure-functional activity in inflammation, apoptosis, and chemotaxis.* (1938-3673 (Electronic)).
75. Yang, H., et al., *High Mobility Group Box Protein 1 (HMGB1): The Prototypical Endogenous Danger Molecule.* (1528-3658 (Electronic)).
76. Tang, D., T.R. Billiar, and M.T. Lotze, *A Janus Tale of Two Active High Mobility Group Box 1 (HMGB1) Redox States.* Molecular Medicine, 2012. **18**(1): p. 1360-1362.
77. Zhu, L., et al., *Redox status of high-mobility group box 1 performs a dual role in angiogenesis of colorectal carcinoma.* Journal of Cellular and Molecular Medicine, 2015. **19**(9): p. 2128-2135.
78. Frank, M.G., et al., *The redox state of the alarmin HMGB1 is a pivotal factor in neuroinflammatory and microglial priming: a role for the NLRP3 inflammasome.* Brain, behavior, and immunity, 2016. **55**: p. 215-224.
79. Venereau, E., et al., *Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release.* (1540-9538 (Electronic)).
80. Janko, C., et al., *Redox Modulation of HMGB1-Related Signaling.* Antioxidants & Redox Signaling, 2014. **20**(7): p. 1075-1085.
81. Kang, R., et al., *HMGB1 in Cancer: Good, Bad, or Both?* Clinical cancer research : an official journal of the American Association for Cancer Research, 2013. **19**(15): p. 4046-4057.
82. Andersson, U., H. Yang, and H. Harris, *Extracellular HMGB1 as a therapeutic target in inflammatory diseases.* Expert Opinion on Therapeutic Targets, 2018. **22**(3): p. 263-277.
83. Kang, R., et al., *HMGB1 in Health and Disease.* Molecular aspects of medicine, 2014. **0**: p. 1-116.
84. He, S.-J., et al., *The dual role and therapeutic potential of high-mobility group box 1 in cancer.* Oncotarget, 2017. **8**(38): p. 64534-64550.
85. Musumeci, D., G.N. Roviello, and D. Montesarchio, *An overview on HMGB1 inhibitors as potential therapeutic agents in HMGB1-related pathologies.* Pharmacology & Therapeutics, 2014. **141**(3): p. 347-357.
86. Li, J., et al., *Structural Basis for the Proinflammatory Cytokine Activity of High Mobility Group Box 1.* Molecular Medicine, 2003. **9**(1-2): p. 37-45.
87. Yang, H., et al., *Reversing established sepsis with antagonists of endogenous high-mobility group box 1.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(1): p. 296-301.
88. Gong, Q., et al., *Protective Effect of Antagonist of High-mobility Group Box 1 on Lipopolysaccharide-Induced Acute Lung Injury in Mice.* Scandinavian Journal of Immunology, 2008. **69**(1): p. 29-35.

89. Davé, S.H., et al., *Ethyl pyruvate decreases HMGB1 release and ameliorates murine colitis.* Journal of Leukocyte Biology, 2009. **86**(3): p. 633-643.
90. Li, M.L., et al., *Ethyl pyruvate administration suppresses growth and invasion of gallbladder cancer cells via downregulation of HMGB1-RAGE axis.* (0394-6320 (Print)).
91. Pellegrini, L., et al., *HMGB1 targeting by ethyl pyruvate suppresses malignant phenotype of human mesothelioma.* Oncotarget, 2017. **8**(14): p. 22649-22661.
92. Wang, L., et al., *Atorvastatin protects rat brains against permanent focal ischemia and downregulates HMGB1, HMGB1 receptors (RAGE and TLR4), NF-kappaB expression.* (1872-7972 (Electronic)).
93. Liu, M., et al., *Simvastatin suppresses vascular inflammation and atherosclerosis in ApoE(−/−) mice by downregulating the HMGB1-RAGE axis.* Acta Pharmacologica Sinica, 2013. **34**(6): p. 830-836.
94. Kuroiwa, Y., et al., *Identification and Characterization of the Direct Interaction between Methotrexate (MTX) and High-Mobility Group Box 1 (HMGB1) Protein.* PLoS ONE, 2013. **8**(5): p. e63073.
95. Mollica, L., et al., *Glycyrrhizin Binds to High-Mobility Group Box 1 Protein and Inhibits Its Cytokine Activities.* Chemistry & Biology, 2007. **14**(4): p. 431-441.
96. Gong, G., et al., *Glycyrrhizin attenuates rat ischemic spinal cord injury by suppressing inflammatory cytokines and HMGB1.* Acta Pharmacologica Sinica, 2012. **33**(1): p. 11-18.
97. Wu, X., et al., *Glycyrrhizin Suppresses the Growth of Human NSCLC Cell Line HCC827 by Downregulating HMGB1 Level.* BioMed Research International, 2018. **2018**: p. 6916797.
98. Su, X., et al., *Glycyrrhizic acid: A promising carrier material for anticancer therapy.* Biomedicine & Pharmacotherapy, 2017. **95**: p. 670-678.
99. Li, W., H. Sama Ae Fau - Wang, and H. Wang, *Role of HMGB1 in cardiovascular diseases.* (1471-4892 (Print)).
100. Wang, H., A.E. Ward Mf Fau - Sama, and A.E. Sama, *Targeting HMGB1 in the treatment of sepsis.* (1744-7631 (Electronic)).
101. Wang, Y., et al., *The Role of HMGB1 in the Pathogenesis of Type 2 Diabetes.* Journal of Diabetes Research, 2016. **2016**: p. 2543268.
102. Tang, D., et al., *High-mobility Group Box 1 [HMGB1] and Cancer.* Biochimica et biophysica acta, 2010. **1799**(1-2): p. 131.
103. Tang, D. and R. Kang, *HMGB1 in Cell Death*, in *Cell Death - Autophagy, Apoptosis and Necrosis*, T.M. Ntuli, Editor. 2015, InTech: Rijeka. p. Ch. 18.
104. Gnanasekar, M., K. Thirugnanam S Fau - Ramaswamy, and K. Ramaswamy, *Short hairpin RNA (shRNA) constructs targeting high mobility group box-1 (HMGB1) expression leads to inhibition of prostate cancer cell survival and apoptosis.* (1019-6439 (Print)).
105. Kang, R., et al., *The HMGB1/RAGE inflammatory pathway promotes pancreatic tumor growth by regulating mitochondrial bioenergetics.* Oncogene, 2014. **33**(5): p. 567-577.
106. He, S., et al., *HMGB1 released by irradiated tumor cells promotes living tumor cell proliferation via paracrine effect.* Cell Death & Disease, 2018. **9**(6): p. 648.
107. Ni, P., et al., *HMGB1 silence could promote MCF-7 cell apoptosis and inhibit invasion and metastasis.* International Journal of Clinical and Experimental Pathology, 2015. **8**(12): p. 15940-15946.
108. Huber, R., et al., *Tumour hypoxia promotes melanoma growth and metastasis via High Mobility Group Box-1 and M2-like macrophages.* Scientific Reports, 2016. **6**: p. 29914.
109. Chen, R.-C., et al., *The role of HMGB1-RAGE axis in migration and invasion of hepatocellular carcinoma cell lines.* Molecular and Cellular Biochemistry, 2014. **390**(1-2): p. 271-280.

110. Shaobo Ke, H.S., Wei Shi, Yongshun Chen, *Down-regulation of HMGB 1 induces apoptosis and inhibits invasion and migration of MCF-7 breast cancer cells through targeting SMARCC 1.* International Journal of Clinical and Experimental Pathology, 2017.
111. Jiang, W., et al., *Reduced High-Mobility Group Box 1 Expression Induced by RNA Interference Inhibits the Bioactivity of Hepatocellular Carcinoma Cell Line HCCLM3.* Digestive Diseases and Sciences, 2012. **57**(1): p. 92-98.
112. Tohme, S., et al., *Neutrophil Extracellular Traps Promote the Development and Progression of Liver Metastases after Surgical Stress.* Cancer research, 2016. **76**(6): p. 1367-1380.
113. van Beijnum, J.R., et al., *Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature.* (0006-4971 (Print)).
114. Yang, S., et al., *High-mobility group box-1 and its role in angiogenesis.* (1938-3673 (Electronic)).
115. Decker, W.K., et al., *Cancer Immunotherapy: Historical Perspective of a Clinical Revolution and Emerging Preclinical Animal Models.* Frontiers in Immunology, 2017. **8**: p. 829.
116. Ventola, C.L., *Cancer Immunotherapy, Part 3: Challenges and Future Trends.* Pharmacy and Therapeutics, 2017. **42**(8): p. 514-521.
117. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* (1097-4172 (Electronic)).
118. Burnet, M., *Cancer—A Biological Approach: I. The Processes Of Control. II. The Significance of Somatic Mutation.* British Medical Journal, 1957. **1**(5022): p. 779-786.
119. Schreiber, R.D., M.J. Old Lj Fau - Smyth, and M.J. Smyth, *Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion.* (1095-9203 (Electronic)).
120. Mittal, D., et al., *New insights into cancer immunoediting and its three component phases — elimination, equilibrium and escape.* Current opinion in immunology, 2014. **27**: p. 16-25.
121. Kim, R., M. Emi, and K. Tanabe, *Cancer immunoediting from immune surveillance to immune escape.* Immunology, 2007. **121**(1): p. 1-14.
122. Beatty, G.L. and W.L. Gladney, *Immune Escape Mechanisms as a Guide for Cancer Immunotherapy.* Clinical Cancer Research, 2015. **21**(4): p. 687.
123. Martinotti, S., M. Patrone, and E. Ranzato, *Emerging roles for HMGB1 protein in immunity, inflammation, and cancer.* Immunotargets and Therapy, 2015. **4**: p. 101-109.
124. Campana, L., P. Bosurgi L Fau - Rovere-Querini, and P. Rovere-Querini, *HMGB1: a two-headed signal regulating tumor progression and immunity.* (0952-7915 (Print)).
125. Dumitriu, I.E., et al., *Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products.* (0022-1767 (Print)).
126. Sundberg, E., et al., *High mobility group box chromosomal protein 1 acts as a proliferation signal for activated T lymphocytes.* (1878-3279 (Electronic)).
127. Li, Y., et al., *A Possible Role of HMGB1 in DNA Demethylation in CD4(+) T Cells from Patients with Systemic Lupus Erythematosus.* Clinical and Developmental Immunology, 2013. **2013**: p. 206298.
128. Aguilar-Cazares, D., et al., *Relationship of dendritic cell density, HMGB1 expression, and tumor-infiltrating lymphocytes in non-small cell lung carcinomas.* (1533-4058 (Electronic)).
129. Park, I.A., et al., *Endoplasmic reticulum stress induces secretion of high-mobility group proteins and is associated with tumor-infiltrating lymphocytes in triple-negative breast cancer.* Oncotarget, 2016. **7**(37): p. 59957-59964.
130. Lee, H.J., et al., *Cytoplasmic expression of high mobility group B1 (HMGB1) is associated with tumor-infiltrating lymphocytes (TILs) in breast cancer.* (1440-1827 (Electronic)).
131. Peng, R.-Q., et al., *Co-expression of nuclear and cytoplasmic HMGB1 is inversely associated with infiltration of CD45RO+ T cells and prognosis in patients with stage IIIB colon cancer.* BMC Cancer, 2010. **10**: p. 496-496.

132. He, Y., et al., *Tissue damage-associated "danger signals" influence T-cell responses that promote the progression of preneoplasia to cancer.* (1538-7445 (Electronic)).
133. Melis, M.H.M., et al., *The adaptive immune system promotes initiation of prostate carcinogenesis in a human c-Myc transgenic mouse model.* Oncotarget, 2017. **8**(55): p. 93867-93877.
134. Awasthi, S., *Toll-Like Receptor-4 Modulation for Cancer Immunotherapy.* Frontiers in Immunology, 2014. **5**: p. 328.
135. Riehl, A., et al., *The receptor RAGE: Bridging inflammation and cancer.* Cell Communication and Signaling : CCS, 2009. **7**: p. 12-12.
136. Anderson, A.C., *Tim-3: an emerging target in the cancer immunotherapy landscape.* (2326-6074 (Electronic)).
137. Luo, X., S.D. Miller, and L.D. Shea, *Immune Tolerance for Autoimmune Disease and Cell Transplantation.* Annual review of biomedical engineering, 2016. **18**: p. 181-205.
138. Waldmann, H., *Mechanisms of immunological tolerance.* Clinical Biochemistry, 2016. **49**(4): p. 324-328.
139. Rodríguez-Perea, A.L., et al., *Phenotypical characterization of regulatory T cells in humans and rodents.* Clinical and Experimental Immunology, 2016. **185**(3): p. 281-291.
140. Wildin, R.S. and A. Freitas, *IPEX and FOXP3: Clinical and research perspectives.* Journal of Autoimmunity, 2005. **25**: p. 56-62.
141. Caridade, M., L. Graca, and R. Ribeiro, *Mechanisms Underlying CD4+ Treg Immune Regulation in the Adult: From Experiments to Models.* Frontiers in Immunology, 2013. **4**: p. 378.
142. d'Hennezel, E. and C.A. Piccirillo, *Functional plasticity in human FOXP3+ regulatory T cells.* Human Vaccines & Immunotherapeutics, 2012. **8**(7): p. 1001-1005.
143. d'Hennezel, E., et al., *Single-cell analysis of the human T regulatory population uncovers functional heterogeneity and instability within FOXP3+ cells.* (1550-6606 (Electronic)).
144. Bin Dhuban, K., et al., *Functional dynamics of Foxp3(+) regulatory T cells in mice and humans.* (1600-065X (Electronic)).
145. Zou, W., *Regulatory T cells, tumour immunity and immunotherapy.* (1474-1733 (Print)).
146. Rech, A.J., et al., *CD25 Blockade Depletes and Selectively Reprograms Regulatory T Cells in Concert with Immunotherapy in Cancer Patients.* Science translational medicine, 2012. **4**(134): p. 134ra62-134ra62.
147. Shang, B., et al., *Prognostic value of tumor-infiltrating FoxP3(+) regulatory T cells in cancers: a systematic review and meta-analysis.* Scientific Reports, 2015. **5**: p. 15179.
148. Huang, L.F., et al., *The effect of high-mobility group box 1 protein on activity of regulatory T cells after thermal injury in rats.* (1540-0514 (Electronic)).
149. Zhu, X.M., et al., *High mobility group box-1 protein regulate immunosuppression of regulatory T cells through toll-like receptor 4.* (1096-0023 (Electronic)).
150. Liu, Z., L.D. Falo, and Z. You, *Knockdown of High Mobility Group Box 1 in Tumor Cells Attenuates Their Ability to Induce Regulatory T Cells and Uncovers Naturally Acquired CD8 T Cell-dependent Antitumor Immunity.* Journal of immunology (Baltimore, Md. : 1950), 2011. **187**(1): p. 118-125.
151. Luo, C., et al., *Toll-Like Receptor 4 Signaling in High Mobility Group Box-1 Protein 1 Mediated the Suppression of Regulatory T-Cells.* Medical Science Monitor : International Medical Journal of Experimental and Clinical Research, 2017. **23**: p. 300-308.
152. Gabrilovich, D.I. and S. Nagaraj, *Myeloid-derived-suppressor cells as regulators of the immune system.* Nature reviews. Immunology, 2009. **9**(3): p. 162-174.
153. Kortylewski, M., et al., *Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity.* (1078-8956 (Print)).

154. Parker, K.H., D.W. Beury, and S. Ostrand-Rosenberg, *Myeloid-Derived Suppressor Cells: Critical Cells Driving Immune Suppression in the Tumor Microenvironment.* Advances in cancer research, 2015. **128**: p. 95-139.
155. Toh, B., et al., *Mesenchymal Transition and Dissemination of Cancer Cells Is Driven by Myeloid-Derived Suppressor Cells Infiltrating the Primary Tumor.* PLOS Biology, 2011. **9**(9): p. e1001162.
156. Najjar, Y.G., et al., *Myeloid derived suppressor cell subset accumulation in renal cell carcinoma parenchyma is associated with intratumoral expression of IL-1ß, IL-8, CXCL5 and Mip-1α.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2017. **23**(9): p. 2346-2355.
157. Li, W., et al., *HMGB1 recruits myeloid derived suppressor cells to promote peritoneal dissemination of colon cancer after resection.* Biochemical and Biophysical Research Communications, 2013. **436**(2): p. 156-161.
158. Su, Z., et al., *Bio-HMGB1 from breast cancer contributes to M-MDSC differentiation from bone marrow progenitor cells and facilitates conversion of monocytes into MDSC-like cells.* (1432-0851 (Electronic)).
159. Parker, K.H., L.A. Horn, and S. Ostrand-Rosenberg, *High-mobility group box protein 1 promotes the survival of myeloid-derived suppressor cells by inducing autophagy.* Journal of Leukocyte Biology, 2016. **100**(3): p. 463-470.
160. Parker, K., et al., *HMGB1 enhances immune suppression by facilitating the differentiation and suppressive activity of myeloid-derived suppressor cells.* Cancer research, 2014. **74**(20): p. 5723-5733.
161. Solinas, G., et al., *Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation.* (1938-3673 (Electronic)).
162. Shand, F.H.W., et al., *Tracking of intertissue migration reveals the origins of tumor-infiltrating monocytes.* Proceedings of the National Academy of Sciences of the United States of America, 2014. **111**(21): p. 7771-7776.
163. Abraham, D., et al., *Stromal cell-derived CSF-1 blockade prolongs xenograft survival of CSF-1-negative neuroblastoma.* International journal of cancer. Journal international du cancer, 2010. **126**(6): p. 1339-1352.
164. Zhang, W., Q. Tian J Fau - Hao, and Q. Hao, *HMGB1 combining with tumor-associated macrophages enhanced lymphangiogenesis in human epithelial ovarian cancer.* (1423-0380 (Electronic)).
165. Rojas, A., et al., *HMGB1 enhances the protumoral activities of M2 macrophages by a RAGE-dependent mechanism.* (1423-0380 (Electronic)).
166. Su, Z., et al., *HMGB1 Facilitated Macrophage Reprogramming towards a Proinflammatory M1-like Phenotype in Experimental Autoimmune Myocarditis Development.* Scientific Reports, 2016. **6**: p. 21884.
167. Schaper, F., et al., *High mobility group box 1 skews macrophage polarization and negatively influences phagocytosis of apoptotic cells.* (1462-0332 (Electronic)).
168. Karuppagounder, V., et al., *Modulation of Macrophage Polarization and HMGB1-TLR2/TLR4 Cascade Plays a Crucial Role for Cardiac Remodeling in Senescence-Accelerated Prone Mice.* PLoS ONE, 2016. **11**(4): p. e0152922.
169. Guerriero, J.L., et al., *DNA Alkylating Therapy Induces Tumor Regression through an HMGB1-Mediated Activation of Innate Immunity.* Journal of immunology (Baltimore, Md. : 1950), 2011. **186**(6): p. 3517-3526.
170. Ma, Y., et al., *Dendritic Cells in the Cancer Microenvironment.* Journal of Cancer, 2013. **4**(1): p. 36-44.

171. Enk, A.H., et al., *Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma.* (0020-7136 (Print)).
172. Shurin, M.R., et al., *Regulatory dendritic cells: new targets for cancer immunotherapy.* (1555-8576 (Electronic)).
173. Demoulin, S., et al., *HMGB1 secretion during cervical carcinogenesis promotes the acquisition of a tolerogenic functionality by plasmacytoid dendritic cells.* (1097-0215 (Electronic)).
174. Zhang, Y., et al., *Tumor-derived high-mobility group box 1 and thymic stromal lymphopoietin are involved in modulating dendritic cells to activate T regulatory cells in a mouse model.* (1432-0851 (Electronic)).
175. Cerwenka, A. and L.L. Lanier, *Natural killer cell memory in infection, inflammation and cancer.* (1474-1741 (Electronic)).
176. Nair S Fau - Nair, S. and M.V. Dhodapkar Mv Fau - Dhodapkar, *Natural Killer T Cells in Cancer Immunotherapy.* (1664-3224 (Electronic)).
177. Nicholson, S.E., N. Keating, and G.T. Belz, *Natural killer cells and anti-tumor immunity. LID - S0161-5890(17)30595-3 [pii] LID - 10.1016/j.molimm.2017.12.002 [doi].* (1872-9142 (Electronic)).
178. Parodi, M., et al., *Natural Killer (NK)/melanoma cell interaction induces NK-mediated release of chemotactic High Mobility Group Box-1 (HMGB1) capable of amplifying NK cell recruitment.* Oncoimmunology, 2015. **4**(12): p. e1052353.
179. DeMarco, R.A., M.T. Fink Mp Fau - Lotze, and M.T. Lotze, *Monocytes promote natural killer cell interferon gamma production in response to the endogenous danger signal HMGB1.* (0161-5890 (Print)).
180. Cantoni, C., et al., *NK Cells, Tumor Cell Transition, and Tumor Progression in Solid Malignancies: New Hints for NK-Based Immunotherapy?* Journal of Immunology Research, 2016. **2016**: p. 4684268.
181. Powell, D.R. and A. Huttenlocher, *Neutrophils in the tumor microenvironment.* Trends in immunology, 2016. **37**(1): p. 41-52.
182. Houghton, A.M., et al., *Neutrophil Elastase-Mediated Degradation of IRS-1 Accelerates Lung Tumor Growth.* Nature medicine, 2010. **16**(2): p. 219-223.
183. Eruslanov, E.B., et al., *Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer.* The Journal of Clinical Investigation, 2014. **124**(12): p. 5466-5480.
184. Takeshima, T., et al., *Key role for neutrophils in radiation-induced antitumor immune responses: Potentiation with G-CSF.* Proceedings of the National Academy of Sciences of the United States of America, 2016. **113**(40): p. 11300-11305.
185. Patnaik, A., et al., *Cabozantinib Eradicates Advanced Murine Prostate Cancer by Activating Antitumor Innate Immunity.* (2159-8290 (Electronic)).
186. Tadie, J.-M., et al., *HMGB1 promotes neutrophil extracellular trap formation through interactions with Toll-like receptor 4.* American Journal of Physiology - Lung Cellular and Molecular Physiology, 2013. **304**(5): p. L342-L349.
187. Dine, J., et al., *Immune Checkpoint Inhibitors: An Innovation in Immunotherapy for the Treatment and Management of Patients with Cancer.* Asia-Pacific Journal of Oncology Nursing, 2017. **4**(2): p. 127-135.
188. Tang, D. and M.T. Lotze, *Tumor immunity times out: TIM-3 and HMGB1.* Nature immunology, 2012. **13**(9): p. 808-810.
189. Chiba, S., et al., *Tumor-infiltrating DCs suppress nucleic acid–mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1.* Nature immunology, 2012. **13**(9): p. 832-842.

190. Li, G., X. Liang, and M.T. Lotze, *HMGB1: The Central Cytokine for All Lymphoid Cells.* Frontiers in Immunology, 2013. **4**: p. 68.
191. Yang, D., et al., *High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin.* (0741-5400 (Print)).
192. Abe, A., et al., *High Mobility Group Box1 (HMGB1) released from cancer cells induces the expression of pro-inflammatory cytokines in peritoneal fibroblasts.* (1440-1827 (Electronic)).
193. Yan, W., et al., *High Mobility Group Box 1 Activates Caspase-1 and Promotes Hepatocellular Carcinoma Invasiveness and Metastases.* Hepatology (Baltimore, Md.), 2012. **55**(6): p. 1863-1875.
194. Yun, J., et al., *The HMGB1–CXCL12 Complex Promotes Inflammatory Cell Infiltration in Uveitogenic T Cell-Induced Chronic Experimental Autoimmune Uveitis.* Frontiers in Immunology, 2017. **8**: p. 142.
195. Russo, V. and M.P. Protti, *Tumor-derived factors affecting immune cells.* Cytokine & Growth Factor Reviews, 2017. **36**: p. 79-87.
196. Shabsigh, A., et al., *Defining early morbidity of radical cystectomy for patients with bladder cancer using a standardized reporting methodology.* (1873-7560 (Electronic)).
197. Mak, R.H., et al., *Long-Term Outcomes in Patients With Muscle-Invasive Bladder Cancer After Selective Bladder-Preserving Combined-Modality Therapy: A Pooled Analysis of Radiation Therapy Oncology Group Protocols 8802, 8903, 9506, 9706, 9906, and 0233.* Journal of Clinical Oncology, 2014. **32**(34): p. 3801-3809.
198. Shrivastava, S., et al., *The Role of HMGB1 in Radioresistance of Bladder Cancer.* (1538-8514 (Electronic)).
199. Gurtler, A., et al., *Stain-Free technology as a normalization tool in Western blot analysis.* (1096-0309 (Electronic)).
200. Wang, W.E.I., et al., *Overexpression of high mobility group box 1 and 2 is associated with the progression and angiogenesis of human bladder carcinoma.* Oncology Letters, 2013. **5**(3): p. 884-888.
201. Luo, J., et al., *LncRNA UCA1 promotes the invasion and EMT of bladder cancer cells by regulating the miR-143/HMGB1 pathway.* Oncology Letters, 2017. **14**(5): p. 5556-5562.
202. Yin, H., et al., *HMGB1-mediated autophagy attenuates gemcitabine-induced apoptosis in bladder cancer cells involving JNK and ERK activation.* Oncotarget, 2017. **8**(42): p. 71642-71656.
203. Wang, L., et al., *Ionizing Radiation Induces HMGB1 Cytoplasmic Translocation and Extracellular Release.* Guo ji fang she yi xue he yi xue za zhi = International journal of radiation medicine and nuclear medicine, 2016. **40**(2): p. 91-99.
204. Jiang, H., et al., *Down-regulation of LncRNA TUG1 enhances radiosensitivity in bladder cancer via suppressing HMGB1 expression.* Radiation Oncology, 2017. **12**(1): p. 65.
205. Pasi, F., et al., *Effects of single or combined treatments with radiation and chemotherapy on survival and danger signals expression in glioblastoma cell lines.* (2314-6141 (Electronic)).
206. Yang, L. and Y. Zhang, *Tumor-associated macrophages: from basic research to clinical application.* Journal of Hematology & Oncology, 2017. **10**(1): p. 58.
207. Wang, G., et al., *Targeting YAP-dependent MDSC infiltration impairs tumor progression.* Cancer discovery, 2016. **6**(1): p. 80-95.
208. Kachikwu, E.L., et al., *Radiation Enhances Regulatory T Cell Representation.* International journal of radiation oncology, biology, physics, 2011. **81**(4): p. 1128-1135.
209. Ahn, G.O., et al., *Inhibition of Mac-1 (CD11b/CD18) enhances tumor response to radiation by reducing myeloid cell recruitment.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(18): p. 8363-8368.

210. Shabason, J.E. and A.J. Minn, *Radiation and Immune Checkpoint Blockade: From Bench to Clinic.* (1532-9461 (Electronic)).
211. Zhao, Y., et al., *Wnt signaling induces radioresistance through upregulating HMGB1 in esophageal squamous cell carcinoma.* Cell Death & Disease, 2018. **9**(4): p. 433.
212. Ke, S., et al., *Downregulation of high mobility group box 1 modulates telomere homeostasis and increases the radiosensitivity of human breast cancer cells.* (1791-2423 (Electronic)).
213. Venereau, E., et al., *HMGB1 as biomarker and drug target.* Pharmacological Research, 2016. **111**: p. 534-544.
214. Liikanen, I., et al., *Serum HMGB1 is a predictive and prognostic biomarker for oncolytic immunotherapy.* OncoImmunology, 2015. **4**(3): p. e989771.
215. Fahmueller, Y.N., et al., *Immunogenic cell death biomarkers HMGB1, RAGE, and DNAse indicate response to radioembolization therapy and prognosis in colorectal cancer patients.* (1097-0215 (Electronic)).