HMGB-1 Release and the CD8⁺ T Cell Response Elicited by Radiation Treatment in Malignant Pleural Mesothelioma

by

Matthew Wu

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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2015

Abstract

Malignant pleural mesothelioma (MPM) is a cancer of the pleura that is associated with the inhalation of asbestos. Treatments, such as radiation therapy, have direct cytotoxic effects on cancer cells in addition to immune activating effects through the release of pro-inflammatory molecules associated with cancer cell death. The goal of this project was to investigate the role of High Mobility Group Box 1 (HMGB-1), a typical Danger Associated Molecular Pattern (DAMP) protein released after radiation treatment, in CD8$^+$ T Cell mediated tumor killing and in patient survival. Radiated tumor cells release HMGB-1 in vitro. Furthermore, radiation treatment leads to HMGB-1 release and correlates with higher survival in vivo. This thesis demonstrates that radiation leads to HMGB-1 release which is correlated to MPM cell death and increased survival in vivo. The findings of this project could be used to develop new therapies in combination with radiation.
Acknowledgments

Graduate school is an experience. Dr. Heath McMillan, a PhD student at the time whom I was working with, once told me: “research is like a drug; the lows are very low, but the highs are incredible”. At this point I can absolutely say that he was right. Sometimes research can be disheartening as one failure is met with the next. However, finding a truly amazing result and knowing that I’m the first person to be uncovering this individual piece of a complex biological puzzle, is something that has kept me coming back for more. I have numerous people to thank for this tremendous opportunity and for their support.

Firstly I would like to thank my supervisor Dr. Marc de Perrot for his unparalleled support and guidance. From my time as a summer student he instilled in me a passion for cancer research, a subject in which I had little previous experience. Now as I finish my Master of Science degree, that passion continues. Marc has been a great mentor for me and I hope that I can inspire others as he has inspired me.

The members of the lab have been huge supporters of my work. Dr. Licun Wu, Ms. Hana Yun, and Dr. Yidan Zhao have taught me everything from cell culture, to flow cytometry and I am incredibly thankful for their guidance. I appreciate all the administrative help from Ms. Corrina Cufaro, Ms. Susan Beaudoin, and Ms. Ivone Ornelas. A big thank you to Dr. Luis De La Maza who has not only collaborated and shared in the graduate experience with me but has also become a great friend.

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Contributions

This work was supported by the Swiss National Science Foundation (SNSF), Toronto General Hospital Foundation, and Princess Margaret Hospital Foundation Grants. Dr. Luis de la Maza performed the image-guided irradiation of mice using the X-Rad 225Cx irradiator. Dr. Licun Wu performed some of the injections of anti-HMGB-1 into the mice.
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CRT</td>
<td>Calreticulin</td>
</tr>
<tr>
<td>CTCF</td>
<td>Corrected Total Cell Fluorescence</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-Lymphocyte-Associated Antigen 4</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger Associated Molecular Pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EPD</td>
<td>Extended Pleurectomy-Decortication</td>
</tr>
<tr>
<td>EPP</td>
<td>Extrapleural Pneumonectomy</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Related Kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Fv</td>
<td>Variable Fragment</td>
</tr>
<tr>
<td>H2SeO3</td>
<td>Selenious Acid</td>
</tr>
<tr>
<td>HMGB-1</td>
<td>High Mobility Group Box 1</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular Adhesion Molecule</td>
</tr>
<tr>
<td>ICD</td>
<td>Immunogenic Cell Death</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte Function Associated Antigen</td>
</tr>
<tr>
<td>LRT</td>
<td>Local Radiation Therapy</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Mac-1</td>
<td>Macrophage Receptor 1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility</td>
</tr>
<tr>
<td>MPM</td>
<td>Malignant Pleural Mesothelioma</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD-like Receptor Family Pyrin Domain Containing 3</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed Death-1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed Death Ligand 1</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen Recognition Receptor</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation End Products</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SMART</td>
<td>Surgery for Mesothelioma After Radiation Therapy</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>VLA</td>
<td>Very Late Antigen</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilms Tumor Protein</td>
</tr>
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</table>
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Chapter 1

1 General Background: Malignant Pleural Mesothelioma; Epidemiology, Development, Pathogenesis, and Treatment

1.1 Malignant Pleural Mesothelioma Background

Malignant pleural mesothelioma (MPM) is a cancer that affects the serous membrane of the pleura (Jaurand, et al. 2009). Known for its lack of response to treatment, MPM has a median survival from presentation of 9-12 months (Armstrong, et al. 1984, Robinson, et al. 2005). The incidence of MPM has increased by 65 percent over the past two decades worldwide and is projected to continue to increase. MPM is associated with asbestos exposure and while asbestos use has been controlled in North America, the incidence of disease is predicted to continue to increase for at least the next 5 to 10 years (Peto, et al. 1995, Spirtas, et al. 1986, Walker, et al. 1983). The continued use of asbestos in the developing world suggests that the incidence of MPM will continue to increase worldwide for the foreseeable future.

1.1.1 The Mesothelium

MPM affects the cells of the mesothelium. The concept of the mesothelium was first proposed in 1890 from observations of epithelial-like cells coating the organs of the body (Minot 1890). The mesothelium, consisting of a mono-layer of mesothelial cells, displays a morphological cobblestone-like appearance. It is a functionally complex tissue, providing a non-adherent
surface for organs through fluid secretion. In addition to preventing organ abrasion, the mesothelium also mediates many inflammatory processes such as antigen presentation and chemokine production (Mossman, et al. 2013, Mutsaers 2004, Yung and Chan 2007). Furthermore, the mesothelium mediates other functions such as the transportation of fluids across serous membranes, and is essential to tumor cell adhesion in MPM (Mutsaers 2004).

1.1.2 Presentation

Mesotheliomas can affect the serous surfaces of the pleura, peritoneum, and pericardium. While pleural mesotheliomas have been reported to represent around 73% of all mesothelioma diagnoses, it is important to note that with progression of disease, this neoplasm can expand to the other cavities of the body (Suzuki 2001).

MPM is a disease that affects more males than females with males reported to represent 68% to 79% of all MPM cases (Adams, et al. 1986, Antman, et al. 1988, Brenner, et al. 1982, Ratzer, et al. 1967, Ruffie, et al. 1989). Many clinical studies universally confirm the greater incidence of disease in males with history of asbestos exposure being a common factor. The mean age of disease diagnosis is around 55. However, a wide range of diagnoses in older individuals as well as in children has been reported (Fraire, et al. 1988).

Symptoms of MPM occur very slowly. Patients typically present with a characteristic unexplained shortness of breath related to excess pleural fluid, known as pleural effusion, in addition to chest wall pain (Ismail-Khan, et al. 2006). Pleural effusion in the pleural space reduces the lungs capacity to expand and leads to breathing difficulty and chest pain localized to the affected side (Chahinian, et al. 1982). The right side is more commonly affected,
representing around 60% of cases due to asbestos concentrating more readily in the right lung (Pass, et al. 2005).

MPM progression and growth results in the gradual thickening of the pleura, leading to a loss of the pleural space. The lung on the affected side is unable to inflate, resulting in progressing symptoms of dyspnea and chest pain. MPM may invade surrounding areas such as the lung, chest wall, and diaphragm. Additionally, MPM has been observed to expand to the contralateral side and the peritoneum (Chahinian, et al. 1982).

1.2 Asbestos and Other Risk Factors for MPM

The toxicity of asbestos has been known much before its association with mesothelioma. In 1935, asbestos was first implicated in the development of lung carcinomas (Lynch and Smith 1935). In the 1950s, asbestos was being consistently shown to induce lung cancers in asbestos workers (Braun and Truan 1958, Doll 1993).

The first link between asbestos and mesotheliomas was established by a fundamental study that noted that while pleural mesothelioma is an uncommon tumor, 45 of the 47 identified cases of mesothelioma in the north west of Cape Province, South Africa had a history of crocidolite asbestos fiber exposure (Wagner, et al. 1960). In 1964 this finding was supported by a study that found 4 mesotheliomas in 255 deaths among insulation workers in the Asbestos Workers Union in New York (Selikoff, et al. 1964). In both cases, the authors described the number of MPM cases as exceedingly high for such a rare tumor.
**Figure 1. Asbestos carcinogenesis.** Inhaled asbestos penetrates through the lung and becomes lodged in visceral pleura where it may cause cells to undergo transformation into cancer.

MPM’s known association with asbestos exposure is unique as it is one of the few cancers with a known causative agent. Asbestos is a naturally occurring mineral with common uses including insulation for buildings. Its malleability, tensile strength, and heat resistance render it useful in industrial processes. There is some evidence to suggest that exposure to asbestos can occur due to naturally occurring asbestos-containing land forms (Pan, et al. 2005). However, occupation remains the primary risk factor for developing MPM (Agudo, et al. 2000, Godleski 2004, Goldberg, et al. 2000, Howel, et al. 1999, Iwatsubo, et al. 1998, Kishimoto, et al. 2004, Zellos and Christiani 2004).

There is a higher incidence of MPM in males compared to females as a result of asbestos-related work being traditionally performed by men. In addition, the husband’s profession remains a risk factor for women who present with MPM as asbestos fibers brought home on clothing often lead to low-levels of domestic exposure (Huncharek, et al. 1989, Roggli, et al. 1997). The mean time between exposure to asbestos and onset of disease is around 30 to 40 years. While the risk of
mesothelioma 10 years after exposure to asbestos is virtually non-existent, the probability of developing mesothelioma increases over time (Bianchi, et al. 1997, Boffetta 1998, Hillerdal 1999, Mossman and Gee 1989).


1.3 MPM Development

It is thought that the development of MPM is mediated by the asbestos fibers which penetrate deep into the lung and scratch the mesothelial cells lining the lung. The asbestos fibers that lodge in the lung are able to repeatedly damage the mesothelium and result in chronic inflammation; a hallmark cause of cancer development (Hanahan and Weinberg, Thompson, et al. 2014).

While the immune response is able to target and destroy cancerous cells, it is often aberrant immune activation that leads to cancer development (Hanahan and Weinberg). Inflammatory
infiltrates from persistent inflammation consist primarily of macrophages that release pro-inflammatory cytokines such as IL (Interleukin)-1β and TNF (Tumor Necrosis Factor)-α in addition to reactive oxygen species (ROS). Asbestos results in reactive oxygen species generation and aberrant growth receptor signaling from macrophages attempting to digest the fibers (Scott, et al. 2012).

NLRP3 (NOD-like receptor family pyrin domain containing) inflammasome is a DAMP sensing mechanism that may be important aspect of mesotheliogenesis. The NLRP3 inflammasome is triggered by extracellular DAMP (Danger Associated Molecular Pattern) molecules as well as silicates such as asbestos. Macrophages are unable to breakdown asbestos or other silicate fibers due to the high aspect ratio (the ratio of length to diameter); leading to “frustrated” phagocytosis (Hornung, et al. 2008). The lysosome, which provides a wide range of degrading enzymes to aid in phagocytic breakdown, swells and ruptures when trying to destroy asbestos fibers. This damage triggers the cathepsin-B pathway and subsequent activation of the NLRP3 inflammasome. The importance of the NLRP3 inflammasome is demonstrated by its activation of IL-1β secretion. In non-infectious or injury settings, IL-1β is not secreted by tissue macrophages. However, the presence of asbestos fibers can lead to the secretion of the pro-inflammatory IL-1β; providing a setting for chronic inflammation and transformation of mesothelial cells.

Recently, the NLRP3 inflammasome has been suggested as being dispensable in MPM development as NLRP3−/− mice and wildtype mice exposed to asbestos showed similar survival times (Chow, et al. 2012). This indicates that the NLRP3 inflammasome is not essential for tumor development in mice exposed to asbestos. However, this NLRP3 has not been precluded
from being a non-essential contributor to mesothelioma development. Furthermore, other pathways of inflammation may function as redundancy when NLRP3 genes are knocked out.

The inflammation from damage and repair contributes to DNA damage and subsequent transformation of cells into cancerous cells. Asbestos fibers have also been implicated in disturbing mitosis by damaging the mitotic spindle apparatus that occurs during cell division, resulting in chromosomal damage and the potential for mutation (Dinarello 2009).

1.3.1 Histological Subtypes


Epithelioid MPM is typically identified by immunohistochemical staining which is able to distinguish between epithelioid MPM, sarcomatoid MPM, Biphasic MPM, sarcoma, adenocarcinoma, and carcinosarcoma (Inai 2008). Markers for epithelioid MPM include positive staining for Calretinin (Doglioni, et al. 1996, Gotzos, et al. 1996, Ordonez 2005, Ordonez 2007), WT-1(Ordonez 2007), Podoplanin (Kimura and Kimura 2005, Ordonez 2005, Ordonez 2007), and Keratin 5/6 (Ordonez 2007). Sarcomatoid MPM is not as easily identified and may be

Table I. Positive staining patterns of epithelioid and sarcomatoid MPM.

<table>
<thead>
<tr>
<th></th>
<th>Epithelioid</th>
<th>Sarcomatoid</th>
</tr>
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<tbody>
<tr>
<td>Calretinin</td>
<td>92-100%</td>
<td>18-100%</td>
</tr>
<tr>
<td>WT-1</td>
<td>69-99%</td>
<td>10%</td>
</tr>
<tr>
<td>Podoplanin</td>
<td>66-93%</td>
<td>30-72%</td>
</tr>
<tr>
<td>Keratin 5/6</td>
<td>92-100%</td>
<td>0%-29%</td>
</tr>
<tr>
<td>AE1/AE3</td>
<td>100%</td>
<td>77-100%</td>
</tr>
<tr>
<td>CAM5.2</td>
<td>97-100%</td>
<td>90-94%</td>
</tr>
</tbody>
</table>

Approximately 60% of all MPM cases are of the epithelioid subtype (Husain, et al. 2009). Biphasic and sarcomatoid subtypes are less common and comprise of about 30% and 10% of cases respectively. Biphasic disease is diagnosed when at least 10% of the visible cells are epithelioid cells in sarcomatoid disease or when 10% of cells in a predominantly epithelioid tumor are sarcomatoid in nature (Pass, et al. 2005, Tischoff, et al. 2011).

The epithelioid subtype of disease typically provides the best prognosis while biphasic and sarcomatoid subtype of disease present shorter survival times and are significantly negative prognostic factors (Neumann, et al. 2013). While MPM is resistant to the typical cancer
treatments chemotherapy, radiation, and surgery, better outcomes from chemotherapeutic treatment are observed in the epithelioid subtype. In contrast, sarcomatoid disease is ordinarily unresponsive to chemotherapy (van Zandwijk, et al. 2013). Furthermore, multimodality therapy presents better outcomes for epithelioid disease than for biphasic or sarcomatoid subtypes (Ceresoli, et al. 2001, Richards, et al. 2006).

**Figure 2. H & E staining of patient biopsies.** Epithelioid disease, sarcomatoid MPM, and the biphasic subtypes are displayed. Biphasic MPM possesses cells of both the epithelioid and sarcomatoid morphology.

### 1.4 Current Treatment Options

MPM is challenging to treat and presents a median survival time after diagnosis of less than 12 months (Vogelzang, et al. 2003). Patient prognoses after standard therapeutic interventions such
as surgery, radiation, and chemotherapy have remained bleak. There is a substantial need to investigate new avenues of treatment to bring about positive change for MPM patients.

1.4.1 Surgery


Extended pleurectomy-decortication (EPD) is a radical surgery that involves the removal of the parietal and visceral pleura, diaphragm, and sometimes the pericardium leaving the lung denuded but remaining in the body (Rusch 2012). EPD may be advantageous for older individuals as the removal of the lung in EPP may not be well tolerated for individuals over 65. While EPP offers better potential clearance, EPD alone offers similar survival to EPP (Nakas and Waller 2014).
1.4.2 Chemotherapy

Non-surgically resectable disease is treated with the first-line chemotherapy regimen of Pemetrexed and Cisplatin. Cisplatin is a platinum-based drug that causes apoptosis through the cross-linking of DNA (Tanida, et al. 2012). Pemetrexed acts by inhibiting the enzyme thymidylate synthase, effectively preventing DNA synthesis (Vogelzang, et al. 2003). In a phase III clinical trial, Vogelzang and colleagues found median survival time of patients receiving Pemetrexed and Cisplatin of 12.1 months compared to 9.3 months in patients receiving only Cisplatin. Currently, there is no standard second-line chemotherapy regimen (Ceresoli, et al. 2011, Pasello, et al. 2011, Stebbing, et al. 2009, Xanthopoulos, et al. 2008, Zauderer and Krug 2012, Zucali, et al. 2008). Moderate increase in survival times have been reported in the use of second-line chemotherapeutics, however, low patient numbers in these studies warrants further investigation.

A previous study involving the use of bevacizumab, an anti-VEGF antibody, in addition to the Pemetrexed and Cisplatin combination failed to demonstrate any benefit in survival of mesothelioma patients (Dowell, et al. 2012). However, a randomized phase 3 trial with a greater number of patients presented at the 2015 American Society of Clinical Oncology (ASCO) annual meeting found a significant increase in overall survival of patients receiving Pemetrexed, Cisplatin and bevacizumab triplet therapy (median: 18.8 months) compared to Pemetrexed and Cisplatin only (median: 16.1 months) (Zalcman, et al. 2015). These results indicate that triplet therapy may become the new first line treatment for unresectable MPM.
1.4.3 Radiation Therapy

Radiation therapy is typically provided as part of multimodality treatment including surgery and radiation. While there have been no randomized trials demonstrating its effectiveness, in a phase I/II trial, Surgery for Mesothelioma After Radiation Therapy (SMART) has displayed promising results with epithelial MPM patients experiencing a 3-year survival of 84% up from 30% with previous radiotherapy regimens (Cho, et al. 2014, Haas and Sterman 2013). This is in sharp contrast to a previous report on 140 asbestos workers with pleural mesothelioma in which only one patient survived longer than 3 years after diagnosis without treatment (Ribak and Selikoff 1992). Furthermore, median survival of patients with biphasic disease without treatment is estimated to be around 4-5 months (Moore, et al. 2008).

Figure 3. Overall survival of patients with epithelial and biphasic subtypes of disease. At 3 years, overall survival in epithelioid MPM patients was 84% (Figure reproduced from previous article in our lab (Cho, et al. 2014)).
1.4.4 SMART Treatment

SMART is a new methodology of administering a short course of high-dose radiation before surgery. This method differs from previous approaches in which surgery is used to remove macroscopic disease followed by adjuvant radiation or chemotherapy given after surgical resection in order to remove remaining microscopic disease.

The increase in survival is thought to occur as a result of the specific immune activation effects (Lee, et al. 2009, Levy, et al.). A short course of high dose radiation can activate the immune system by antigen release from dying tumor cells. A negative aspect of radiation treatment is that radiation also kills CD8$^+$ T Cells. However, hypofractionated radiation, as used in SMART, does not deplete CD8$^+$ T cells for as great a time as longer low dose regimens do, potentially allowing for more CD8$^+$ T cell mediated tumor cell killing (Siva, et al. 2015).

![Image](image-url)

**Figure 4.** Radiation treatment of cancers leads to DNA damage. DNA damage may be repaired and result in tumor cell survival. Greater number of DNA double-stranded breaks leads to irreparable damage and either programmed cell death or necrosis.
1.4.4.1 Abscopal Effect and Immunogenic Radiation

A strong indicator of the immune system activating effects of radiation is the presence of the abscopal effect; the shrinkage of a distant tumor outside the field of treatment after radiating a tumor mass (Antoniades, et al., Cotter, et al. 2011, Isobe, et al. 2009, Lakshmanagowda, et al. 2009, Takaya, et al. 2007). In clinical reports and animal studies, distant tumors can regress as a result of irradiation of the primary tumor. The radiation induced abscopal effect is thought to occur due to immune system activation towards antigens shared by both local and distant tumors. This may allow for the killing of distant tumors and the prevention of recurrence. This effect has been observed clinically in many cancers including lymphoid malignancies, hepatocellular carcinoma, uterine cervical carcinoma, Merkel cell carcinoma, uterine cervical carcinoma, and melanoma.

A study from our lab demonstrated the immunogenic aspect of radiation by reproducing the abscopal effect in a concomitant immunity model where two MPM tumors were injected concurrently on the leg and on the flank (Wu, et al. 2015). While the irradiated leg tumor shrank in size due to radiation-induced DNA damage and cell death, the flank tumor outside of the field of radiation also shrunk presumably as a result of immune system involvement. This idea is supported by the observation that the abscopal effect is not present in immunodeficient NOD-SCID mice. Furthermore, this effect was enhanced by the immune activating anti-CTLA-4 checkpoint antibody (Wu, et al. 2014, Wu, et al. 2015).

It is thought that radiation is able to overcome the pro-growth tumor microenvironment by re-initiating immune recognition of the tumor through release of danger signals also known as DAMP molecules (Matzinger 1994, Matzinger 2002). This may allow immune cells to recognize and destroy radiated as well as distant tumors in an in vivo vaccination-like effect.
Ionizing radiation is able to elicit the release of DAMPs such as HMGB-1, ATP, and Calreticulin. These molecules are able to promote antigen processing and presentation by Dendritic Cells (DC)s and thus the priming of anti-tumor T cells (Apetoh, et al. 2007, Ghiringhelli, et al. 2009). In humans, radiation treatment is able lead to CD8$^{+}$ T cell recruitment and to the elimination of tumors (Lugade, et al. 2008).

1.5 Future Treatment Options: Immunotherapy

Immunotherapy is a growing field in which the patient’s immune system is used to destroy cancer. Our lab has sought to improve upon MPM treatment by combining radiation and immunotherapy.

There has been a paradigm shift in goals for treating cancer. While cancer cell death is the ultimate objective, the activation of the immune system towards cancer may be more valuable than the immediate killing effects of treatments such as surgery, radiation, or chemotherapy as they may leave microscopic disease behind. While cancers display various other hallmark behaviours including evasion of cell death and self-sufficient growth signals, evasion of the immune system is a large part of tumor progression (Hanahan and Weinberg). Immune surveillance is a well-known theory that posits that the immune system continually monitors and eliminates cells that have undergone mutations towards a cancerous phenotype. This idea has been supported by observation that various cancers are highly prevalent in immunocompromised people (Vajdic and van Leeuwen 2009). Furthermore, genetically engineered mice that are deficient in CD8$^{+}$ T cells, CD4$^{+}$ T helper cells, or NK cells, also demonstrate increased tumor incidence compared to wildtype mice (Kim, et al. 2007, Teng, et al. 2008). In our lab, we have also observed that tumor incidence is much higher in engineered mice whose entire CD8$^{+}$ T cell
repertoire binds only to ovalbumin peptide; perhaps impairing its ability to recognize other tumor antigens (Unpublished Data).

The goal of immunotherapy is to eradicate this immune evasion and promote tumor cell killing. Immunotherapy can function through passive immunity, involving monoclonal antibodies against specific tumor antigens, or active immunity in which the host immune system is educated to recognize and destroy cancerous cells that have previously become invisible to immune defenses.

1.5.1 Cytokines

Cytokines are secreted proteins that affect neighbouring cells with the appropriate receptors. Cytokines comprise of a large part of the immune response and thus have been used in treatments to sway the immune system against cancer (Murphy 2012).

IFN-α was the first immunotherapeutic agent used in cancer for the treatment of advanced melanoma. After approval as adjuvant treatment, many groups have shown that IFN-α administration is able to reduce the risk of recurrence and improve long-term survival rates. As a result, this cytokine has also been used in other cancers including renal carcinoma and various leukemias (Baxevanis, et al. 2009, Smyth, et al. 2004).

In MPM, IFN-α alone has little to no benefit (Ardizzoni, et al. 1994, Sterman, et al. 2011). However, there have been promising results demonstrating increased disease free survival and survival time when administering IFN-α intrapleurally before surgery (Sterman, et al. 2011).
1.5.2 Monoclonal Antibodies

Monoclonal antibodies directed against tumor-specific antigens have shaped oncologic treatment. Antibodies against tumor associated antigens in melanoma and prostate cancer have paved the way for use in other cancers (Baxevanis, et al. 2009). In addition, the HER2 binding trastuzumab is a well-known mAb used in the treatment of HER2+ breast cancers. Furthermore, antibodies are currently in pre-clinical and clinical phases for cancers such as lymphoma, leukemia, and others (Perez, et al. 2007).

mAbs in cancer work through a wide range of methods. They may induce apoptosis or neutralize growth receptors and arrest tumor cell growth (Murphy 2012). Abs may also be conjugated to toxins that localize cytotoxic treatments to the tumor (Hughes 2010). The binding of antibodies to tumor antigens may also elicit the immune response through recruitment of immune cells such as monocytes and macrophages by Fc receptor binding the Fc region of the Ab. Furthermore, tumor cell death may occur by Ab-mediated complement deposition and cell death by membrane rupture (Scott, et al. 2012). There also exist mAbs that target the tumor microenvironment through mechanisms such as targeting stromal cells or preventing angiogenesis (Deckert 2009, Schliemann and Neri 2010, Welt, et al. 1994).

mAbs towards Mesothelin have been used in mesothelin expressing MPM cases (Kreitman, et al. 2009). A phase I clinical trial in which anti-mesothelin antibody variable fragments (Fv) were administrered to mesothelioma patients with known mesothelin expression found partial response in 4 patients and disease stability in 19 patients out of 34 total patients. Further studies have investigated combination therapy of anti-mesothelin mAb with Gemcitabine chemotherapy. While mesothelin may be a potential therapeutic target, MPM currently lacks any reliable tumor associated antigens. Furthermore mesothelin expression may be present in some patients with
the epithelial subtype of disease, biphasic disease is much more varied in expression and sarcomatoid typically does not express mesothelin (Hassan, et al. 2010).

1.5.3 Immune Modulating Antibodies

1.5.3.1 Anti-CTLA-4

Antibodies may also act through immunomodulation by binding to checkpoint molecules and swaying the immune system towards an anti-cancer response. One method includes blocking surface receptors that normally function to inhibit the immune response. In the three signal hypothesis, Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA-4) is present on T Cells and is able to bind to the co-stimulatory signal B7.1 and B7.2 on dendritic cells and inhibit the adaptive immune response. The blocking of this interaction with an antibody prevents T-cell inhibition and enables T-cell activating interactions to occur (Murphy 2012).

Anti-CTLA-4 antibody has demonstrated efficacy in the clinical setting. It was first approved in 2011 for use in treatment of metastatic melanoma and has since demonstrated an increase in survival when compared to chemotherapy treatment (Robert, et al. 2011). A phase I trial of ipilimumab, an anti-CTLA-4 antibody, on prostate, and ovarian cancer has showed some efficacy through reduction of tumor size and lymphocyte infiltration (Hodi, et al. 2003, Ribas 2007, Small, et al. 2007). Furthermore, combination with chemotherapy has shown an improvement in the rate of complete response (Goff, et al. 2009, Yuan, et al. 2011).

There are few studies investigating CTLA-4 in MPM treatment. A recent phase II clinical trial studied the use of CTLA-4 for patients with un-resectable disease and disease progression after chemotherapy. CTLA-4 in this study was administered alone once every 90 days and
demonstrated limited efficacy with no patients demonstrating complete response and 2 out of 29 patients displaying a partial response (Calabro, et al. 2013). Data from mouse studies in our lab suggest that anti-CTLA-4 mAb alone does not offer prolonged survival. However, synergistic effects were observed when combined with radiation therapy, leading to greater survival than radiation alone (Wu, et al. 2015).

1.5.3.2 Anti-PD-1

The Programmed Death-1 (PD-1) Programmed Death Ligand 1 (PD-L1) pathway has garnered interest as another potential therapeutic target. PD-1 is expressed on T cells and triggers T cells to undergo apoptosis when binding and interacting with PD-L1. PD-L1 is expressed on various cell types including epithelial cells and hematopoietic cells in response to the cytokine IFN-γ. It is thought that this mechanism of T cell down-regulation prevents excess damage of surrounding tissues during inflammation. Tumor cells are able to hijack this mechanism by expressing PD-L1 in order to evade T cell-mediated destruction by triggering T cell programmed cell death. Binding of PD-1 blocks this interaction and counteracts this evasion mechanism (Carreno and Collins 2002, Greenwald, et al. 2005, Keir, et al. 2008, Pentcheva-Hoang, et al. 2009).

Clinically, anti PD-1 mAb has been used in melanoma patients. In one phase I clinical study, objective responses were achieved in 28% of the 94 total patients that had advanced melanoma (Topalian, et al. 2012). PD-1 has been studied in MPM as well. PD-L1 expression is correlated with decreased survival as patients with PD-L1 positive tumors show a median survival of 4.79 months compared to 16.3 months in patients with PD-L1 negative tumors. It has also been reported that at least 20% of MPM tumors are PD-L1 positive (Cedres, et al. 2015). While this molecule may be an important therapeutic pathway for some, there still remains a number of MPM patients that do not have PD-L1 expressing tumors.
Studies in our lab have demonstrated limited therapeutic effect of anti PD-1 mAb alone on subcutaneous MPM tumors (Unpublished Data). However, tumor shrinkage or slowed tumor growth is observed when combined with local radiation therapy, similar to the combination of radiation and CTLA-4 inhibition.

1.6 Cancer Treatment and Immunogenic Cell Death

Treatments such as radiation, chemotherapy, and immunotherapy are able to elicit cell death of tumor cells. However, an important aspect in successful treatment involves immunogenic cell death (ICD), in which tumor cell death triggers a lasting immune response through the release of DAMP molecules.

1.6.1 DAMPs

DAMP molecules are a major concept in understanding how immune responses are elicited in the absence of pathogens in conditions such as injury, autoimmunity, and cancer (Matzinger 1994). DAMPs are also important in rendering normally immunogenically silent forms of cell death immunogenic. The DAMP theory builds upon the Pathogen Recognition Receptors (PRRs) model. PRRs describe a diverse family of receptors that bind to Pathogen Associated Molecular Pattern (PAMP) molecules. PAMPs are a broad term defining evolutionarily conserved pathogen molecules that bind to PRRs to elicit a host immune response against the invading pathogen (Murphy 2012). The PAMP-PRR theory is a fundamental theory in immunology that explains how the inflammation in infection arises.

The DAMP theory, proposed by Matzinger, describes self-molecules that have normal intracellular functions apart from their extracellular immunostimulatory effects. Upon release of
these molecules by dying or stressed cells, they are liberated and thus able to bind to PRR receptors and begin the pathways to inflammation in the absence of infection (Matzinger 1994).

Among the numerous DAMPs that exist, the most well-studied include Calreticulin (CRT), adenosine triphosphate (ATP), and High-Mobility Group Box 1 (HMGB-1) protein (Kroemer, et al. 2013, Krysko, et al. 2012, Matzinger 2002).

1.6.2 DAMPs in Cancer Therapy

The potential importance of DAMPs has been observed in numerous mouse animal models where radiation or chemotherapy are used to induce ICD.

1.6.2.1 Calreticulin

Apoptosis occurs constantly in normal tissue turnover and does not result in inflammation. CRT is a DAMP that may be important in cancer therapy by rendering apoptotic cancer cells immunogenic. UV radiation and various chemotherapy drugs, such as anthracyclines, are able to cause immunogenic apoptosis through the shuttling of CRT to the surface of the dying cell (Apetoh, et al. 2007, Ghiringhelli, et al. 2009, Obeid, et al. 2007). Surface CRT promotes DC phagocytosis, tumor antigen processing, presentation, and the incitement of an immune response towards the tumor cells. CRT’s importance may be evidenced by animal experiments in which inhibiting CRT in anthracycline treated tumor cells weakens its immunogenicity in mice (Obeid, et al. 2007).
1.6.2.2 Adenosine Triphosphate

ATP release has been demonstrated in separate experiments of tumor cells being treated with chemotherapy or radiation (Martins, et al. 2009, Ohshima, et al. 2010). ATP is another DAMP that, upon release into the extracellular environment, is recognized by P2Y2 receptors on phagocytes that result in recruitment into sites of inflammation (Elliott, et al. 2009). In an experiment demonstrating ATP released by tumor cells undergoing autophagy in response to chemotherapy, RNA interference of pathways required for ATP release during autophagy abolished the anti-tumor response (Michaud, et al. 2011). Thus ATP is a key player in ICD.

1.6.2.3 HMGB-1

HMGB-1 release is associated with multiple forms of cell death including, sustained autophagy, late apoptosis, and necrosis (Tang, et al. 2010, Tang, et al. 2010). These forms of ICD occur as a result of DNA-damaging treatments such as radiation or chemotherapy. HMGB-1 release is crucial for antigen presenting cell (APC) activation and subsequent adaptive immune response towards tumor cells (Lotze and Tracey 2005). Vaccination of mice with tumor cells undergoing ICD is able to provide a protective effect upon re-challenging the mouse with live tumor cells. Conversely, in this prophylactic vaccination model, live tumor cells are able to grow on re-challenge if HMGB-1 in the ICD tumor vaccine is blocked (Apetoh, et al. 2007).

HMGB-1 is a key molecule in MPM that may contribute to the success of SMART radiation treatment by mediating ICD. Thus the focus of this project is on HMGB-1 in radiation treatment for MPM.
1.7 HMGB-1

1.7.1 Background

HMGB-1 is a DAMP protein with effects in both the development of various cancers as well as in promoting the host anti-cancer immune response. The duality of HMGB-1 necessitates the elucidation of its role in the context of mesothelioma.


HMGB-1 is a highly conserved protein belonging to the HMGB family of proteins with a greater than 99% sequence homology among all mammals studied (Ferrari, et al. 1996, Paonessa, et al. 1987, Rauvala, et al. 1988). This protein is composed of HMG boxes A-box (amino acids 1-79) and B-box (amino acids 89-163) as well as a glutamate and aspartate rich negatively charged C-terminal domain (Bustin, et al. 1990, Goodwin, et al. 1973). HMGB-1 uses these domains to bind non-specifically to the minor grooves of DNA (Agresti and Bianchi 2003, Bustin 1999). This binding is reported to bend DNA and promote transcription factor assembly on DNA targets (Agresti and Bianchi 2003). In addition, HMGB-1 is able to associate with histones and unravel chromatin coils to allow transcription factors to interact with uncoiled DNA (He, et al. 2000).

![HMGB-1 protein domains diagram]

**Figure 5. HMGB-1 protein domains.** HMGB-1 consists of multiple domains that confer specific functions including intracellular DNA binding and other nuclear functions as well as extracellular RAGE receptor binding.

### 1.7.2 HMGB-1 Signaling Pathways

HMGB-1 is known to mediate effects through a variety of receptors: the Receptor for Advanced Glycation End Products (RAGE), Toll-like receptor (TLR) 2, TLR 4, and TLR 9.

RAGE was the first receptor found to bind HMGB-1 (Neeper, et al. 1992). The HMGB-1-RAGE interaction is a mediator of inflammation. Additionally, HMGB-1-RAGE mediates a broad range of immune effects through chemotaxis, proliferation, and differentiation, of immune cells (Ryckman, et al. 2003). HMGB-1-RAGE results in NF-κB activation through either the prototypical MyD88 pathway or through extracellular signal-related kinase (ERK) 1, ERK2, and p38. This leads to pro-inflammatory cytokine production such as TNF, IL-6, and IFN-γ. HMGB-
1 binding to TLR-2 and TLR-4 leads to NF-κB translocation into the nucleus and translation of inflammatory genes through the MyD88 pathway (Neeper, et al. 1992).

TLR-9 is known to bind CpG DNA that is normally found in pathogen organisms such as bacteria. Recent research has demonstrated that HMGB-1 is able to preferentially bind to CpG DNA. This complex is able to enhance the immunostimulatory effect of CpG DNA when detected by TLR-9 in macrophages, DCs, and B cells. Released HMGB-1 is able to enter endosomes and accelerate the formation of the CpG DNA and TLR-9 complex. This then results in the secretion of pro-inflammatory cytokines IL-6, IL-12, IFN-α, and TNF-α (Ivanov, et al. 2007, Tian, et al. 2007).
Figure 6. HMGB-1 binds RAGE receptor or TLR 2 or TLR 4. The signalling pathways functions through the typical MyD88 pathway or through a pathway yet to be characterized; involving ERK 1/2 and p38 (Lotze and Tracey 2005).
1.8 The Pro-Cancer Side of HMGB-1

1.8.1.1 The Role of HMGB-1 in Development

In development of disease, asbestos has been demonstrated to cause mesothelial cell necrosis and the release of HMGB-1 from the nucleus into the extracellular milieu (Yang, et al. 2010). A possible pathway of development involves macrophage TNF-α release in response to HMGB-1 in the extracellular environment. TNF-α is thought to mediate mesothelial cell survival by causing NF-κB translocation into the nucleus. Additionally, mesothelial cells are normally quiescent with only 0.16% to 0.5% of cells undergoing mitosis (Mutsaers 2004). However, in the presence of inflammation from injury, 30% to 80% of mesothelial cells undergo cell division and proliferation (Mutsaers, et al. 2002). Cells that are stimulated to survive and proliferate can accumulate DNA damage, divide, and may eventually give rise to a cancerous MPM cell (Yang, et al. 2006). The HMGB-1-TNF-α pathway may play a central role in MPM development as TNF-α receptor knockout mice exposed to asbestos do not develop the fibroproliferative lesions observed when wild-type mice are exposed to asbestos.

1.8.1.2 HMGB-1 in Disease Progression

After transformation, HGMB-1 has been described as having a role in the progression of disease. MPM patients have been shown to have higher serum HMGB-1 concentrations than individuals exposed to asbestos who have not developed MPM (Tabata, et al. 2013). Furthermore, a recent study demonstrated that treatment of mice with anti-HMGB-1 antibody slows the growth of the intraperitoneal injected human epithelioid malignant mesothelioma cell line, REN (Jube, et al. 2012). Thus HMGB-1 may promote the progression of disease.
Interestingly, in pancreatic tumor cell lines it has been shown that HMGB-1-RAGE signaling enhances the activity of mitochondrial complex I in tumor cells, thus promoting greater ATP production (Kang, et al. 2014). This ATP production is thought to allow for proliferative and migratory abilities of tumor cells. This finding supports the observation of greater RAGE expression in gastric and colorectal cancer patients being associated with greater invasiveness and metastasis (Kuniyasu, et al. 2002, Sasahira, et al. 2005). HMGB-1 may play a similar role in MPM and thus blocking this protein may lead to lower ATP production and slower growth and prolonged survival as observed in MPM patients (Tabata, et al. 2013).

During treatment, HMGB-1 also has roles in rendering cancerous cells resistant to therapy. Studies in other cancers such as leukemia and osteosarcoma have demonstrated that HMGB-1 suppression by RNAi increases the anti-cancer effectiveness of cytotoxic agents such as chemotherapy and radiation (Huang, et al. 2012, Liu, et al. 2011, Livesey, et al. 2012). In addition, HMGB-1 over expression has rendered certain cell types resistant to these treatments. HMGB-1 in pancreatic cancer cells is able to counter cytotoxic agents by interfering with cell death pathways (Tang, et al. 2010). This protein is able to confer survival to cancer cells exposed to chemotherapy by inducing a Beclin-1 dependent autophagy response. Autophagy is the degradation of organelles and is important in tumor cell survival as it allows for recycling of ATP and biosynthetic molecules under conditions of nutrient limitation or metabolic stresses such as chemotherapy and radiation (Degenhardt, et al. 2006). Autophagy allows for cancer cells to enter a state of dormancy and resume growth after treatment has finished; leading to the recurrence of disease. Intrinsic and extrinsic apoptosis is inhibited by HMGB-1; further supporting the role of HMGB-1 in the survival of cancerous cells.
1.9 The Anti-Cancer Side of HMGB-1

1.9.1 HMGB-1 Release

HMGB-1 has a diverse range of functions on the immune system. Apart from its intranuclear role, HMGB-1 can be released extracellularly and function as a DAMP to trigger the immune response (Andersson, et al. 2000, Wang, et al. 1999). This protein can facilitate a multitude of immunogenic effects through both active and passive release.

1.9.2 Active Release of HMGB-1

Active release of HMGB-1 occurs primarily through secretion by immune cells. HMGB-1 active release has been best characterized in macrophages and monocytes stimulated by TNF which were shown to release HMGB-1 in the absence of cell death. Actively secreted HMGB-1 has a critical role in sepsis (Abraham, et al. 2000, Qin, et al. 2006, Suda, et al. 2006, Wang, et al. 1999, Yang, et al. 2004). Sepsis, systemic inflammation against pathogen infection, can result in considerable damage and hypoperfusion to the host’s organs through the increase in vascular permeability required for immune cell recruitment. HMGB-1 has been shown to be released as a late proinflammatory mediator in a variety of lipopolysaccharide sepsis models. In fact, the blocking of HMGB-1 with neutralizing antibodies is able to rescue mice and rats dose-dependently from lethal sepsis incurred by Cecal Ligation Puncture model.

Active HMGB-1 release has also been demonstrated in DCs (Lotze and Tracey 2005) pituicytes (glial cells of the posterior pituitary) and human umbilical venous endothelial cells(Wang, et al. 1999). Additionally, HMGB-1 has been shown to be actively released by dendritic cells in order to stimulate clonal expansion and polarization of naïve CD4+ T cells (Dumitriu, et al. 2005).
The mechanism of active release is best characterized in macrophages and monocytes. Active release involves the endolysosome secretory pathway (Gardella, et al. 2002). While HMGB-1 is normally able to travel in between the nucleus and cytoplasm through nuclear pores, inflammatory signals such as LPS or TNF prevent the re-entry of HMGB-1 to the nucleus by the acetylation of specific lysine amino acids. HMGB-1 is then taken up by endolysosomes through an unknown mechanism. Endolysosomes then fuse with the membrane and release their contents. It has been previously demonstrated that the released HMGB-1 is pre-synthesized and that new HMGB-1, shown by the incorporation of radio-labelled peptides, is translated approximately 16 hours after the original stimuli (Wang, et al. 1999).

1.9.3 Passive Release of HMGB-1

Passive HMGB-1 release occurs through diffusion out of the cell due to the loss of membrane integrity most often related to cell death. HMGB-1 has been known to be released by cells that are permeabilized, undergoing necrosis, or late apoptosis (Degryse, et al. 2001, Falciola, et al. 1997, Muller, et al. 2001). Regulated necrosis, referred to as necroptosis, also leads to HMGB-1 release (Kaczmarek, et al. 2013) and sustained autophagy is yet another mechanism of HMGB-1 to be released (Tang, et al. 2010).

The immunogenic potential of HMGB-1 was demonstrated in a study that measured HMGB-1 release in culture supernatant after triggering necrosis through repeatedly freeze-thawing fibroblasts (Scaffidi, et al. 2002). Further experiments by this group displayed HMGB-1’s ability to stimulate macrophage TNF release with necrotic fibroblasts but abrogating this effect with HMGB-1 deficient fibroblasts (Scaffidi, et al. 2002).
Apoptosis is considered to be immunogenically silent (Bianchi and Manfredi 2007). Supporting this idea, HMGB-1 remains bound to DNA and histones during due to underacetylation of histones during apoptosis. During apoptosis, HMGB-1 remains in the nucleus and is unable to stimulate an immune response. Thus, the passive release of HMGB-1 through cell death is a crucial aspect of the immune response.

While both mechanisms of HMGB-1 release are pro-inflammatory in nature, actively released HMGB-1 tends to be a late mediator of inflammation while passive HMGB-1 by cells undergoing cell death is an early mediator. Furthermore, HMGB-1 is hyper-acetylated as part of the active secretion pathway by immune cells and non-acetylated when liberated passively (Bonaldi, et al. 2003). The functional significance of this difference remains unclear.
Figure 7. **HMGB-1 and its multitude of intracellular and extracellular roles.** HMGB-1 has normal intracellular DNA-binding activity. It also has effects on tumor cell metastasis. Its release is an important mediator of inflammation through RAGE or TLR signalling. (Figure reproduced with permission from Nature Reviews Immunology).

### 1.10 The Immune Response and HMGB-1

HMGB-1 plays a broad role in generating immune responses. The signaling cascades described in the previous section are important for immune system activation against pathogens and cancer. In addition, aberrant HMGB-1-mediated inflammation contributes to the pathogenesis of various immunological conditions including septic shock (Abraham, et al. 2000, Qin, et al. 2006, Suda,

The immune system is divided into the innate and adaptive systems (Murphy 2012). The innate defense system provides us with immediate and rapid immunity against a broad range of pathogens by recognizing evolutionary conserved molecules. The adaptive immune system requires time for an antigen-specific response to be generated against pathogen molecules not previously encountered by the host. In the absence of pathogens, HMGB-1 and other DAMPs released by dying cells provide a mechanism of sterile immune activation. This project is focused on the adaptive system as it provides a specific response as well as immune memory, which prevents further disease by the same pathogen. This idea can also be applied to cancer in which a successful adaptive response can prevent the recurrence of a cancer that is removed or killed with treatment.

1.10.1 T Cells

The immune system consists of a wide range of cells that derive from the pluripotent hematopoietic stem cells of the bone marrow (Murphy 2012). Cells of the lymphoid and myeloid lines undoubtedly play a diverse range of roles in cancer. T cells, deriving their name from their maturation in the thymus, are broadly delineated into cytotoxic, helper, and regulatory T cells.

In cancer, T cells remain important for the large role they play in the immune response. CD8+ cytotoxic T cells, named for the CD8 surface receptor they possess, are effector cells that are able to directly kill virally infected cells to prevent viral spread to surrounding cells. They are
also paramount in destroying cancerous cells. Numerous studies have examined and found positive correlations between the number of tumor-infiltrating CD8\(^+\) T cells and better patient prognosis. Greater levels of tumor-infiltrating CD8\(^+\) T cells have been associated with better prognoses in colorectal cancer, head and neck cancers, ovarian, and breast cancers (Bachmayr-Heyda, et al. 2013, Balermpas, et al. 2014, Gisterek, et al., Nosho, et al. 2010).

Our lab has similarly found that high levels of CD8\(^+\) tumor-infiltrating lymphocytes after Pemetrexed chemotherapy is associated with longer progression-free survival as well as delayed recurrence in malignant pleural mesothelioma patients (Anraku, et al. 2008). Many immune checkpoint therapies in cancer attempt to achieve this CD8\(^+\) T cell infiltration and tumor cell killing in order to bring greater survival rates to patients. Blocking PD1 and TIM3 with neutralizing antibodies has been investigated as a way to re-activate exhausted CD8\(^+\) T cells in melanoma and colon cancer (Fourcade, et al. 2010, Sakuishi, et al. 2010). Bringing better prognosis and the chance to permanently eliminate tumors, the focus of this work is on CD8\(^+\) T cells and their ability to directly kill MPM cells.

1.10.2 The T Cell Immune Response

In an adaptive T cell response, APCs such as Immature DCs in peripheral tissue constantly uptake antigens from the extracellular environment through micropinocytosis (Murphy 2012). Binding of PAMPs or DAMPs to PRRs on immature DCs act stimulates their maturation.

HMGB-1 released through both active and passive mechanisms are able to stimulate the immune response through PRR signaling on DC. HMGB-1 secreted by Natural Killer (NK) cells lead to DC maturation (Semino, et al. 2005). In HeLa cells, HMGB-1 was shown to act as an immune adjuvant as stimulating DCs with wild-type necrotic cells induced maturation while HMGB-1
deficient necrotic cells did not (Rovere-Querini, et al. 2004). Furthermore, *in vitro* recombinant HMGB-1 is able to induce DC maturation (Messmer, et al. 2004).

DC maturation involves cleaving and processing previously up taken antigens for loading onto the Major Histocompatibility (MHC) molecule (Murphy 2012). This binding also stimulates up-regulation of chemokine receptor CCR7 which recognizes CCL 19 and CCL 21; targeting the DC to the draining lymph node. MHC I-Peptide complexes are shuttled to the surface of the DC, allowing it to present this complex to the T Cell Receptor (TCR) on T-cells.

**Figure 8.** DAMPs released by secondary apoptotic or necrotic are sensed by DCs. DCs migrate to the lymphnode and present their MHC-Ag complex to T cells and trigger the adaptive immune response.
1.10.3 The Three Signal Hypothesis

The three signal hypothesis is a fundamental concept in immunology involving three stimulatory events that must occur to generate a T cell immune response (Murphy 2012). The first signal involves the MHC-TCR interaction which is stabilized by either CD4 or CD8 molecules on T cells. The second signal involves the costimulatory interaction of B7.1 and B7.2 on DCs binding to CD28 on T cells. The last aspect involves cytokines secreted by APCs that differentiate T-cells into one of many T cell subsets that include but are not limited to CD8 cytotoxic, Th1, Th2, Th17, and T regulatory cells. Crucially, DAMPs such as HMGB-1 play a role in every aspect of the three signals. DAMP signaling leads to antigen processing and DC maturation that is responsible for antigen presentation on MHC molecules. TLR signaling has a significant role in upregulating the costimulatory signal. NFκB signaling described previously leads to cytokine secretion that affects differentiation of T cells.
Figure 9. The 3-signal hypothesis in immunology involves the activation of the adaptive response by signals provided by APCs. These signals include the activation signal from the MHC-Ag-TCR interaction, the costimulatory signal of B7.1 B7.2 binding CD28, and the cytokine differentiation signal.
HMGB-1 induces IL-2 up-regulation by DCs that allows for CD8 T cell licensing (Murphy 2012, Zhu, et al. 2009). This process allows CD8+ T cells to exit into the periphery and mediate specific cell killing. Once licensed CD8+ T cells exit into the bloodstream, HMGB-1-activated endothelial cells are able to recruit circulating T cells by up-regulating ICAM-1, VCAM-1, and E-selectin (Treutiger, et al. 2003). This allows circulating immune cells to bind and ultimately extravasate into the inflamed tissue (Murphy 2012). ICAM-1 binds to LFA-1 or Mac1, VCAM-1 binds to VLA-4, and E-selectin binds to the ligand Sialyl-Lewis.

Figure 10. CD8+ T cell licensing. CD4+ T Cells and DCs function in concert to provide specific activation signals to CD8+ T Cells, allowing them to travel to the periphery and perform specific cell killing (Kurts, et al. 2010).
1.11 Summary

MPM is a cancer of the lung lining that is associated with the inhalation of asbestos fibers, a fibrous compound still found in the insulation of older buildings. While asbestos use in North America has been controlled, there still remains a risk of MPM development as older buildings containing silicates are renovated or demolished. Due to a long incubation time between exposure and onset of disease, the incidence of disease in North America has increased by 65 percent over the past two decades worldwide and is projected to continue to increase until at least the year 2020. Moreover, the continued use in the developing world suggests that MPM will continue to rise worldwide for the foreseeable future. Non-resectable MPM is treated with a chemotherapy regiment that increases median survival from 9 to 12 months in epithelial disease. Sarcomatoid and biphasic (a mix of epithelial and sarcomatoid disease) MPM subtypes offer worse overall survival. Thus there is a substantial need to understand the different factors that mediate treatment success in the MPM subtypes for novel treatments to be developed.

The de Perrot lab has developed a new methodology of administering short, but high-dose, radiation fractions before surgery which differs from previous approaches in which radiation is given after surgical resection. A feasibility study of Surgery for Mesothelioma After Radiation Therapy (SMART) has demonstrated that this procedure results in a significant increase in 3-year survival in patients with epithelial disease from 30% to 84% compared to previous treatment modalities. However, biphasic overall survival was significantly lower at 13%. There is therefore a substantial need to understand how different subtypes respond to radiation in the context of host immune system stimulation.
New research suggests that high-dose hypofractionated radiation is able to stimulate the immune system in addition to mediating direct tumor killing. Cytotoxic T lymphocytes circulate through the body and are able to destroy tumors. However, an inhibitory tumor microenvironment renders T cells unable to inhibit tumor growth. Furthermore, MPM has few known tumor associated antigens; making it difficult to target treatment using engineered T-cell receptors or neutralizing monoclonal antibodies. DAMP molecules offer a new avenue of treatment. DAMPs, released from cells undergoing immunogenic cell death, are implicated in stimulating the antigen uptake, maturation, and antigen presentation of dendritic cells; crucial activators of cytotoxic T cells that mediate tumor killing. HMGB-1, a prototypical DAMP, has been demonstrated as necessary to stimulate the immune system after chemotherapy in many animal models of cancer and we are keen to observe its role in MPM.

In this chapter we have discussed the dual nature of the protein HMGB-1 in both development and treatment of disease. We have studied HMGB-1 in tumor development and progression but also in its role as a DAMP and as a mediator of the immune response. We have also examined, in depth, the immunostimulatory effects of radiation through DAMP release and the benefit of the CD8⁺ T cell immune response in eliminating MPM cells. The following chapter will outline the hypothesis and aims of this study.
Hypothesis and Aims

Research in support of pro and anti-tumor roles of HMGB-1 has been increasing. Broadly, HGMB-1 has been suggested to play a dual role in both cancer cell survival and death. HMGB-1 has been described as a negative prognostic marker in several cancers including MPM. However after treatment, HMGB-1 is suggested to aid in the recruitment of the immune response. The goal of this study was to elucidate the role of HGMB-1 in mediating an anti-tumor immune response in radiation treatment of malignant pleural mesothelioma. The hope of this translational research project was to use the findings of this study to understand and improve treatment given to MPM patients.

The objectives of this study were threefold: 1) to determine if the different subtypes of MPM differ in HMGB-1 release, 2) to correlate HMGB-1 release in paraffin-embedded patient samples to survival, 3) to investigate the CD8+ T cell immune response elicited by HMGB-1 release. As epithelial disease offers a greater survival than sarcomatoid disease, it is hypothesized that epithelial disease releases more HMGB-1 by immunogenic cell death after radiation treatment which leads to the generation of a specific CD8+ T cell response. This CD8+ T cell response leads to specific tumor killing and results in greater overall and disease free survival in patients after SMART.

To tackle the first objective, I postulated that HMGB-1 released as a result of radiation treatment leads to an immune response that aids in improving patient survival. I suggest that epithelioid subtypes which are commonly responsive to SMART treatment release more HMGB-1 by cell death. Passively liberated HMGB-1 could then bind to RAGE and TLR molecules on DCs and stimulate a T Cell immune response. This immune involvement would lead to specific tumor
cell killing and therefore longer survival. Conversely, I propose that sarcomatoid cells do not release as much HMGB-1 in response to radiation induced cell death and therefore recruit the immune system to a lesser extent, leading to a shorter patient survival time. For the second objective, I postulated that higher staining intensity of HMGB-1 after treatment would correlate with higher patient survival as HMGB-1 would recruit the immune system through binding to APCs. In studying the last objective, I hypothesized that radiation in vivo would lead to tumor cell death which would result in HMGB-1 release. This release would be responsible APC maturation, tumor antigen presentation, and a CD8+ T Cell immune response measured by CD8+ T Cell infiltration.
3 Materials and Methods

3.1 Human and Murine Cell Lines

All cell lines used, with the exception of CRL-9444 (MeT-5A), were grown in RPMI 1640 culture media (Life Technologies Inc., Burlington ON, CAN) supplemented with 10% FBS. CRL-9444 was grown in Medium 199 with 1.5g/L sodium bicarbonate, 3.3 nM EGF, 400 nM hydrocortisone, 870 nM bovine insulin, 20 mM HEPES, 3.87 µg/L H2SeO3, and 10% FBS. Additionally, cultures were grown in a 37°C and 5% CO₂ environment.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H226 (CRL-5826)</td>
<td>Human Epithelioid Mesothelioma</td>
<td>Mesothelioma derived from pleural effusion</td>
</tr>
<tr>
<td>NCI-H2052 (CRL-5915)</td>
<td>Human Epithelioid Mesothelioma</td>
<td>Stage 4 mesothelioma derived from lymph node metastasis</td>
</tr>
<tr>
<td>NCI-H28 (CRL-5820)</td>
<td>Human Sarcomatoid Mesothelioma</td>
<td>Stage 4 mesothelioma derived from pleural effusion</td>
</tr>
<tr>
<td>NCI-H2452 (CRL-5946)</td>
<td>Human Sarcomatoid Mesothelioma</td>
<td>Derived from lung tissue</td>
</tr>
<tr>
<td>MeT-5A (CRL-9444)</td>
<td>Human Immortalized Mesothelial Cell</td>
<td>Mesothelium cells derived from pleural fluids of non-cancerous individual</td>
</tr>
<tr>
<td>6E6 (PTA-5433)</td>
<td>Murine BALB/c anti-HMGB-1 Hybridoma</td>
<td>Hybridoma line generated in BALB/c mice in response to vaccination with HMGB-1 protein</td>
</tr>
<tr>
<td>AB12</td>
<td>Murine BALB/c Epithelial Mesothelioma</td>
<td>Malignant mesothelioma from BALB/c mice exposed to crocidolite asbestos</td>
</tr>
<tr>
<td>AE-17-OVA</td>
<td>Murine C57BL/6 Ovalbumin-transfected Epithelial Mesothelioma</td>
<td>Malignant mesothelioma cells from C57BL/6 mice exposed to crocidolite asbestos</td>
</tr>
</tbody>
</table>
3.2 Cell Line Radiation

2 million cells of the cell line of interest are plated in a 75 mm² flask. Adherent cells are irradiated for the time corresponding to the desired dose (Gy) in a Gammacell 40 Extactor Caesium-137 irradiator (Best Theratronics Ltd., Ottawa ON, CAN). Radiation was given in 1 fraction.

3.3 Flow Cytometry Cell Death Quantification

2 million cells of the cell line of interest are plated in a 75 mm² flask. Adherent cells are irradiated for the time corresponding to the desired dose (Gy) in a Gammacell 40 Extactor Caesium-137 irradiator (Best Theratronics Ltd., Ottawa ON, CAN).

After 1 fraction of 25Gy radiation, adherent cell lines were incubated for 1, 2, 4, 24, or 48 hours before staining with 5µL/100µL of cells Annexin V Ab (Biolegend Inc., San Diego CA, USA) and 1µL/1mL of cells eFluor450 viability dye (eBioscience Inc., San Diego CA, USA). Cells were measured for fluorescence on a BD LSR II flow cytometer (BD Biosciences, Mississauga ON, CAN). Analysis was performed using FlowJo V10 (FlowJo LLC, Ashland, USA) software. Annexin V– eFluor450– cells were deemed to be viable cells and Annexin V+ eFluor450– cells were considered apoptotic in nature. Annexin V+ eFluor450+ and Annexin V– eFluor 450+ cells were considered to be necrotic cells.
Figure 11. **Quantification of cell death by flow cytometry and gating strategy.** Annexin V–eFluor450– cells were considered viable cells and Annexin V+ eFluor450– cells were considered apoptotic. Annexin V+ eFluor450+ and Annexin V– eFluor 450+ cells were considered to be necrotic cells.
3.4 HMGB-1 concentration of Mouse Serum or Cell Culture Supernatant by ELISA

Cell culture supernatants were collected every 1, 2, 4, 24, or 48 hours after the single 25Gy radiation fraction. Supernatant was collected from the corresponding time points in non-treated cell lines. Mouse blood was collected in EDTA tubes to prevent coagulation and centrifuged at 3000 RPM for 15 minutes. Serum was collected to be used in HMGB-1 measurement. Media or serum was placed in wells coated with anti-HMGB-1 antibody from a sandwich ELISA kit (IBL International, Hamburg, GER). HMGB-1 in the samples were left overnight to bind to anti-HMGB-1 antibodies. Secondary antibody conjugated to an enzyme was allowed to bind to the captured HMGB-1 protein. Substrate was added and colour change reaction was observed. Concentrations were determined by four-parameter logistic test using a standard curve. Samples were measured in duplicate.

3.5 Mice

Eight to twelve week old female C57BL/6 and BALB/c wildtype mice were purchased from The Jackson Laboratory (Maine, USA).

All mice were housed at the Toronto Medical Discovery Tower’s Animal Resource Centre under pathogen-free conditions in accordance with institutional and national animal care and ethics protocols.
3.6 Radiation Mouse Model

Eight to twelve week old C57BL/6 mice were injected subcutaneously in the right flank with 1 x 10^6 AE-17-OVA cells in 100µL of PBS. Mice were divided into two groups: 1) no treatment and 2) local radiation therapy involving a total dose of 15 Gy over 3 days (3 fractions). At least 3 mice were used in each group. Radiation occurred on days 8, 9, and 10 after cell injection. 225 kV 13 mA radiation was given to mice under isofluorane anaesthesia using the X-Rad 225Cx (Precision X-Ray, Branford CT, USA) in which flank tumors were visually targeted using X-ray images in x, y, and z dimensions. Imaging resolution was 0.2mm with an image acquisition dose of 1-10 cGy. On day 12, 17, and 22, mice were sacrificed from each group and blood and tissue samples were collected. Blood was taken by cardiac puncture method under isofluorane for analysis of serum HMGB-1 level. Mice were then sacrificed by cervical dislocation. Mouse tumors were measured and resected for use in immunohistochemical and immunofluorescent staining. The longest two perpendicular measurements were taken and tumor volume was calculated using the formula:

\[ \text{Length} \times \text{Width}^2 \times \left( \frac{\pi}{6} \right) \]

In a similar experiment, mice were measured for tumor growth and sacrificed when tumors reached a volume of 250 mm³. In this experiment mice were either 1) untreated, 2) received local radiation therapy of 15Gy over 3 days, or 3) received 15Gy local radiation therapy over 3 days and anti-HMGB-1 antibody (intraperitoneal 100µg injection per day for 5 days; days 8-12). There were at least 3 mice per group.
Figure 12. Schematic of untreated, radiated, and radiation + anti-HMGB-1 treatment in tumor bearing mice. In the treated group, days 12, 17, and 22 correspond to 2, 7, and 12 days after LRT respectively.

3.7 Hybridoma Preparation and anti-HMGB-1 Ab Purification

BALB/c mice were given a 0.5mL Pristane (Sigma-Aldrich, Oakville ON, CAN) intraperitoneal (IP) injection one week before IP injection of 5 x 10^6 cells in 0.5mL phosphate buffered saline (PBS) of the 6e6 HMGB-1 hybridoma cell line (PTA-5433 from American Type Culture Collection, Manassas VA, USA). One week after cell injection, mice are sacrificed and ascites fluid with antibody, produced by the hybridoma tumor, is drained using a cannula into tubes with EDTA to prevent coagulation.

Cell culture media or ascites fluid generated from the 6e6 hybridoma cell line was purified for anti-HMGB-1 Abs through a protein A antibody purification kit (Abcam Inc., Toronto ON,
Desired sample is mixed with a protein A resin which binds to the Ab constant domain. The protein A beads bound to the Ab are collected in a filter tube. Anti-HMGB-1 Abs are then eluted and collected.

### 3.8 Hybridoma Anti-HMGB-1 Antibody Blocking Test

Anti-HMGB-1 isolated from ascites fluid generated by the anti-HMGB-1 6E6 hybridoma was purified and tested for binding ability through ELISA assay. Recombinant HMGB-1 protein (80 ng/mL, HMGBiotech Srl., Milano, ITA) was allowed to bind to hybridoma-generated anti-HMGB-1 antibody in a 1:1 or 1:2 ratio (80 ng/mL or 160 ng/mL Ab) for 30 minutes in PBS. Ab-recombinant HMGB-1 mixture or recombinant HMGB-1 alone was measured for HMGB-1 concentration by ELISA kit (IBL International, Hamburg, GER) to determine the 6E6 antibody’s ability to bind HMGB-1. Lack of signal in the mixture group is indicative of 6E6 antibody binding.

### 3.9 Patient Sample HMGB-1 Immunohistochemistry

Mesothelioma tumor tissue samples were acquired and sent to pathology after patients underwent biopsy, extrapleural pneumonectomy, decortication, or pleurectomy with decortication. Some samples were snap frozen in liquid nitrogen and kept at −80°C on dry ice however most were immediately fixed in paraformaldehyde for 48 hours before being embedded in paraffin blocks. Frozen and paraffin embedded samples were sliced into 5µm thick sections using a microtome. Frozen sections were fixed with 1% paraformaldehyde for 1 hour before staining. Paraffin sections were deparaffinised with Xylene, 100% ethanol, 95% ethanol, and 70% ethanol respectively. Paraffin sections underwent antigen retrieval by treating with 100°C citrate buffer for 20 minutes. Sections were blocked with serum (5% BSA in Tris-buffered saline) for one
hour before the addition of primary anti-HMGB-1 antibody (Abcam Inc., Toronto ON, CAN) at a 1:100 dilution. After incubating overnight at 4°C, sections were washed in TBS+0.2% Tween 20. Slides were subsequently incubated for 1 hour at room temperature with anti-rabbit-HRP secondary Ab kit (Vector Laboratories Inc., Burlington ON, CAN). After washing, the HRP substrate DAB (3, 3-diaminobenzidine) (Vector Laboratories Inc., Burlington ON, CAN) was added to each slide at 100 µL per section. Sections were then counterstained with hematoxylin, dehydrated, and mounted with mounting media (Fischer Scientific, Ottawa ON, CAN).

Immunostained slides were imaged at 200x magnification with the Aperio ImageScope digital scanner and visualized with Aperio ImageScope Viewer version 12.1 (Vista, CA, USA). Scanning was provided by the Advanced Optical Microscopy Facility (Toronto ON, CAN). Semi-quantification of nuclear staining into high, medium and weak positive as well as negative was performed using the Aperio nuclear v9 algorithm (Leica Microsystems Inc., Concord ON, CAN). Distinction of nuclear staining intensity was based on observation and modelled using the Aperio algorithm which applied observational parameters of all tissues studied.

**Figure 13. Quantification of staining using image analysis software.** Quantification of high, medium, and low intensity staining is detected as well as negative cells. Image analysis can then be applied to an entire tissue sample.
3.10 Immunofluorescence

Cell lines used for immunofluorescence were grown on glass slides and underwent the designated treatment. Cell lines or frozen tissue samples on slides were fixed with cold acetone for 10 minutes. Paraffin embedded samples were deparaffinised with Xylene, 100% ethanol, 95% ethanol, and 70% ethanol respectively and antigen retrieval was performed by immersing samples in 100°C citrate buffer for 20 minutes. Samples were blocked with 5% BSA in Tris-buffered saline for one hour before the addition of primary Ab. After incubating overnight at 4°C, sections were washed in TBS+0.2% Tween 20. Slides were subsequently incubated for 1 hour at room temperature with the appropriate fluorescently labelled secondary Ab. Slides were further washed with TBS+0.2% Tween 20 before adding mounting media with DAPI nuclear stain. Cover slips were placed on top and sealed with nail polish.

Fluorescently labelled cells or tissues were visualized with the WaveFX (Quorum Technologies Inc, Guelph ON, CAN) confocal microscope system. Pictures were analyzed using ImageJ V1.47 (National Institute of Health, USA). Corrected total cell fluorescence (CTCF) was calculated by the formula CTCF = Integrated Density – (Area of cell x mean fluorescence of background reading).

3.11 Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Inc, La Jolla CA, USA). More than 2 groups were compared using one-way ANOVA analysis. Student’s T-test was used to analyze two groups. A P-value of less than 0.05 was considered statistically significant.

Results have been presented as Mean ± Standard Error of the Mean (SEM). * indicates p<0.05, ** indicates p<0.01, and *** indicates p<0.001 in all results figures.
4 Results

4.1 *In vitro* HMGB-1 characterization

4.1.1 *Epithelioid and sarcomatoid human MPM cell lines differ in intracellular HMGB-1 expression 24 hours after radiation treatment*

To determine the effect of $\gamma$-radiation on intracellular HMGB-1 expression, human epithelioid MPM cell line H226 and human sarcomatoid MPM cell line H28 were irradiated over a dosage range of 5-75 Gy in a single fraction. Staining of HMGB-1 by immunofluorescence was performed and radiated cells were compared in fluorescent intensity to untreated cells.

Immunofluorescent staining of human epithelioid (H226) and sarcomatoid (H28) MPM cell lines was performed 24 hours after the designated radiation doses were given (Figure 14A). In this experiment it was found that fluorescently labelled intracellular HMGB-1 displayed decreasing fluorescence intensity (488 nm) levels as radiation dose increased in the H226 subtype (Figure 14B) while fluorescent intensity increased across dose in H28 (Figure 14C). These findings suggest that either HMGB-1 expression is affected by radiation or that intracellular HMGB-1 is being lost or gained. HMGB-1 may be lost to the extracellular environment in epithelioid H226 while buildup or new synthesis of HMGB-1 may be occurring in sarcomatoid H28.
Figure 14. Expression of HMGB-1 in human MPM cell lines 24 hours after radiation treatment. A) Immunofluorescent microscopy images (405 and 488 nm) of nuclei (DAPI 405 nm) and immunolabelled HMGB-1 (488 nm). Quantification of cell fluorescence at 488 nm for B) H226 and C) H28.
4.1.2 Epithelioid MPM cell line H226 releases more HMGB-1 than sarcomatoid line H28 in response to γ-radiation

While intracellular HMGB-1 is hidden from the immune system, extracellular HMGB-1 functions as a pro-inflammatory DAMP. In order to confirm the differences observed in immunofluorescent staining of H226 and H28 cell lines and their relation to extracellular release, media HMGB-1 concentration was measured. HMGB-1 concentration in the media and therefore release from cells was determined by HMGB-1 ELISA analysis of culture media 24 hours after each treatment. Radiation was given in a single fraction.

In the no treatment group, HMGB-1 release from H226 (7.113 ± 0.4929 ng/mL) and H28 (4.844 ± 1.567 ng/mL) was not significantly different (P=0.2165). 24 hours after treatment with 5 Gy γ-radiation, HMGB-1 in the media was significantly higher in H226 (8.847 ± 0.2496 ng/mL) than H28 (7.155 ± 0.0925 ng/mL, P=0.0238). At 25Gy, H226 (24.99 ± 6.066 ng/mL) released significantly more HMGB-1 into the media than H28 (8.090 ± 0.5087 ng/mL, P=0.0213). This trend was also seen at 50Gy (H226, 60.99 ± 4.601 ng/mL, vs. H28, 6.001 ± 0.5240 ng/mL, P=0.0070). Lastly, 24 hours after 75 Gy γ-radiation treatment, H226 media HMGB-1 concentration was significantly higher than H28 (37.95 ± 3.733 ng/mL vs. 8.061 ± 0.5600 ng/mL, P=0.0156).

Overall, this data displayed a trend that in response to radiation, epithelioid H226 cells release more HMGB-1 into the media at all doses compared to sarcomatoid H28 cells. As extracellular HMGB-1 release has been reported to occur as a result of necrotic cell death, this data suggests that until 24 hours, epithelioid H226 is more susceptible to radiation than sarcomatoid H28.
4.1.3 Epithelioid lines H226 and H2452 release more HMGB-1 into culture medium 24 and 48 hours after 25Gy γ-radiation than sarcomatoid lines H28 and H2052

In this experiment, the effect of radiation on HMGB-1 release was measured in epithelioid and sarcomatoid cell lines to observe the effect of a single dose of radiation on HMGB-1 release over time. 25Gy radiation is the typical clinical dose given to patients with MPM undergoing SMART therapy. Thus a single dose of 25Gy radiation was chosen to observe HMGB-1 release. As H226 and H28 demonstrated differences in HMGB-1 release, additional epithelioid (H2452) and sarcomatoid (H2052) cell lines were included to observe whether this trend was dependent on subtype. Lastly, HMGB-1 release over time was measured to later identify the expected HMGB-1 release time points in vivo.
Media release of HMGB-1 was measured by ELISA at each time point after 25Gy radiation treatment or at the same point in untreated cells. We found no significant differences at time points before 24 hours. Media HMGB-1 was significantly increased in treated H226 cells (18.61 ± 0.9562 ng/mL) compared to untreated H226 cells (5.807 ± 0.4632 ng/mL) 24 hours after radiation (P< 0.0001). Similarly, HMGB-1 concentration was significantly increased 24 hours after radiation in radiated H2452 cells (29.50 ± 1.500 ng/mL) compared to untreated (4.917 ± 0.5290 ng/mL) H2452 cells (P=0.0042). No significant difference was observed between untreated and radiated H28 cells at 24 hours (7.054 ± 0.5747 vs. 9.654 ± 2.693 ng/mL, P=0.4008). No significant difference was observed between untreated and radiated H2052 cells at the 24 hour time point (3.478 ± 0.3181 vs. 3.545 ± 0.8395 ng/mL, P=0.9446).

At the 48 hour time point, a significant increase was observed in treated H226 cells (42.88 ± 4.240 ng/mL) compared to untreated H226 cells (8.371 ± 0.6406, P<0.0001). Similarly, treated H2452 (91.50 ± 11.50 ng/mL) cells showed greater media HMGB-1 concentrations than untreated H2452 cells (8.447 ± 1.923 ng/mL, P=0.0191). Conversely, there was no significant difference between untreated and treated H28 cells (9.524 ± 0.1926 vs. 11.57 ± 0.9960 ng/mL, P=0.0538) or between untreated and treated H2052 cells (4.062 ± 0.3347 vs. 7.048 ± 1.617 ng/mL, P=0.1448).

These findings overall suggest that, at least until 48 hours after treatment, epithelioid MPM cell lines are more susceptible to radiation in terms of HMGB-1 release while sarcomatoid MPM cell lines are less susceptible; unchanging in HMGB-1 release after radiation treatment.
Figure 16. HMGB-1 release into culture medium by non-treated or radiated epithelioid lines H226 and H2452 and sarcomatoid cell lines H28 and H25052. ELISA measurement of medium HMGB-1 concentration at time points (1-48 hours) after either no treatment (NoRx) or 25 Gy.
4.1.4 **Radiation induced cell death in human cell lines**

To test the susceptibility of epithelioid MPM and sarcomatoid MPM to radiation, cell death was measured in untreated and single fraction 25Gy radiated cells. After each time point, floating and adherent cells were collected for analysis. Cell death was measured by flow cytometric analysis of Annexin V and eFluor450 viability staining.

The percentage of apoptotic cells (Annexin V$^+$ efluor 450$^-$) did not change significantly over time. At 48 hours after 25Gy radiation, radiated H226 cells displayed significantly higher percentage of necrotic cells (Annexin V$^+$ efluor450$^+$ and Annexin V$^-$ efluor450$^+$) than untreated H226 cells out of total events measured (21.28 ± 0.7804 % vs. 48.41 ± 2.109 %, P=0.0003). 48 hours after treatment, 25Gy radiated H226 cell culture demonstrated a higher percentage of necrotic cells than 25Gy radiated H28 (48.41 ± 2.109% vs. 12.52 ± 0.8741 %, P<0.0001). The difference between treated and untreated H28 cells 48 hours after radiation was not significant (9.047 ± 1.448 vs. 12.52 ± 0.8741, P=0.1094).

These findings suggest that in H226, necrotic cell death is the predominant form of cell death elicited by 25Gy radiation treatment and occurs at least 48 hours after treatment. Conversely, sarcomatoid H28 is resistant to 25Gy radiation by not undergoing necrotic cell death at least until 48 hours after treatment.
Figure 17: Percentage cell death after no treatment or 25Gy radiation. A) Apoptotic cell death and B) necrotic cell death was measured for H226 and H28 untreated or 25Gy radiated cell lines.
4.1.5  **AE-17-OVA epithelioid mouse cell line behaves similarly to H226 in terms of HMGB-1 release after 25Gy radiation**

This experiment investigated HMGB-1 release after one dose of 25Gy radiation in AE-17-OVA to determine if it behaved similarly to human epithelioid MPM cell lines. HMGB-1 release from AE-17-OVA cells was determined by ELISA of culture media. Cells were either untreated or subjected to 25Gy radiation by a Caesium-137 irradiator. Culture media was collected 1, 2, 4, 24 and 48 hours after treatment. Media was then analyzed by an HMGB-1 ELISA kit to determine supernatant concentration.

At the 4 hour time point, HMGB-1 in radiated AE-17-OVA (12.08 ± 3.840 ng/mL) was increased compared to no treatment (2.622 ± 0.4585 ng/mL, P=0.1283). At 24 hours, media HMGB-1 concentrations were significantly higher in radiated AE-17-OVA than untreated cells (84.630 ± 14.3500 ng/mL, P=0.0484). Lastly, 48 hours after treatment, media HMGB-1 concentration was higher in radiated cells (140.800 ± 14.9200 ng/mL) than in unirradiated cells (34.021 ± 1.4110 ng/mL, P=0.0191).

The results demonstrate that murine epithelioid AE-17-OVA behaves similarly to human epithelioid H226 as radiated groups release more HMGB-1 than untreated cells. However AE-17-OVA differs in that HMGB-1 release appears to begin 4 hours after radiation while radiated H226 begins releasing HMGB-1 around the 24 hour time point.
Figure 18. HMGB-1 release into culture medium by non-treated or radiated human epithelioid line H226 and H2452 and murine epithelioid cell line AE-17-OVA. ELISA measurement of medium HMGB-1 concentration at time points (1-48 hours) after either no treatment (NoRx) or 25 Gy.
4.1.6 **AE-17-OVA epithelioid mouse cell line behaves similarly to human epithelioid cell line H226 in terms of cell death when treated with 25Gy radiation**

The purpose of this experiment was to investigate whether, similar to H226, murine epithelioid MPM cell line AE-17-OVA cell death occurs at the same time as HMGB-1 release. Radiated cells were given 25Gy in a single fraction.

After each time point, floating and adherent cells were collected for analysis. Cell death was measured by flow cytometric analysis of Annexin V and eFluor450 viability staining. Apoptotic cells were considered as Annexin V\(^+\) efluor 450\(^-\). Annexin V\(^+\) efluor450\(^+\) and Annexin V\(^-\) efluor450\(^+\) cells were considered necrotic.

We found that the percentage of apoptotic cells did not change significantly over time in AE-17-OVA. Basal rate of apoptosis in AE-17-OVA of both untreated and radiated cells is increased compared to H226. 4 hours after treatment, the percentage of AE-17-OVA cells undergoing necrosis in the radiated group was increased compared to untreated (15.05 \(\pm\) 3.126\% vs. 23.82 \(\pm\) 1.040\%, \(P=0.0562\)). 24 hours after treatment, necrotic cell death in radiated AE-17-OVA (38.33 \(\pm\) 4.410\%) was significantly higher than in untreated AE-17-OVA cells (10.18 \(\pm\) 7.410\%, \(P=0.0309\)).

At 48 hours after 25Gy radiation, radiated H226 cells displayed significantly higher percentage of necrotic cells than untreated H226 cells out of total events measured (21.28 \(\pm\) 0.7804 \% vs. 48.41 \(\pm\) 2.109 \%, \(P=0.0003\)). Similarly, in AE-17-OVA radiated cells necrotic cell death (69.78 \(\pm\) 8.851\%) was significantly higher than in untreated murine cells (14.29 \(\pm\) 3.873\%, \(P=0.0046\)).
Overall, AE-17-OVA behaves similarly to H226 in that radiated cells undergo more necrotic cell death compared to untreated cells. Differently however, AE-17-OVA demonstrates increased necrotic cell death at 24 hours after treatment in radiated cell lines. These data suggest that AE-17-OVA behaves similarly to H226 in terms of necrotic cell death but succumbs to radiation-induced cell death at an earlier time point.

**Figure 19: Percentage cell death after no treatment or 25Gy radiation.** A) Apoptotic cell death and B) necrotic cell death was measured for murine epithelioid AE-17-OVA and human epithelioid H226 untreated or 25Gy radiated cell lines.
4.2 Patient Sample and *in vivo* HMGB-1

4.2.1 **No significant difference in HMGB-1 positivity by IHC staining between untreated and SMART treated epithelioid MPM paraffin embedded patient samples**

Epithelioid patient samples were stained for HMGB-1 to investigate whether HMGB-1 staining in treated patients correlated with longer survival times of MPM patients.

Using Aperio Image Scope nuclear analysis software, the strength of HMGB-1 nuclear staining in paraffin embedded patient samples was measured. Cells were designated by the software as being either high, medium, or low strength in staining. Furthermore, percent of HMGB-1 positive nuclei was measured by counting positive nuclei regardless of strength of staining. Epithelioid MPM samples were used for this experiment.

The percentage of highly positive nuclei in non-treated or SMART treated MPM patients did not differ significantly (32.47 ± 5.59% vs 31.69 ± 5.44%, P=0.9372). No treatment (24.44 ± 2.48%) and SMART treatment (19.95 ± 1.91%) patient samples did not differ significantly in the percentage of medium intensity positive nuclei (P=0.2216). Similarly, percent low intensity positive nuclei of no treatment (19.74 ± 3.30%) and SMART treated (17.76 ± 1.95%) samples did not differ significantly (P=0.6107). Finally, the overall percentage of positive nuclei in the samples of each group did not display any significant difference (76.64 ± 2.41% vs 69.40 ± 5.229%, P=0.4328). The no treatment group was N=5 while the SMART treated group was N=14.
While MPM patients receiving SMART treatment have significantly higher survival than untreated patients, the lack of difference in HMGB-1 staining intensities between the groups suggest HMGB-1 immunohistochemistry alone is not an adequate predictor of survival in untreated or SMART treated MPM patients.

Table III. Patient Demographics.

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>66 ± 9.7</th>
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<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
</tr>
<tr>
<td>No Treatment</td>
<td>N=5</td>
</tr>
<tr>
<td>SMART</td>
<td>N=14</td>
</tr>
</tbody>
</table>
Figure 20. High, medium, and low intensity nuclear staining of HMGB-1 in paraffin embedded patient samples. Percentage of total nuclei with A) high, B) medium, and C) low intensity staining was quantified in MPM patient samples. D) Total positively stained nuclei was also measured. Typical images of patient samples from epithelioid MPM patients who received E) no treatment or F) SMART treatment are displayed.
4.2.2 Serum HMGB-1 is significantly higher 2 days after treatment in mice receiving local radiation therapy.

Mice with subcutaneous epithelioid MPM from AE-17-OVA inoculation were either untreated or radiated to observe whether HMGB-1 is released \textit{in vivo} in response to radiation. Mice in the 15Gy treatment group had their tumors locally irradiated by image guidance. Mice received 3 fractions of 5Gy (1 per day) for a total dose of 15 Gy. HMGB-1 ELISA was performed on mouse serum on days 12 (2 days after LRT), 17 (7 days after LRT), and 22 (12 days after LRT) after subcutaneous MPM tumor inoculation.

At the time point 2 days after completing LRT, there was a significant increase in mean serum HMGB-1 concentration of radiated mice (N=5, 52.77 ± 8.380 ng/mL) compared to mice who did not receive treatment (N=9, 11.51 ± 3.381 ng/mL, P=0.0002). At 7 days after completion of LRT, there was no significant difference in the mean serum HMGB-1 concentration of non-treated (N=8, 84.61 ± 37.41) and radiation treated (N=8, 47.95 ± 19.59) mice (P=0.3999). Similarly there was no significant difference in mean serum HMGB-1 concentration between radiated mice (N=7, 133.9 ± 51.30) 12 days after completing LRT and no treatment mice (N=6, 85.38 ± 32.14) at the same time point (P=0.4579). These results suggest that radiating the tumor leads to an acute release of HMGB-1 into the serum 2 days after treatment and that this effect disappears at later time points.
Figure 21. Serum HMGB-1 and tumor growth curves of non-treated and mice treated with 15 Gy LRT. A) at days 12, B) 17, and C) 22, serum HMGB-1 was measured. 15Gy LRT mice had significantly greater serum HMGB-1 than untreated mice at day 12 (2 days after LRT).

4.2.3 Tumor Growth Curve

Tumor size measurements were taken to observe the effect of radiation on tumor size. Tumor growth was measured for untreated and 15 LRT treated mice. At day 12 there was no significant difference between untreated (218.6 ± 55.30 mm$^3$, N=6) and radiated (78.8 ± 15.43 mm$^3$, N=3) mice (P=0.2162). At day 17, untreated mice (425.3 ± 122.4 mm$^3$, N=4) were significantly higher in tumor volume than treated mice (64.9 ± 24.99 mm$^3$, N=4, P=0.0279). Finally on day 22, untreated mice (898.3 ± 473.70 mm$^3$, N=3) displayed a significantly greater tumor volume than LRT treated mice (66.85 ± 22.81 mm$^3$, N=6, P=0.0106). Collectively, these findings demonstrate that radiation is able to slow tumor growth while in untreated groups, tumors continue to increase in size over time.
Figure 22. Tumor volume increases more in untreated mice compared to mice receiving LRT. Measurements of tumor size after LRT were taken on days 12 (2 days after LRT), 17 (7 days after LRT), and 22 (12 days after LRT).
4.2.4 Percent HMGB-1 positive nuclei in tissues of mice receiving no treatment or 15Gy radiation.

The purpose of this experiment was to quantify HMGB-1 staining of untreated and radiated murine epithelioid MPM tumors. The goal was to study whether radiation treatment has an effect on HMGB-1 expression in tissue. The percentage of HMGB-1 positive cells of untreated and radiated tumor tissue at each time point was measured. 2 days after the completion of LRT, there was no significant difference in the mean percent positive cell staining of NoRx (88.97 ± 2.157%) and 15Gy (93.89 ± 1.347%) groups (P=0.1016). NoRx (81.84 ± 3.792%) and 15Gy (95.20 ± 1.101%) groups showed a significant difference in percent HMGB-1 positive nuclei 7 days after completing LRT (P=0.0148). Lastly, 12 days after completion of LRT, NoRx (89.27 ± 2.758%) and 15Gy (95.71 ± 1.081%) did not significantly differ in mean percent positive nuclei (P=0.0724). Despite the lack of significant difference at 2 days and 12 days after LRT, there was a trend towards increased percent positive nuclei in 15Gy radiated tumors. These results propose that radiation may increase tumor HMGB-1 expression.

Figure 23. Percent positive nuclei in tumor tissue without treatment or with 15Gy radiation. Staining was performed on tissues A) 2 days, B) 7 days, or C) 12 days after completing radiation treatment or on non-treated tissues of the same time point.
4.2.5 Percent HMGB-1 high, medium, and low positivity in mouse tissues from untreated and 15Gy radiated mice

Percent positive cells were divided into high, medium and low intensity staining to model our observations of tumor tissue in which there are varying degrees of positive staining intensity. Staining of tissues for HMGB-1 was performed by immunohistochemistry and analyzed using Aperio image analysis software.

Significant differences were observed in low intensity staining 2 days after LRT. Tissues treated with LRT were significantly lower (7.323 ± 1.538 %) in percent low intensity stained cells than untreated tissues (13.63 ± 1.958 %, P=0.0446). At 7 days after LRT, there was a significant increase in high intensity stained cells in radiated tumors (65.28 ± 5.285%) compared with untreated tumors (24.16 ± 8.434%, P=0.0061). High intensity staining is higher in radiated tumors at 2 and 7 days after treatment (P=0.0750 and P= 0.0061 respectively) while low intensity staining is lower in radiated tumors at the 2, 7, and 12 day time points (P=0.0446, P= 0.0534, and P=0.0788 respectively).

Overall, low and medium intensity staining tends to be lower in radiated tumors while high intensity staining tends to be higher in radiated tumors at 2 and 7 days after treatment. These trends tend to be less clear at day 12 after treatment. This suggests radiation may have a role in either increasing HMGB-1 expression of tumor cells, thus leading to higher levels of high intensity staining. Alternatively, radiation may cause HMGB-1 release, leading to lower levels of low and medium intensity staining.
Figure 24. Percent nuclei with low, medium, and high staining of tissues 2, 7, and 12 days after LRT. 12 days after LRT, A) low, B) medium, and C) high positive nuclei are displayed. 7 days after the completion of LRT, D) low, E) medium, and F) high IHC staining is quantified. Finally, 2 days after the completion of LRT, the mean percentage of G) low, H) medium, and I) high positively stained nuclei is reported for untreated and 15Gy LRT treated mouse tissues.
4.2.6 **Quantification of tumor infiltrating CD8+ T Cells in untreated and radiated mouse tumor tissue**

CD8+ T Cells are responsible for mediating antigen-specific tumor cell killing. Staining for CD8+ T Cells was performed on untreated and radiated tumors to study the immunogenic effect of radiation in terms of CD8+ T Cell recruitment. The recruitment of the CD8+ T Cell immune response was measured using immunofluorescent staining of tumor tissue for CD3+CD8+ cells by staining CD3 (488 nm) and CD8 markers (633nm). Untreated and 15Gy radiated tumor samples from mice were taken 2, 7, and 12 days after LRT.

Quantification at each time point was performed by averaging the cell counts of 5 random fields. The number of CD3+CD8+ cells counted 2 days after LRT was not found to be significantly different between untreated and radiated tissues (18.40 ± 4.739 vs. 15.20 ± 2.818 cells, P=0.5775). The number of CD3+CD8+ cells counted 7 days after LRT was significantly increased in radiated tissue (32.20 ± 8.225 cells) compared to untreated tissue (10.60 ± 3.076 cells, P=0.0398). 12 days after LRT, radiated tissue was similarly increased in the number of CD3+CD8+ cells compared with untreated tissue (55.60 ± 13.07 vs. 1.600 ± 0.5099 cells, P=0.0033). Overall, 2 days after LRT, untreated and radiated tumors have similar staining for CD3 and CD8 markers However over time, CD3 and CD8 staining decreases in the untreated group while CD3 and CD8 staining increases in the 15Gy radiated group. The peak staining for CD3 and CD8 occurs 12 days after receiving radiation.

In summary, these findings indicate that local radiation treatment of tumors is able to stimulate the recruitment of CD8+ T Cells to tumor tissue.
Figure 25. Immunofluorescent staining of untreated or 15Gy radiated mouse tissue. A)
Immunofluorescent images of DAPI, CD3, and CD8 merged staining (405 blue, 488 green, and
633nm red respectively). B) Quantification of CD3⁺ CD8⁺ cells per x200 magnified field.
4.2.7 **6E6 hybridoma anti-HMGB-1 antibody is able decrease the signal of recombinant HMGB-1* *in vitro* by ELISA**

The purpose of this experiment was to observe whether anti-HMGB-1 antibody is able to bind recombinant HMGB-1 for later *in vivo* use. Anti-HMGB-1 Abs were generated from ascites fluid after injection of the hybridoma cell line 6E6. Recombinant HMGB-1 (80 ng/mL) was measured by ELISA. Recombinant HMGB-1 was also mixed with anti-HMGB-1 Ab generated from the 6E6 hybridoma in a 1:1 ratio (80 ng/mL of recombinant HMGB-1 mixed with 80 ng/mL of anti-HMGB-1 Ab). The mixture was then plated on the ELISA kit to measure signal abrogation and the ability of anti-HMGB-1 to bind recombinant HMGB-1.

The recombinant HMGB-1 and anti-HMGB-1 Ab mixture (6.835 ± 1.714 ng/mL) measured a significantly lower concentration of HMGB-1 than recombinant HMGB-1 alone (74.83 ± 1.146 ng/mL, *P*=0.0009). The ability of anti-HMGB-1 to block the recombinant HMGB-1 signal in the ELISA kit indicates that it is able to bind and neutralize HMGB-1.

![Figure 26. Anti-HMGB-1 Ab abrogates recombinant HMGB-1 signal by ELISA. Wells were measured in duplicate.](image)
4.2.8 Anti-HMGB-1 Antibody combined with LRT increases tumor growth compared to LRT alone

In this experiment, the goal was to determine the role of HMGB-1 in mediating an anti-tumoral effect. Since HMGB-1 is hypothesized to mediate anti-tumoral immunity it was predicted that if HMGB-1 was blocked during radiation the immune response would not be formed against the tumor. AE-17-OVA mice either received no treatment (N=5), local radiation therapy (N=4), or local radiation therapy in combination with anti-HMGB-1 antibody (N=5, 100µg per day on days 8-12 after tumor inoculation). Radiation was given in 3 5Gy fractions (1 fraction per day) for a total of 15Gy. Tumor volume was measured using the longest length and width measurements and calculated with the formula: Length x Width² x (π/6).

NoRx showed the most rapid tumor growth and greatest tumor volumes. LRT+anti-HMGB-1 and LRT alone groups displayed lower tumor volumes than NoRx (Figure 29A). In observing just LRT+anti-HMGB-1 and LRT groups, there was a trend towards greater tumor volume at days 11 (P=0.0535) and a weaker trend at 14 (P=0.2669) in the LRT+HMGB-1 group. Tumor growth is trending towards being greater in the LRT+anti-HMGB-1 over time. This trend is greatest at days 38 (P=0.0769) and 40 (P=0.0441).

Overall, blockade of HMGB-1 during radiation treatment displayed a trend of greater tumor volume over time compared to LRT alone. The results of this experiment suggest that HMGB-1 is an important beneficial aspect of radiation therapy as its neutralization during LRT increases tumor growth.
Figure 27. LRT in combination with HMGB-1 demonstrates greater tumor growth compared with LRT alone. A) Untreated mice (NoRx) demonstrate the greatest tumor growth compared to LRT + anti-HMGB-1 and LRT groups. B) Mice receiving LRT + anti-HMGB-1 exhibit greater tumor volume than mice treated with LRT alone.
5 Discussion

The aims of this study were to study radiation treatment in MPM and factors that are crucial to successful treatment. As radiation causes cell death, HMGB-1 is an important molecule released from dying cells that is responsible for triggering inflammation. Thus 1) I investigated the release of HMGB-1 as a result of radiation treatment in different MPM subtypes. 2) I examined whether HMGB-1 release is related to the difference in survival observed in patients by staining paraffin-embedded patient samples for HMGB-1. 3) I studied the CD8+ T cell immune response generated by HMGB-1 release in radiation treatment.

The first set of experiments set out to characterize HMGB-1 release by radiation in vitro using MPM cell lines. As there is a worse prognosis for MPM patients with biphasic and sarcomatoid subtypes of disease compared with epithelioid disease, these experiments aimed to determine if this difference relates to their response to radiation treatment. Based on the SMART protocol and other groups using radiation therapy, epithelioid disease responds better than the other subtypes. Indeed in this study the in vitro model mimicked what is observed in the clinic. Radiation treatment resulted in more necrotic cell death and HMGB-1 release in epithelioid lines than sarcomatoid lines.

In tackling the second goal, the HMGB-1 staining of patient samples was performed. Chemotherapy has been shown to increase survival by a few months. However, the SMART feasibility study demonstrated a significant increase in 3 year survival from 30% to 84% in epithelioid patients. Thus, the HMGB-1 staining was compared between the two patient groups and compared to survival. No significant difference was found in the intensity of staining of either treatment group, suggesting a much more complex clinical picture.
The last group of experiments focused on in vivo models to observe radiation treatment, HMGB-1 release, and the potential for CD8+ T cell recruitment and tumor cell killing. Significant differences were found in HMGB-1 release and CD8+ T cell recruitment in untreated and radiated mice. This study indicates that HMGB-1 may be a major player in generating an anti-cancer immune response after radiation therapy.

An in-depth view of each of the aims of this project is discussed below.

5.1 In vitro radiation-mediated HMGB-1 release

HMGB-1 Immunofluorescence Expression

The H226 epithelioid and H28 sarcomatoid cell lines were studied for differences in HMGB-1 immunofluorescent staining after no treatment or 5–75 Gy radiation (Figure 15). Quantification of fluorescent intensity revealed opposing patterns of each cell line. There was a trend towards decreasing intensity across dose in the H226 line while the H28 cell line displayed an increase in intensity. HMGB-1 immunofluorescence after radiation treatment has received limited study and may identify behaviours that can be applied to clinical treatment options.

Epithelioid MPM is more susceptible to SMART treatment involving radiation as well as chemotherapeutic treatment in the clinical setting. Furthermore, sarcomatoid disease is a significant negative prognostic factor (Neumann, et al. 2013). Decreasing fluorescence may indicate release of HMGB-1 into the extracellular environment. Liberated HMGB-1 could then bind to APCs and trigger an anti-cancer immune response. The release of HMGB-1 in H226 may be responsible for the better outcomes seen in patients with epithelioid disease.
The greater fluorescent intensity in the H28 sarcomatoid cell line may represent a mechanism of survival. In other studies, RNAi suppression of HMGB-1 renders leukemia and osteosarcoma cell lines more susceptible to chemotherapy and radiation (Huang, et al. 2012, Liu, et al. 2011, Livesey, et al. 2012). Conversely, pancreatic tumor cell lines have been shown to be more resistant to cytotoxic therapies when HMGB-1 is overexpressed (Tang, et al. 2010). In the H28 cell line, increased expression of HMGB-1 may be a survival mechanism used to counteract the radiation treatment. With its roles in non-specific DNA binding and DNA transcription promotion, there remains many possibilities in terms of up-regulation of other pro-survival proteins such as DNA repair proteins. Based on these findings HMGB-1 release was studied.

Cell death will also be discussed later.

**HMGB-1 media release**

It was hypothesized that the decreasing intensity in H226 epithelioid line was due to release of HMGB-1 into the extracellular milieu. Complementing this, the increase in staining of the H28 sarcomatoid cell line was predicted to correlate with a lack of release of HMGB-1 into the media; instead remaining intracellular. The next experiment employed ELISA to measure media HMGB-1 concentration of the same cell lines 24 hours after no treatment or 5-75Gy γ-radiation (Figure 16). We found a significant trend across the dosing range in which the H226 epithelioid line released more HMGB-1 into the media than the H28 sarcomatoid cell line at every radiation dose. While higher doses tended to increase HMGB-1 release in H226, H28 remained similar in HMGB-1 release over the entire dosing range.

These findings complement the previous experiment. In both cell lines, higher HMGB-1 fluorescence corresponded with lower media release and vice versa. Even at high doses, H28 displayed high fluorescent intensity and low release of HMGB-1.
As was previously covered, HMGB-1 release occurs during late apoptotic or necrotic cell death when the membrane of the cell becomes permeable (Degryse, et al. 2001, Falciola, et al. 1997, Muller, et al. 2001). Thus the decreasing fluorescent intensity and increasing HMGB-1 release observed in the H226 cell line may relate to increasing levels of necrotic cell death. Therefore high intensity H28 staining and low HMGB-1 release could indicate a lack of cell death and the sequestration of HMGB-1.

Radiation results in DNA damage which can either be repaired, resulting in cell survival, or remain irreparable leading to cell death by apoptosis. Higher doses lead to necrosis and uncontrolled cell death (Tesniere, et al. 2008). As observed in H226, the decrease in fluorescent intensity with increasing radiation dose and the concomitant increase in HMGB-1 observed in the media indicates greater levels of necrotic cell death. Conversely in H28, the high fluorescent intensity and the lack of HMGB-1 media increase 24 hours after treatment indicates cell death is not occurring and HMGB-1 is remaining intracellular. In these initial experiments H226 and H28 differ in their response to radiation in terms of HMGB-1 release. However, we wanted to observe the response to radiation over time. Furthermore, as these are just 2 cell lines, we wanted to confirm that the differences observed were as a result of differing subtypes. Thus the next experiment focused on observing the radiation response of more cell lines as well as the examining the release of HMGB-1 over time.

**HMGB-1 release over time by multiple cell lines**

Patients undergoing SMART treatment often receive 25Gy hypofractionated radiation treatment before surgery and surgery is typically performed 2-7 days after radiation. Hence there is a short
window in which the immune response is formed. To study whether the differences in response to radiation by H226 and H28 were based on their subtype differences rather than just chance, multiple cell lines were added. To better model the clinical dose, H226 and H2452 epithelioid MPM cell lines and H28 and H2052 sarcomatoid lines were observed at various time points until 48 hours after receiving 25Gy radiation.

At the early time points of 1-4 hours after treatment, radiated lines tended to release similar levels of HMGB-1 as their un-irradiated counterparts. However at 24 and 48 hours, the greatest differences are found. At 24 hours, both radiated the epithelioid lines differed significantly in HMGB-1 release compared to untreated epithelioid cells as well as untreated and radiated sarcomatoid cells. Furthermore, radiated sarcomatoid lines release more HGMB-1 than untreated sarcomatoid cells. A similar trend occurs at 48 hours after treatment. There is a difference in HMGB-1 concentration in media of untreated and treated sarcomatoid cells. While treated sarcomatoid cells release more HMGB-1 than untreated, they release significantly less than radiated epithelioid cell lines. Radiated epithelioid lines release 5 to 8 fold more HMGB-1 than untreated epithelioid cells. This is starkly contrasted with radiated sarcomatoid releasing at most 1.5 fold more HGMB-1 than untreated sarcomatoid cells.

These findings may be important to what occurs in vivo. Rapid growth and the lack of vasculature support often leads to cell death by necrosis in the center of tumors. As discussed above, necrosis leads to HMGB-1 release which may facilitate tumor survival through neo-angiogenesis mediated by recruited pro-angiogenic macrophages (Yang, et al. 2014). While chronic release is thought to promote angiogenesis, the sudden acute release of HMGB-1 by cytotoxic therapies is able to trigger the immune response (Venneri, et al. 2007). It is therefore possible that while radiated sarcomatoid cell lines release more HMGB-1 than untreated, the
difference is quite small compared to the potential 8 fold increase seen in epithelioid cells. It is conceivable that in patients with sarcomatoid MPM, radiation triggers a slight increase in HMGB-1 by cell death, but not enough to overcome the chronic HMGB-1 release that promotes tumor growth. On the contrary, epithelioid MPM may release a large acute burst of HMGB-1, as seen in these in vitro experiments, thus overcoming the pro-tumor microenvironment and promoting a host anti-tumor response. These in vitro differences may provide insight into clinical disease.

Interestingly, the findings of this set of experiments disagree with an article studying H28, H2452 and H2052 basal HMGB-1 release. The authors claim that HMGB-1 release is highest in H28 and H2052 while lower in H2452. However the differences were marginal and differently from this study, cell culture conditions in their work did not include serum (Tabata, et al. 2013).

**Murine epithelioid line AE-17-OVA HMGB-1 Release**

AE-17-OVA cells were observed for release of HMGB-1 after LRT. As AE-17-OVA was later used in in vivo experiments, the purpose of this experiment was to determine whether the in vitro activity of murine epithelioid and human epithelioid cell lines were similar. The ultimate goal was to ensure consistency in vitro so that later in vivo results could potentially be extrapolated to human interventions.

AE-17-OVA cells were treated with 25Gy radiation and observed over 48 hours. Similar to human epithelioid cell lines H226 and H2452, AE-17-OVA treated with radiation at 24 and 48 hours after treatment showed the greatest media release of HMGB-1 compared to untreated cells. Despite the lack of significant difference, there is a trend towards higher HMGB-1 release 4
hours after treatment. At 24 and 48 hours there is a significantly higher media HMGB-1 concentration in radiated AE-17-OVA compared to untreated cells.

Similar to human cell lines, the hypothesis for this finding was that radiation resulted in necrotic cell death, leading to an acute release of HMGB-1 in radiated cell cultures. Discussed above was the hypothesis that human epithelioid MPM cell acute release of HMGB-1 results in an immune response that translates into longer survival. The goal of the findings with AE-17-OVA, given that they behave similarly to human epithelioid MPM in vitro, was to later investigate the HMGB-1-mediated immune response in mice.

**Human cell line In vitro cell death**

The purpose of this aspect of study was to determine whether HMGB-1 release is correlated to necrotic cell death. Epithelioid cell line H226 was found to undergo a significant increase in necrotic cell death 48 hours after treatment compared to untreated cells. Conversely, sarcomatoid H28 did not demonstrate an increase in necrotic cell death. Furthermore, apoptotic cell death was consistent at all time points. Thus HMGB-1 release appears to correlate with necrotic cell death as the maximum HMGB-1 concentration and maximal necrotic cell death levels occur 48 hours after treatment.

An interesting trend to note is that HMGB-1 release tends to appear in the epithelioid cell lines around 24 hours after radiation while necrotic cell death differences are not apparent until 48 hours after treatment. This may be a result of HMGB-1 being released by necrosis before staining for necrosis by eFluor viability dye is possible. Additionally, there may be other forms of cell death that are resulting in HMGB-1 release. Sustained autophagy and necroptosis were
not measured in this experiment but have been reported to result in HMGB-1 release (Tang, et al. 2010).

These in vitro experiments may build the initial parts of a larger picture. MPM patients receiving radiation differ in their prognoses depending on the subtype of disease present. Epithelioid patients tend to experience longer survival after treatment. The SMART feasibility study did not demonstrate any survival benefit for biphasic patients. Other studies have had similar findings in which biphasic and sarcomatoid MPM disease does not respond as well to chemotherapy or radiation. What has been demonstrated here is that, in vitro, both epithelioid cell lines behave similarly by responding to radiation, in terms of HMGB-1 release, while the sarcomatoid subtypes both do not respond as strongly to radiation. This may represent an inherent subtype similarity. Furthermore this may offer insight into clinical cases. Epithelioid disease may be more susceptible to radiation treatment. Epithelioid disease may be more likely to undergo uncontrolled necrosis and release HMGB-1 into the extracellular environment and possibly the blood (more on serum release discussed later). This would enable patient antigen presenting cell activation and an anti-cancer response. While some cell death does occur in sarcomatoid cells, there may not be enough HMGB-1 released to trigger an immune response. The difference in survival in the clinic may be dependent on the immune response in addition to the immediate cytotoxic effects of treatment.

In the sarcomatoid type, lower levels of necrosis suggests a natural resistance to radiation and lower levels of cell death compared to epithelioid disease. Thus sarcomatoid disease may survive, keeping HMGB-1 sequestered intracellularly and hidden from the immune system. This dissimilarity from epithelioid cell lines may be due to the cell lines’ differing abilities to resist radiation treatment or to repair DNA damage. In other studies, sarcomatoid H28 was shown to
be more resistant to chemotherapy as a result of higher basal mRNA levels of DNA synthesis enzymes thymidylate synthase (TS). Epithelioid H2052 was shown to be more sensitive to chemotherapeutic agents as a result of low TS mRNA level (Giovannetti, et al. 2011). Furthermore, high expression of TS has been attributed to radiation resistance in human uterine cervical cancer cell lines (Saga, et al. 2002). This is one option among many potential pathways that may render sarcomatoid cells resistant to radiation.

**Murine AE-17-OVA in vitro Cell Death**

With the goal of moving to animal studies, the murine epithelioid cell line AE-17-OVA was used to determine if, similar to human epithelioid cell lines, HMGB-1 release was correlated to cell death. After 25Gy radiation, apoptotic cell death and necrotic cell death were observed over time. Differently from H226, AE-17-OVA demonstrated a trend towards increasing apoptotic cell death in radiated cells 48 hours after treatment. However, the difference was not significant. Furthermore, at 24 hours after treatment, radiated AE-17-OVA cells displayed a trend towards more necrotic cell death than untreated cells while H226 did not. Similar to the human epithelioid cell line H226, necrotic cell death was increased in radiated AE-17-OVA cells 48 hours after treatment compared to untreated cells.

While the trends are similar, AE-17-OVA and human epithelioid cell lines there are subtle differences to note. AE-17-OVA appears to have a higher basal level of apoptotic cell death which may represent a faster natural turnover rate as AE-17-OVA was observed to have a much more rapid division time than all human MPM cell lines. Furthermore, the high division rate may explain the higher percentage of necrotic cell death starting 24 hours after radiation as the effects of DNA damage potentially manifest more quickly in rapidly dividing cells.
5.2 Paraffin-embedded Patient Samples.

This study of HMGB-1 was geared towards translating findings into clinical use. Therefore, the next area of interest was the use of MPM patient samples. Paraffin-embedded epithelioid MPM samples were stained by immunohistochemistry for HMGB-1 expression (Figure 21). The hypothesis was that high expression after treatment would give rise to the potential for greater HMGB-1 release. Extracellular HMGB-1 would trigger APC maturation and the recruitment of the immune response. In studying the effect of radiation on HMGB-1 release, samples from patients who did not receive treatment were compared to those who received SMART treatment.

We did not observe any significant difference in percent positive nuclei between the treatment groups. Furthermore, no trends were observed when splitting the overall percent positive nuclei into high, medium, and low staining intensities. The means of untreated and SMART treated patient samples were relatively similar with a high variability among the SMART treated samples.

A significant challenge to this area of the study was the timeline between when patients receive surgery and radiation. Tumor samples were either removed by biopsy or through surgical intervention such as EPP. Patients receiving SMART underwent radiation before having the tumor removed by EPP. Since surgery occurred between 2 and 7 days after radiation, it became difficult to compare staining; especially since the in vitro experiments demonstrated that levels of HMGB-1 measured depend on the time point observed after treatment.

The clinical picture is also more complex than in vitro analysis. While HMGB-1 was a potential measure for response to radiation in cell culture, tumors are extremely heterogeneous and may contain multiple cell types that may respond differently to radiation; they may have tumor stem
cells and multiple lineages of differentiated cells. Furthermore, stromal cells and immune cells are a large part of tumors that HMGB-1 staining alone can not differentiate between. The heterogeneity of the tumor cells and tumors between patients may make it difficult to discern HMGB-1’s by patient staining alone.

The composition of the tumors was also very different. Tumors differed in cellularity from very cellular to virtually acellular. Furthermore, within subtypes, such as epithelioid and sarcomatoid, there are a variety of other classifications that may influence tumor behaviour (Arrossi, et al. 2008). Percent positive nuclei was used to standardize samples that varied in cellularity. However, it is possible that relatively acellular tumors act differently than tumors with less connective tissue and more cells. This may be one of many factors that lead to the lack of trends observed.

In the next set of experiments mouse models were used to ensure that treatment timelines and tumor sizes were standardized. Furthermore, tumor pathology was consistent as tumors were generated in mice by injecting a syngeneic tumor cell lines.

5.3 In vivo mouse model

HMGB-1 has a dual role. High HMGB-1 in the blood has been demonstrated as a negative prognostic factor in cancer. In mesothelioma, HMGB-1 was been shown to be elevated in the blood of patients with tumor and not in non-tumor bearing patients exposed to asbestos (Tabata, et al. 2013). However, HMGB-1 is also implicated in generating an immune response by being released from cells undergoing necrotic cell death. In this set of experiments, the aim was to investigate the role of HMGB-1 in generating an immune response against radiation-treated
tumors. Mice were irradiated with a maximum tolerable dose of 3 doses of 5Gy radiation which is similar to the SMART study in which 5 doses of 5Gy was used in humans.

**Serum HMGB-1 Measurement**

The first part of this experiment involved observing whether HMGB-1 is released into the blood after radiating subcutaneous mouse tumors. Subcutaneous injection of the epithelioid AE-17-OVA tumor cell line allowed for the measurement of the tumor and easy targeting of the radiation. Mice were then sacrificed 2 days, 7 days, and 12 days after completion of LRT.

We found no significant difference in serum HMGB-1 between untreated and radiated mice at 7 and 12 days post LRT. However, 2 days after LRT, there was a significant increase in serum HMGB-1 concentration in mice receiving 15Gy LRT to the tumor. Similar to the *in vitro* studies of radiation induced cell death, the acute HMGB-1 release 2 days after treatment may have been due to the direct cytotoxic effects of radiation treatment. Tumor cells would have then released HMGB-1 into the local extracellular environment and eventually into the bloodstream.

At later time points, there is a greater concentration of HMGB-1 in the bloodstream of both untreated and radiated mice. Previous research has noted the dual role of HMGB-1 in both facilitating tumor growth as well as recruiting the immune system. While there are high levels of HMGB-1 in the blood of untreated and treated mice, their impact may differ vastly. In untreated mice, it is possible that HMGB-1 is being actively released by the tumor in order to enable ATP production in neighbouring tumor cells and ultimately cell division and tumor progression. It’s also possible that while untreated tumors did not visually display any necrosis during surgery, at even the latest time point of this experiment, it remains possible that the anoxic center of the
tumor was undergoing necrotic cell death and releasing HMGB-1. The high levels in treated mice may relate more to tumor cell death and the recruitment of the immune system.

Tumor volumes in untreated and radiated tumors may support these ideas. Tumor volume was measured over the course of treatment (Figure 23). Tumor volume and HMGB-1 concentration in tended to increase together; supporting the idea that untreated tumor HMGB-1 may be necessary for the tumor’s life cycle. Conversely, radiated mice have increased serum HGMB-1 in the absence of tumor volume increase. In the radiated mice, tumors are actively shrinking or stable in size, decreasing the likelihood of active secretion from the tumor.

Radiation has a large impact on tumor growth as untreated mice had tumors that were significantly greater in volume than radiated tumors, especially at later time points (Figure 23). High levels of HMGB-1 in radiated tumors may have been released by direct cytotoxicity of radiation as well as due to immune system recruitment and its release by cells such as monocytes and macrophages (Gardella, et al. 2002). In addition, CD8\(^+\) T Cell-mediated tumor killing may also result in HMGB-1 release by killed tumor cells. CD8\(^+\) T Cell killing of tumor cells may explain the lack of increasing tumor volume in radiated mice. The increase in CD8\(^+\) T cells may support this hypothesis (discussed below).

This study demonstrates that the setting of observation is important for determining the pro or anti-tumor effects of HMGB-1. Previous studies in gastric cancer, non-small cell lung cancer, hepatocellular carcinoma, leukemia and MPM have correlated serum HMGB-1 to increasing tumor volume and disease progression (Cheng, et al. 2008, Chung, et al. 2009, Jube, et al. 2012, Kang, et al. 2007, Shang, et al. 2009, Sheng, et al. 2009). This study establishes that serum HMGB-1 can increase in the absence of tumor volume increase; as in the case of mice receiving LRT that have stable tumor growth, longer survival, and high serum HMGB-1 concentration.
**T Cell Tumor Infiltration**

There is a striking difference between the tissues of mice having remained untreated or receiving 15Gy radiation. The staining for CD3 and CD8 markers demonstrate T Cell recruitment at later time points in radiated mice. Untreated mice present some T Cells at 2 days and less at 7 days. By 12 days, there are few to no T Cells in the entire tissue section of untreated tumor.

To tackle the observation of disappearing T Cells in untreated mice there are several hypotheses. The initial injection of tumor cells may have triggered the immune response, leading to similar levels of T Cells at the first time point (2 days after LRT). However, immunoediting and an immunosuppressive microenvironment may have led to the downregulation and apoptosis of tumor infiltrating T Cells in untreated mice. In one study on multiple myeloma, CD8\(^+\) T Cells were expanded *ex vivo* and infused into tumor-bearing mice. In mice with recurrent disease, the authors identified specific loss of MHC mutations and loss of tumor associated antigen expression; allowing tumor cells to evade specific immune destruction (Klippel, et al. 2014). Furthermore immunosuppressive tumor environments involving infiltrating T-regulatory Cells, M2 macrophages, PD-L1 expression, IL-10 secretion, CTLA-4 expression, and TGF\(\beta\) expression are known immune downregulators in MPM (Stevenson, et al. 2013, Tey, et al. 2006, Wong, et al. 2014). Radiation is thought to change the stromal environment and remove some of the immune inhibiting factors (Wong, et al. 2014). Thus radiation treatment and the release of pro-inflammatory molecules such as HMGB-1 may serve to stimulate an infiltrating CD8\(^+\) T Cell response by removing the immunosuppressive tumor microenvironment.
It was observed that radiated mice have fewer T Cells initially compared to untreated mice. At early time points even untreated mice are able to recruit TILs. Literature suggests that the initial lower number of CD8$^+$ T Cells in radiated tumors is most likely due to radiation treatment to the tumor which inevitably kills tumor as well as the infiltrated T Cells (Siva, et al. 2015). At 7 days untreated mice have fewer T Cells than treated mice. Treated mice are more numerous in infiltrating T Cells likely due to radiation necrosis releasing antigens and DAMPs such as HMGB-1 that triggered the immune response. As 7 to 9 days are required for the adaptive T Cell immune response to be active in mice, this may provide reasons as to why T Cells appeared the most in the day 7 and 12 time points (Busch, et al. 1998).

Radiated tumor mice demonstrated an acute HMGB-1 release into the serum 2 days after LRT that is higher than in untreated mice. In radiated tumors, the serum HMGB-1 level 7 and 12 days after LRT is not significantly different from serum HMGB-1 levels of untreated mice. However, radiated mice have much smaller tumors at the same time points as untreated mice. Furthermore, radiated mice display significant CD8$^+$ T Cell tumor infiltration which likely aids in slowing tumor growth. There are several hypotheses for this finding. 1) The acute and gradual release of HMGB-1 have different effects. Gradual HMGB-1 release by tumor cells during development (as seen in untreated mice) may act to down regulate or de-sensitize the immune response. Furthermore, tumors often have necrotic centers that release HMGB-1 and do not recruit the immune response, providing another potential mechanism of immune de-sensitization. Radiation treatment result in an acute release that may be necessary to overcome inhibitory or de-sensitization effects of high HMGB-1 levels. The literature suggests that while HMGB-1 has pro-tumor effects in growth and macrophage inhibition, pulsatile release by chemotherapy or radiation therapy promotes antigen processing and tumor-specific T Cell responses (Apetoh, et al. 2007, Campana, et al. 2008, Kuniyasu, et al. 2005).
2) The redox state of HMGB-1 may also differ based on the setting of release. Radiation treatment may promote a pro-inflammatory redox state of HMGB-1. The redox state has been reported as being an important factor in determining immunogenic or tolerogenic aspects of HMGB-1. It has been reported that reduced HMGB-1 promotes anti-tumor inflammation while oxidized HMGB-1 promotes immune tolerance. While necrotic cell death can lead to reduced HMGB-1 release, redox state variability in cancer is a factor due to tolerogenic and immunogenic cell death occurring at the same time and due to the tumor’s unique microenvironment (Tang, et al. 2010). These may explain how a poor prognostic marker in the blood may lead to very different results.

While it has been previously reported by Tabata et al. 2013 that high serum HMGB-1 is an indicator of poor prognosis in MPM patients, the findings of this project may suggest otherwise when measured after receiving radiation treatment. Differently from the previous study, radiation treatment provides longer survival and is correlated with high serum HMGB-1. Without treatment serum HMGB-1 may be a poor prognostic marker, however, this study demonstrates for the first time that HMGB-1 release after treatment may be beneficial for survival in MPM as it is correlated with longer survival and T Cell infiltration. While HMGB-1 serum after treatment has not been extensively studied, a study on esophageal squamous cell carcinoma has shown that serum HMGB-1 levels increase in patients who received preoperative chemotherapy and radiation treatment compared to untreated patients. This increase in HMGB-1 was also correlated with tumor antigen-specific T cell responses (Suzuki, et al. 2012). As our study also demonstrates a rise in serum HMGB-1 post radiation being correlated with TILs, there may be a role in detecting serum HMGB-1 as a post-treatment prognostic marker.
HMGB-1 Immunohistochemical Staining

HMGB-1 immunohistochemical staining was observed in order to understand how radiation affects HMGB-1 expression in tumor tissue. HMGB-1 positive nuclei were significantly increased in 15Gy radiated mice compared to untreated mice 7 days after completing LRT (Figure 25). While, the other time points were not significant, there is a trend towards increased percent positive nuclei in the radiated group at 2 and 12 days after LRT as well. It’s possible that the greater percent HMGB-1 positive nuclei is indicative of radiation-induced HMGB-1 overexpression. Initial overexpression may be important for later release during cell death when HMGB-1 can then be released. Indeed in breast cancer cells, estrogen treatment is able to increase HMGB-1 mRNA levels which has been demonstrated to sensitize these cells to chemotherapy treatment (He, et al. 2000).

Percent positive nuclei was broken down into intensity of staining (Figure 26) for each time point in order to quantify the different staining levels seen in tumor tissue. Significant differences were found between groups in low staining 2 days after LRT (Figure 26 G). There was also a significant difference in medium and high staining 7 days after LRT (Figure 26 E and F). While other groups did not demonstrate significant differences, there trends were quite consistent and most were approaching significance. Overall, at days 2 and 7 after LRT, 15Gy radiated tumors tended to display a lower percentage of low intensity stained nuclei. This was similarly observed for medium staining. However, in high intensity staining, radiated tumors tended to display a higher percentage of highly stained nuclei at the same time points.

HMGB-1 staining overall increased in radiated tumors compared to untreated tumors. This increased staining may relate to an increasing in expression of the tumor cells which may be necessary for HMGB-1 release. Supporting this idea, a study using glioblastoma cell lines found
that radiation resulted in increased nuclear HMGB-1 staining which was correlated with HMGB-1 media release \textit{in vitro}.

In comparing human and murine HMGB-1 immunohistochemical tumor staining, it was predicted that positive HMGB-1 staining be higher in radiated tumors. While this was the case for murine samples, patient staining did not display any trend in relation to treatment or survival. Murine tumors were generated from an epithelioid cell line while all clinical samples selected were of the epithelioid subtype and either received no treatment or radiation. Patient staining may have differed from the staining observed from mouse tissues due to mouse tissues being subcutaneously injected, differing from the pleural tissue in which MPM is found. As many patients had previous asbestos exposure, they presumably developed MPM from long-term inflammation as a result of asbestos fibers causing HMGB-1 release by damaging mesothelial cells. This is then able to lead to TNF-α release by macrophages and many other pro-transformation events. The long-term pro-inflammatory loop experienced by patients may not be experienced by mice that have tumor cells injected directly. Furthermore, injecting asbestos in order to develop tumors in mice was not feasible for the timeline of this project. Murine human differences may be explained by the standardized radiation timeline as well as the consistency of tumor pathology. Moreover, as injection of cells occurred on the same day, tumor size was very similar until treatment. This differs from the clinic in which different stages of disease and different volumes of tumors were irradiated for SMART treatment. Nevertheless, this experiment may allow us to understand what occurs in radiation treatment of MPM.

\textbf{HMGB-1 Blockade}

LRT was shown to result in serum release of HMGB-1. HMGB-1 is released by necrotic cells and is an important factor in DC maturation, leading to Ag presentation and an adaptive immune
response. LRT was also correlated with CD3+CD8+ T Cell infiltration into the tumor. In order to investigate the role of HMGB-1 in the radiation-induced immune response, tumor growth was measured in untreated mice, radiated mice, and mice that were radiated and given anti-HMGB-1 Ab. As HMGB-1 is an important factor in generating an immune response, it was expected that blocking HMGB-1 would result in lower survival than mice receiving LRT alone. It was predicted that if HMGB-1 was blocked, there would be less HMGB-1 available to bind APCs and the immune response would be less able to be generated.

Untreated mice displayed the fastest tumor growth. Both LRT and LRT+HMGB-1 Ab groups displayed slower growth compared to no treatment. While, LRT and LRT+HMGB-1 Ab groups did not demonstrate a significant difference at most time points, there was nevertheless a trend towards LRT treated mice displaying the slowest tumor growth. At the latest time point, this trend reached statistical significance. Thus it appears that HMGB-1 release during radiation induced cell death plays a major role in inhibiting tumor growth.

A similar theme is emerging in that the scenario of HMGB-1 release is crucial for determining its prognostic value. In animal models of MPM, blocking HMGB-1 was shown to slow tumor growth and correlate with longer survival, thus supporting HMGB-1’s role as a pro-tumor agent (Jube, et al. 2012). However our work demonstrates that during LRT, HMGB-1 may have an effect on tumor size as blocking HMGB-1 during LRT results in faster tumor growth. In the setting of LRT-induced cell death, HMGB-1 plays a role in mediating anti-tumor effects. Similar to our study, higher serum HMGB-1 after chemotherapy has been correlated with longer overall survival in esophageal squamous cell carcinoma patients (Suzuki, et al. 2012). Furthermore in a tumor vaccination model involving a mouse colon cancer and sarcoma cell line, injecting cells exposed to a lethal dose of radiation led to a protective memory response that
rejected a subsequent injection of live tumor cells. However, adding blocking HMGB-1 Ab to the radiation exposed cells led to tumor growth upon subsequent live tumor cell injection (Apetoh, et al. 2007). Similar to our findings, this study supports the idea that HMGB-1 is necessary in forming an immune response against dying tumor cells.

Despite the HMGB-1 blockade, there was still slower tumor growth in LRT+HMGB-1 Ab treated mice than in untreated mice. There are several possibilities for the tumor growth to still be impaired.

1) The immediate effects of radiation may have resulted in cell death. Thus tumor growth was slowed compared to untreated mice. The tumor would then take time to divide and become as large as the tumors in the untreated group.

2) HMGB-1 may have been incompletely blocked in terms of concentration as well as over time. HMGB-1 may have still been able to trigger an immune response if the Ab concentration was not high enough to completely negate its effects. Incomplete blocking may have occurred over time as there was a large spike in growth during and shortly after HMGB-1 Ab was administered however, the tumors shrank extensively after HMGB-1 Ab was no longer being given. The immune response may have been inhibited temporarily during the spike in tumor growth and returned once the Ab was no longer present. As \textit{in vitro} necrosis was high 48 hours after radiation, anti-HMGB-1 Ab was given during LRT and until 48 hours after completion of LRT. However, necrotic cell death may have been occurring extensively past 48 hours after LRT \textit{in vivo}; resulting in the observed decrease in tumor growth following the cessation of HMGB-1 Ab.
3) There are likely multiple other DAMPs released during cell death that are important in immune response generation. It’s possible that other DAMPs could function similarly or as redundancy for HMGB-1, and thus blocking HMGB-1 may not completely abolish the immunogenic effect of radiation. Indeed there are studies that support other molecules as being crucial for the immunogenicity of dying cancer cells. One study found that exposure of calreticulin, an “eat me” signal for DCs, was crucial for immunogenicity in a colon cancer cell line. Dying cancer cells exposed to CRT-inducing anthracycline drugs were able to elicit immune memory and the rejection of live tumor cells upon re-challenge. However, blocking CRT eliminated this effect and live tumor cell rechallenge was able to grow (Obeid, et al. 2007).

It is likely that all of the above reasons contributed to lack of anti-HMGB-1 Ab removing the anti-tumoral effects of radiation. Nevertheless, the increased tumor growth observed in mice receiving anti-HMGB-1 Ab in combination with radiation compared to mice receiving radiation alone suggests that HMGB-1 in the context of cytotoxic therapy is important in generating an anti-tumoral effect.
6 Conclusions

The objective of this project was to understand how radiation in SMART treatment recruits the immune system against malignant pleural mesothelioma and how this can be used to improve treatments given to patients. Based on the findings of SMART treatment, we postulated that the benefits seen in MPM treatment are largely due to the activation of the immune system. We hypothesized that the differences in survival observed in each subtype of disease was the result of different degrees immune avoidance by releasing more or less HMGB-1; lower patient survival relating to low HMGB-1 release and low immune recruitment and longer patient survival correlating with high HMGB-1 release and increase immune involvement.

First, in vitro radiation studies were used to understand HMGB-1 release. In vitro cell cultures were radiated and the greatest release of HMGB-1 tended to be exhibited by epithelioid cell lines around 24 to 48 hours after receiving radiation. Furthermore, the release of HMGB-1 tended to correlate with necrotic cell death which similarly increased 48 hours after radiation treatment. HMGB-1 release is likely related to the loss of membrane integrity in uncontrolled cell death. The mouse cell line AE-17-OVA behaved similarly, allowing this effect to be modelled in the in vivo mouse model. We therefore confirmed that γ-radiation is able to induce cell death and subsequent HMGB-1 release in vitro in human and mouse cell lines.

MPM patient samples were used to correlate HMGB-1 expression with patient survival. While untreated and SMART treated patients experienced a significant difference in survival, their HMGB-1 staining remained similar. HMGB-1 immunohistochemical staining alone may not be representative of the immunological complexity of clinical cases and therefore alone is unlikely to be a predictor of patient outcome.
In vivo modelling of disease and treatment yielded some noteworthy data. Radiated tumors grew more slowly than untreated tumors. Interestingly, radiation resulted in a release of HMGB-1 into the blood stream 2 days after the completion of local radiation therapy. Radiated tumors also stained more intensely for HMGB-1 than untreated tumors by immunohistochemistry. Furthermore, radiation treatment correlated with T-cell recruitment, suggesting an immunogenic role for radiation by HMGB-1 release.

From a basic science standpoint, this project sheds light on the intricacy of the immune system during radiation treatment. HMGB-1, a typically pro-inflammatory molecule, is shown to be concentrated in the serum of tumor bearing untreated and treated mice. However, in untreated mice, there is a distinct lack of tumor-infiltrating T Cells. Conversely T Cell numbers increase in radiated mice. Despite the presence of HMGB-1 in the serum, there is a substantially different effect between treated and untreated mice. HMGB-1 prominently plays a dual role in each system and the setting in which it is released is likely very important.

The goal of this project was to translate findings into clinical practice. In vivo data suggests that radiated tumors release HMGB-1 by necrosis and trigger an anti-tumor T Cell response. We support the idea that radiation is effective outside of direct cytotoxic effects and that patients can benefit from immune involvement. Radiation-induced immunogenicity warrants further investigation and supports the potential benefit of immunomodulating therapy in combination with high dose radiation therapy.
7 Limitations

A challenge in the in vitro cell death experiments of the study is the lack of observation after 48 hours. Further time points were observed however in a cell culture model, the cells quickly overgrew the flask. Furthermore, measuring media ELISA meant that media could not be replaced as it would change the HMGB-1 concentration. After 48 hours, cell death occurred in all cell lines of both untreated and radiated samples due to overgrowth, build-up of metabolites, and lack of nutrients. Furthermore, cell death staining was performed using Annexin V and eFluor viability stain for assessing apoptosis and necrosis. However, there are many other forms of cell death including senescence and necroptosis that may contribute to HMGB-1 release. Nevertheless, the trends seen up to 48 hours were important foundations for the in vivo aspects of this project.

A weakness of this study was that each mouse was sacrificed at the point of measurement of HMGB-1, thus only one measurement exists per mouse. It may be beneficial to be able to draw serum samples over time to measure how serum HMGB-1 changes over time in each mouse. Limited mouse blood volume and the high volume required for the ELISA assay necessitated only one blood draw per mouse. Thus larger animals may be necessary to draw blood over time to correlate individual serum HMGB-1 and tumor size.

In an effort to translate basic science findings into the clinic, we believe that a more clinically relevant mouse model could be used. Subcutaneously injected MPM cells was used in order to easily measure tumor growth and target radiation. However, in observing AE-17-OVA in vivo, the tumor remains encapsulated and does not spread to other areas of the body. Furthermore, in other studies from our lab (unpublished data), we have found that aggressive surgery is often
curative. These features differ from clinical MPM which may spread into the chest wall contralaterally. In addition surgery alone in humans is not usually curative and does not offer any increase in patient survival. While posing significant challenges, an intrapleural tumor model may better display the pertinent aspects of disease in patients.

An inherent weakness in this study is the use of cell lines to simulate MPM. Re-implantation of the cell line AE-17-OVA into syngeneic mice may not yield the same microenvironment as spontaneously developed MPM. Where asbestos requires much time and inflammation to develop a tumor, tumor implantation is immediate and may lead to a less relevant microenvironment. Asbestos leads to uncontrolled inflammation which slowly selects for non-immunogenic cancer cell clones. Conversely, injection of one million tumor cells may overwhelm the immune system by sheer cell number rather than immune evasion. Supporting this idea is the information that AE-17-OVA is transfected with the chicken ovalbumin peptide which has been reported as being an immunogenic antigen that can be rejected by some hosts. Our lab and others have found that injecting high cell numbers supports tumor formation in almost 100% of hosts while the rejection rate tends to increase when injecting lower cell numbers.
8 Future Directions

An important future experiment involves the analysis of cytoplasmic HMGB-1 in tissue stains. While nuclear staining may be important for understanding expression levels, HMGB-1 passes through the cytoplasm before being released to the extracellular milieu during necrotic cell death. While the Aperio algorithm did not offer an accurate means of quantifying cytoplasmic staining, it would be beneficial to work with pathologists in order to assess the role of cytoplasmic HMGB-1 staining in radiation-induced immunogenicity in MPM.

One significant experiment in this project was the generation of hybridoma anti-HMGB-1 Ab. Sufficient Ab was generated for the initial blocking experiment with three groups: 1) No treatment, 2) Radiation and anti-HMGB-1, and 3) Radiation only. However, insufficient Ab was generated for the inclusion of a necessary control. Thus, we would like to explore future experiments in which an additional group is included: 4) anti-HMGB-1 Ab alone. This would ensure that the observed trend is not due to just anti-HMGB-1 but rather a combined effect of LRT and anti-HMGB-1.

To compare epithelioid and sarcomatoid subtypes further, in vivo comparisons could be made between AE-17-OVA and a mouse sarcomatoid cell line. If radiating sarcomatoid tumors results in less HMGB-1 release than radiating epithelioid AE-17-OVA tumors, this could explain the lower survival observed in patients with sarcomatoid MPM. This would be able to confirm the hypothesis that the sarcomatoid MPM patients release less HMGB-1 in response to radiation and therefore do not recruit the immune response as intensely as epithelioid disease.

In vivo HMGB-1 release was predicted to occur as a result of necrosis of tumor tissue. To confirm the mode of cell death that relates to HMGB-1 release, tumor tissue could be stained for
apoptosis and necrosis markers as well as HMGB-1 by immunofluorescence. Co-localized staining of necrosis and HMGB-1 markers would indicate that HMGB-1 release by radiation is indeed mediated by necrosis. Furthering this, an important area of future study is the measurement of multiple other forms of cell death that may contribute to HMGB-1 release. Starting with *in vitro* studies, this could be extended to animal tissue staining.

The cell line AE-17-OVA was used as the subcutaneous MPM model. The transfection of this cell line with OVA chicken peptide gives rise to the potential for tracking tumor specific responses against OVA. As OVA tetramers have been used in our lab, a future experiment could involve staining tumor infiltrating lymphocytes with OVA tetramer, thus identifying whether the infiltrating T Cells are indeed tumor specific.

To achieve a more clinically relevant model, it would be beneficial to establish an intrapleural model of MPM. As discussed above, there are some weaknesses with using a subcutaneous model of MPM. Mapping growth may be addressed using MRI, or fluorescently labelled tumor cells that could be imaged *in vivo*. Similar technologies could also be used to target treatment to the tumor.

In further modelling human MPM, future studies could involve mouse MPM developed from asbestos exposure. Spontaneously developed tumors may be less immunogenic than cell lines due to slow selection of cancer cell clones that escape the immune system, potentially better modelling clinical cases of MPM. Spontaneous development is possible as asbestos tumor development in mice has been extensively demonstrated. However, long incubation period and low number of mice that develop tumors after asbestos exposure still poses a potential challenge.
Studying HMGB-1 levels in patient blood is a promising area of future study. While it has been reported that MPM patients have higher serum HMGB-1 than individuals exposed to asbestos without a tumor, we have demonstrated that HMGB-1 serum levels can increase as a result of radiation. Studying HMGB-1 in serum samples of patients before and after radiation could provide more elucidation of HMGB-1’s role in development and treatment in the context of MPM. HMGB-1 levels after treatment could be an indicator of successful treatment.

New DAMPs are constantly being discovered and it is very likely that many DAMPs play a role in radiation-induced immunogenicity. Our earlier studies (unpublished data) found differences in calreticulin expression between radiated and untreated cells. Calreticulin and ATP have been highly studied as danger signals and further study in MPM may yield novel interventions. DAMPs have been suggested to work as redundancy as well as in concert with other DAMPs. Therefore, studying other DAMPs in addition to HMGB-1 may allow for interventions that amplify the immunogenic effect of radiation treatment for MPM.

SMART treatment in patients involves radiation of the tumor mass before surgical resection. Previous methods involved performing surgery followed by radiation. The hypothesis behind SMART’s success in lengthening patient survival was that the tumor was irradiated first, allowing it to release danger signals and antigens before removal. Conversely, previous methods would remove the tumor and subsequent radiation would not serve to activate the immune system as there would be little danger signals or tumor antigen release. Our lab has previously established a SMART mouse model which finds greater survival in mice treated with SMART in comparison to surgery followed by radiation. To further understand if the increase in survival in patients is immune-mediated, HMGB-1 serum levels and tumor infiltrating lymphocytes could be measured after the completion of each treatment. Furthermore, paraffin-embedded patient
samples could be stained by immunohistochemistry or immunofluorescence for CD8$^+$ T Cells whose presence would suggest specific immune-mediated tumor cell killing. SMART treated and surgery then radiation treated patients could be compared.
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Appendices
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