Radiation epigenetically modulates tumor cells and alters activation and function of effector T cells

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ABSTRACT

Radiation is a common therapeutic modality for cancer however it fails to control advanced and malignant disease. As a result, novel approaches that aim to stimulate immune attack of tumors are currently being investigated and approved by the US FDA. It is clear, however, that no single agent will be responsible for achieving long-term control and treatment of cancer and that combination therapies will be required. Previous studies indicate that colorectal cancer cells that survive radiation up-regulate surface expression of cytotoxic T lymphocyte (CTL) relevant proteins including death receptors, cell adhesion molecules and tumor-associated antigens (TAA). The aim of this dissertation was to investigate the effect of non-cytolytic doses of ionizing radiation (IR) on co-stimulatory molecule expression on tumor cells and evaluate the impact of their modulation on effector T-cell biology and tumoricidal activity. Here, several
human tumor cell lines were exposed to various doses of radiation (0-10Gy) and TAA-specific T-cell tumoricidal activity and expression of effector CTL co-stimulatory molecules were evaluated. I found OX40L and 41BBL to be the most consistently upregulated proteins post-IR by flow cytometry. Furthermore, I saw enhanced survival and activation of human CD8+ T-cells exposed to irradiated tumor cells. Importantly, enhanced killing of irradiated tumor cells by TAA-specific CTLs in cytotoxicity assays was reported. Blocking OX40L and 41BBL reversed radiation-enhanced T-cell killing. My data also indicate that expression of 41BBL and OX40L can be epigenetically regulated, as inhibition of histone deacetylases (HDAC) and of DNA methyltransferases (DNMT) resulted in increased OX40L and 41BBL mRNA and protein expression. Furthermore, chromatin immunoprecipitation experiments revealed increased histone H3 acetylation specifically at the 41BBL promoter following irradiation. Last, I began exploring the ability of IR to reverse immune suppression by evaluating the impact of radiation on regulatory T cells (TREGs) that can suppress the function of effector CTLs. I found that RT could reduce TREG numbers likely by altering their phenotype. Overall, this dissertation demonstrates that radiation can be used to make human tumors more immunogenic through epigenetic modulation of genes stimulatory to effector T-cells and that it may also reverse immune suppression by phenotypically altering TREG cells.

INDEX WORDS: radiation therapy, cancer immunotherapy, ionizing irradiation, immunogenic modulation, CTLs, epigenetic, effector co-stimulation, and regulatory T cells.
RADIATION EPIGENTICALLY MODULATES TUMOR CELLS AND ALTERS ACTIVATION AND FUNCTION OF EFFECTOR T CELLS

by

ANITA KUMARI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2015
RADIATION EPIGENETICALLY MODULATES TUMOR CELLS AND ALTERS
ACTIVATION AND FUNCTION OF EFFECTOR T CELLS

by

ANITA KUMARI

Committee Chair: Charlie Garnett-Benson

Committee:  
Susanna F. Greer  
Andrew Gewirtz  
Zhi-Ren Liu

Electronic Version Approved:  
Office of Graduate Studies  
College of Arts and Sciences  
Georgia State University  
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DEDICATION

This dissertation is dedicated to my son Kush Kamal, and my husband Ram. This work would not have been possible without their perpetual love, faith, support, patience and encouragement.
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I would like to express my deepest appreciation to my advisor, Dr. Charlie Benson for guiding, supporting and encouraging me throughout my PhD study. I would like to thank her for her unconditional availability and scientific inputs for every step in the research and providing an excellent laboratory atmosphere that is positive and very supportive, where an independent thought is encouraged but guidance is always available. Benson lab was an environment that allowed me to grow as a scientist and a person all while getting to work with a fantastic group of people. I would like to also thank my committee members Dr. Susanna F. Greer, Dr. Zhi-Ren Liu and Dr. Andrew Gewirtz for their valuable guidance and input through all the years of my research.
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LIST OF ABBREVIATIONS:

Radiation therapy (RT)
Regulatory T cells (Tregs)
Histone acetyltransferases (HATs)
DNA methyl-transferase inhibitor (DNMTi)
Cytotoxic T-cell (CTL)
Trichostatin (TSA)
5-Aza-2'-deoxycytidine (5Aza)
HDAC inhibitors (HDACi)
Adoptive T cell transfer (ACT)
Major histocompatibility complex class I/II (MHC class I/II)
Intercellular adhesion molecule-1 (ICAM-1)
Cancer immunotherapy (CIT)
Colorectal cancer (CRC)
Immunogenic cells death (ICD)
Dendritic cells (DC)
Tumor associated antigens (TAAs)
T cells receptor (TCR)
Histone deacetylases (HDACs)
DNA methyltransferases (DNMTs)
Chromatin immunoprecipitation (ChIP)
Ionizing radiation (IR)
Carcinoembryonic antigen (CEA)
Mucin-1 (MUC1)
Antigen presenting cells (APC)
Immunogenic modulation (IM)
High mobility group box 1 (HMGB1)
IFN g (interferon gamma)
Monoclonal antibodies (mAbs)
Programmed death-1 (PD-1)
Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)
Forkhead Box P3 (FoxP3)
1 INTRODUCTION

1.1 Colorectal Cancer:

Colorectal cancer (CRC) is the second leading cause of cancer related deaths in United States and the five-year survival rate is less than 30% for advanced colorectal cancer (Siegel, Desantis et al. 2014). According to the American Cancer Society more than 100,000 new cases of colorectal cancer, and around 50,000 deaths, are projected to occur in 2015. These numbers clearly demonstrate the urgent need to develop more efficient treatments and new therapeutic regimens for this disease. The standard therapies in advanced stage CRC include surgery, chemotherapy, targeted therapy, and radiation therapy (RT).

RT is an integral part of cancer treatment and it is estimated that almost half of cancer patients undergo radiotherapy (RT) during the course of their illness (Schmid et al., 2012). In rectal cancer, RT is usually given either before or after surgery. In addition, radiation can be given in colorectal patients to palliate symptoms or to people who are not healthy enough for surgery. Radiation is delivered in three different ways to colorectal patients; these are external beam radiation (EBRT), brachytherapy and endocavitary (cancer.org). EBRT is commonly used in the clinic, in which the radiation source is kept outside the body to irradiate tumors. In contrast, brachytherapy employs the use of small pellets of radioactive material that are placed near or within the cancer tissue. Lastly, in endocavitary a small device is introduced, through the anus into the rectum that delivers high intensity radiation. Traditionally, radiation was thought to mediate its effect by direct killing of tumor cells. Accumulating evidence, however, indicates that radiotherapy triggers pro-immunogenic effects depending upon the dose of radiation, which include immunogenic cell death (ICD) and immunogenic modulation (IM)(Galluzzi, Maiuri et al. 2007).
1.2 RT enhances tumor immunogenicity

Immunogenic cells death (ICD) is defined as radiation-induced tumor cell death that results in release of specific signals, which enhance activation and cross presentation of tumor derived antigens by dendritic cells (DC) to T cells (Kroemer, Galluzzi et al. 2013). ICD induces three different molecular signals, which include: (1) the release of tumor associated antigens (TAAs), (2) the transport of calreticulin to cell surface, and (3) secretion of danger signals such as high mobility group box 1 (HMGB1), heat shock protein (HSP70), and ATP. First, novel TAAs (such as carcinoembryonic antigen (CEA) and mucin-1 (MUC1)) released from tumor cells exposed to lethal dose of irradiation are taken up, processed, and presented by DC (Hannani, Sistigu et al. 2011) to T cells. Second, calreticulin (a chaperone protein normally present in the lumen of ER) translocates to cell surface, which acts as a potent “eat me” signal to DCs facilitating phagocytosis of antigens. Third, the passive release of HMGB1 and HSP 70 (Demaria et al., 2005; Tesniere et al., 2008) from dying tumor cells serves as danger signals that enhance DC activation via TLR receptor signaling. Moreover, tumor cell release of ATP following exposure to radiation activates the DC inflammasome, releasing IL-1β (a mediator of inflammatory response (Riteau, Baron et al. 2012). Overall, ICD is a distinct form of tumor cell death, inducing effective adaptive anti-tumor immune responses.

1.3 Immunogenic modulation of tumor cells

Apart from ICD, radiation causes immunogenic modulation (IM), which is defined as radiation-induced phenotypic changes on the tumor cell surface that can enhance cytolytic immune cell activity (Kwilas, Donahue et al. 2012, Wattenberg, Fahim et al. 2014). Radiation
increases expression of major histocompatibility complex class I and II (MHC class I/II; (Sanda, Restifo et al. 1995)), intracellular adhesion molecule-1 (ICAM-1; (Vereecque, Buffenoir et al. 2000); (Garnett, Palena et al. 2004); (Reits, Hodge et al. 2006); (Ifeadi and Garnett-Benson 2012)), co-stimulatory molecules (B7-1) and death receptors (Fas, DR4/DR5; (Ifeadi and Garnett-Benson 2012)) on tumor cells. MHC class I is required for antigen presentation to T cells and B7-1 serve as a co-stimulatory signal to T cells (Lanier, O'Fallon et al. 1995). Furthermore, ICAM-1 binds to lymphocyte function-associated antigen (LFA3) and enhances the interaction between tumor cells and immune cells and subsequent cytolysis of target cells (ref). Importantly, radiation also increases expression of vascular cell adhesion molecule (VCAM-1; (Lugade, Moran et al. 2005)) and chemokine CXCL16 enhancing infiltration of T cells into the tumor (Matsumura, Wang et al. 2008). These findings support the ability of radiation to modulate the tumor milieu, inducing a better environment for immune attack.

Despite these pro-immunogenic effects, radiotherapy alone fails to eradicate metastasis and to generate effective and long lasting T cell responses in patients. This outcome seems to be the result of multiple immunosuppressive strategies that have evolved in tumors, which reduce the optimal immune activities of radiotherapy in patients. Thus, additional interventions are required in conjunction with RT. Recently several studies have reported that RT enhances antitumor immune responses, particularly when combined with cancer immunotherapy (CIT) strategies, however the underlying mechanisms and optimal way to utilize RT specifically for this purpose are still unclear (Postow, Callahan et al. 2012, Golden, Demaria et al. 2013)
1.4 Cancer immunotherapies

There are several advantages of CIT over standard therapies. Cancer immunotherapies offer the advantage of being systemic and rely on activating the host’s own immune response. Once activated, immune cells can effectively eliminate even microscopic lesions and disseminated metastasis (Dunn, Bruce et al. 2002). Moreover, memory cells can prevent the recurrence of cancer (Dimberu and Leonhardt 2011). Since both chemotherapy and radiation kill actively dividing cancerous cells; cancer stem cells, which usually stay in a quiescent state, are resistant to standard therapies (Moore and Lyle 2011). CITs involve the use of different strategies to enhance and stimulate antitumor immune responses, including monoclonal antibodies, therapeutic vaccination, and T cell transfer based immunotherapies (Dimberu and Leonhardt 2011).

Monoclonal antibodies (mAbs) can have been used therapeutically in diverse ways. In the last decade, the U.S. Food and Drug Administration (FDA) has approved a dozen of mAbs to treat both hematologic and solid malignancies (Scott, Wolchok et al. 2012). mAbs have less side effects than other immunotherapies; however, the rapid clearance from the body requires continued infusion of patients. Antagonist mAbs interfere with tumor cell signaling and tumor stroma interaction and can also cause antibody dependent cell mediated cytotoxicity of specific tumor targets. Antagonist mAbs are also currently being employed as “check-point blockers” to block inhibitory signals into responding T cells. Recently the US FDA has approved two such drugs, which block co-inhibitory receptors on activated T cells. Co-inhibitory receptors, such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA4; (Hodi, O'Day et al. 2010)) and programmed death-1 (PD-1), control unwanted and harmful responses directed against self-antigens, and play an important role in preventing autoimmunity (Sharpe, Wherry et al. 2007).
Within the tumor microenvironment they limit the generation of effective antitumor immune responses. Therefore, current approaches focus heavily on blocking these molecules CTLA-4 and PD-1 to generate more effective antitumor response. Agonist mAbs can be used in a different manner from antagonist Abs to stimulate and promote positive signals into responding T cells. Several preclinical studies are investigating how to combine radiotherapy with T cell inducing immunotherapy such as OX40 and 41BB agonist (Gough, Crittenden et al. 2010, Belcaid, Phallen et al. 2014) Varying degree of benefit have been achieved in phase I clinical trials using 41BB agonist abs. A trial was suspended due to hepatotoxicity associated at higher doses (Ascierto, Simeone et al. 2010). In addition, an antibody to human OX-40 antibody (MEDI6469) that targets an activating receptor on T cells (Curti, Kovacsovics-Bankowski et al. 2013) is under study in a phase I clinical trial study of 30 patients with advanced cancer. This study has reported some clinical activity in 12 out of 30 patients.

Another commonly investigated CIT approach is vaccination, which employs the use of viral vectors, whole tumor cells, peptides, proteins or dendritic cells to elicit host immune responses to tumors (Chiang, Kandalaft et al. 2011). Sipuleucel-T/Provenge (dendritic cell based vaccine), for the treatment of metastatic castration-resistant prostate cancer was the first cell-based immunotherapy to be approved by the FDA (Sims 2012). Therapeutic cancer vaccines, however, have limited success as compared to preventative vaccines, as tumors can actively suppress the immune system. To avoid tumor-mediated suppression of immune cells another immunotherapy, , Adoptive T Cell Transfer (ACT) therapy, involves removal of tumor infiltrating lymphocytes (TILs), expanding them \textit{ex vivo}, and re-infusing them back into the patient (June 2007, Rosenberg and Restifo 2015). The major challenge with this personalized therapy is that it is labor intensive and very expensive. Moreover, infusion of T cells induces
severe cytokines storm and autoimmunity (Tey 2014). In spite of some of the negative side-effects, immunotherapies are promising new strategies against cancer and complete responses have been reported (Acharya and Jeter 2013, Tan and Quintal 2015) and many of the side-effects can be medically managed. At present, however, CIT strategies used clinically seem to benefit only a minority of patients and it is unclear why they work so effectively in some and not others. Further, it seems clear that the most effective therapeutic responses have been observed when CITs were combined with standard therapies. Thus, it seems likely that no single modality is sufficient to successfully eliminate and control advanced stage cancer. Moreover, most successful CIT strategies have been limited to melanoma and lung cancers. New combination approaches are needed to enhance the efficacy of CITs and expand its usage in other types of cancers apart from melanoma. In this regard, the diverse pro-immunogenic effects of RT can be exploited in combination with CIT. In order to achieve the maximal synergistic effect, it is imperative to fully investigate and understand radiation-mediated cellular and molecular changes within tumors.

1.5 Role of CD8 T cells:

At the center of almost all CIT strategies is the desire to raise CD8+ cytotoxic T cells that recognize antigens expressed by transformed cells. CD8+ T cell require two signals to become fully activated; the first signal involves recognition of peptides, displayed on MHC-I on antigen presenting cells (APC), by the T cell receptor (TCR) (Mueller, Jenkins et al. 1989). A second signal comes from the ligation of co-stimulatory molecules, B7-1/B7-2 on APC, with CD28 receptors on naïve T-cells, this signal drives full activation of T cells prompting proliferation and IL-2 secretion (Driessens, Kline et al. 2009). In addition, other costimulatory molecules such as
ICOSL, CD70, OX-40L and 4-1BBL expressed by APC, engage cognate receptors on T cells and augments and amplifies expansion, differentiation and effector function of cytotoxic T cells (Kober, Leitner et al. 2008). Conversely, co-inhibitory molecules (CTLA-4 and PD-1) inhibit the effector activity of T cells. Therefore, to elicit an effective antitumor immune response, the immune system needs to recognize tumor antigens by TCR in conjunction with appropriate co-stimulation (Kroczek, Mages et al. 2004, Jensen, Maston et al. 2010).

Cytotoxic T cells are crucial in killing tumor cells with high specificity and efficiency (Finke, Rayman et al. 1990). CTLs kill target cells by one of the three distinct mechanisms. The first is mediated when Fas ligand, which is expressed on CTLs, binds to its receptor (Fas/CD95) on target cells and triggers apoptosis by activating the caspase pathway (Nagata 1996). The second involves the use of perforin and granzyme. Perforin creates pores in the cell membrane and facilitates the entry of granzymes into the target cells and these lytic granules then lyse the target cells (Lowin, Peitsch et al. 1995). Lysis by granzymes can proceed through either a caspase dependent or caspase independent manner (Trapani and Smyth 2002). The third pathway involves the release of cytokines such as TNFα and IFNγ from CTLs (Ghanekar, Nomura et al. 2001, Wohlleber, Kashkar et al. 2012). TNFα binds to its cognate receptor and triggers apoptosis. IFNγ induces MHC-I and Fas expression on target cells, thus enhancing antigen presentation of endogenous peptides and apoptosis, respectively (Andersen, Schrama et al. 2006).

1.6 **Immunosculpting and tumor immune escape:**

Tumors employ multiple strategies to inhibit attack by immune cells and ultimately evade immune surveillance. For example, tumors can decrease antigen presentation, down-regulate
co-stimulatory molecule expression, or recruit immunosuppressive cells into the tumor microenvironment (Marincola, Jaffee et al. 2000, Fujiwara, Higashi et al. 2004, Liyanange et al. 2002). Decreased antigen presentation and processing can occur via several ways, such as loss of antigens, downregulation of TAP (transports degraded peptides into ER) and LMP2/7 unit (proteasome subunits that help in degradation of peptides), leading to reduced processing of antigen epitopes (Wang, Hu et al. 1993, Benitez, Godelaine et al. 1998). In addition, loss of β2 microglobulin and heavy chain of MHC-I leads to diminished transport of MHC-I from ER through the golgi to the surface. Therefore, both processing and presentation of MHC-I is impaired in tumor cells, leading to tumor escape (Drake, Jaffee et al. 2006). Secondly, tumor cells can up-regulate co-inhibitory (PD-1, CTLA4) and down regulate co-stimulatory molecules (B7-1) (Fujiwara, Higashi et al. 2004). PD-1 is a co-inhibitory receptor on T cells and its ligand is PD-L1. Ligation of PD-1 promotes reduces TCR activation and induces apoptosis of CD8-T cells (Dong, Strome et al. 2002). Importantly, PD-L1 expression is increased in several tumors (Thompson, Gillett et al. 2004, Soliman, Khalil et al. 2014) and is correlated with poor prognosis (Thompson, Gillett et al. 2004). In addition, secretion of soluble ligands such as MICA and MICB can also occur in some tumors and block lymphocyte activation (Groh, Wu et al. 2002). Consequently, because of weak immunogenicity (low expression of tumor associated antigens and MHC-I) and low expression of co-stimulatory molecules on tumor cells, CD8+ T cells undergo poor activation against many tumors (Demaria, Ng et al. 2004).

Recruitment of immunosuppressive cells such as T-regulatory cells (T\textsubscript{REG}) and MDSCs into the tumor further suppresses the effector function of T cells (Jacobs, Nierkens et al. 2012, Lindau, Gielen et al. 2013). In addition to expression of PD-L1, MDSCs upregulate inducible nitric oxide synthase (iNOS), which metabolizes L-arginine, and produce nitric oxide (NO) that
induces apoptosis in activated T cells (Saio, Radoja et al. 2001, Gabrilovich and Nagaraj 2009). Another important immunosuppressive cell type in tumor immunity is the CD4+ T\textsubscript{REG} (Baecher-Allan, Brown et al. 2001, Brunkow, Jeffery et al. 2001). T\textsubscript{REG} infiltration is associated with poor prognosis and reduced survival in patients (Curiel, Coukos et al. 2004, Clarke, Betts et al. 2006). T\textsubscript{REGS} can inhibit killer T cell function by releasing suppressive cytokines (TGF\beta and IL-10) or indirectly by inhibiting antigen presenting cells (Yamagiwa, Gray et al. 2001, Ghiringhelli, Puig et al. 2005, Schmidt, Oberle et al. 2012).

1.7 Role of T\textsubscript{REGS} in cancer

T\textsubscript{REGS} comprise 1-3% of total peripheral CD4+ T cells in humans and 5-10% of mouse splenocytes (Peng, Li et al. 2008). CD4+ T\textsubscript{REGS} express high levels of CD25+ (interleukine-2 receptor) and the forkhead box P3 (FOXP3) transcription factor; FOXP3 is regarded as a specific marker of T\textsubscript{REGS} as it controls transcription of several genes responsible for the suppressive function of T\textsubscript{REGS} (Baecher-Allan, Brown et al. 2001, Brunkow, Jeffery et al. 2001). T\textsubscript{REGS} are important cells of the immune system as they inhibit and control unwanted immune responses against self-antigens, thereby protecting us from autoimmune diseases. On the other hand, T\textsubscript{REGS} inhibit the generation of anti-tumor T cell responses, thereby boosting cancer escape from immune-surveillance (Curiel 2007). In this regard, tumors can secrete different chemokine’s, which recruit T\textsubscript{REGS} into the tumor microenvironment (Wang 2006, Wolf, Rumpold et al. 2006). These recruited T\textsubscript{REGS} suppress the effector function of cytotoxic T cells present within tumor (Curiel 2007).

T\textsubscript{REGS} are comprised of natural T\textsubscript{REGS} (nT\textsubscript{REGS}) and induced T\textsubscript{REGS} (iT\textsubscript{REGS}), both subset of T\textsubscript{REGS} infiltrate into tumors (Baumgartner and McCarter 2009, Bour-Jordan and
Bluestone 2009). nTREGS are thymically derived, constitutively express FOXP3, and suppress effector T cells in a contact-dependent manner, using TRAIL (Ren, Ye et al. 2007) and/or perforin and granzyme (Gondek, Lu et al. 2005). In contrast, iTregs are generated in the periphery from naïve CD4+ T cells under tolerogenic cytokines condition and suppress effector CD8+ T cells without requiring contact by secreting suppressing cytokines such as IL-10 and TGF-β (Loser, Apelt et al. 2007, Strauss, Bergmann et al. 2007). Apart from direct inhibition, TREG suppress effector T cell by inhibition of dendritic cells activation. TREGS can increase indoleamine 2,3-dioxygenase (IDO) expression in DCs by CTLA4 signaling. IDO is enzyme which degrades tryptophan to kynurenine. The depletion of tryptophan causes cell cycle arrest of T cells (Schmidt, Oberle et al. 2012).

Increased numbers of TREGS has been found in different cancers; Woo and colleagues have reported increased number of intra-tumoral TREGS in lung and ovarian cancers (Clarke, Betts et al. 2006, Wolf, Rumpold et al. 2006). In particular, the ratio of CD8+ T cells to TREG cell infiltration in tumors is crucial in determining the prognosis (Curiel, Coukos et al. 2004). In a report by Ling and colleagues, both intra-tumor and systemic accumulation of TREGS has been reported in colorectal cancer patients (Ling, K.L., 2007). In addition, the proportion of TREGS in peripheral blood of CRC patients was four times higher than the control. Additionally, an increased frequency of TREGS was reported within tumor (19.2) as compared to normal colon tissue (9%) (Ling, Pratap et al. 2007). Overall, intra-tumoral infiltration of TREGS is a major obstacle to successful immune control of tumors. Based on these observations, depletion of TREGS has become an attractive approach to enhance antitumor responses (Jones, Dahm-Vicker et al. 2002, Li, Hu et al. 2003).
1.8 Current major gaps in our knowledge:

**I. The mechanisms contributing to enhance CTL activity in tumor cells surviving RT and the impact of irradiated tumor cells on CTL biology.**

IR has the ability to modulate gene expression in tumor cells in ways that make them better stimulators of immune cell function (Wattenberg, Fahim et al. 2014). Garnett el al have reported that sub-lethal irradiation of colorectal carcinoma cells (CRC) lines results in enhanced lysis by tumor specific CTLs following 10Gy of irradiation (Garnett, Palena et al. 2004). Moreover, CTL killing was MHC restricted as LS174 cells which lack HLA-A2 are not killed by these HLA-A2-restricted CTLs. Other studies have also reported enhanced killing post RT against head neck squamous carcinoma (Gelbard, Garnett et al. 2006) and prostate carcinoma cells (Chakraborty, Wansley et al. 2008). Previous studies from our lab reveal that SW620 cells are effectively killed by CTL after RT. Though these cells significantly increase expression of the death receptor Fas, this pathway is non-functional in these cells (Ifeadi and Garnett-Benson 2012). Moreover, the increased lysis by CTLs does not obviously correlate with either enhanced expression of MHC-I, nor expression of the tumor antigens CEA or MUC-1. *Thus one part of this study was designed to investigate the underlying mechanisms of enhanced killing of irradiated tumor by CTLs. Moreover, while enhanced killing by CTLs has been reported, no other aspects of T cell biology have been reported following interaction with irradiated tumor cells.*

**II. How RT modulates gene expression in cells receiving sub-lethal doses:**

Previous studies demonstrate that sub-lethal doses of radiation alter the expression of immune relevant genes within tumor cells making tumor cells more susceptible to cytotoxic T-
Several mechanisms have been described for how IR can modulate expression of immune relevant genes in tumor cells. While LD-RT (<2Gy) has been shown to reduce NF-κB via proteasome inhibition, hypofractionated doses of IR (2-25Gy) have conversely demonstrated increased activity of NF-κB. NF-κB can be activated in different cell types within an irradiated tumor mass including the tumor cells, stromal cells, cells of the vasculature, and immune cells. In tumor cells, genotoxic stress induced NF-κB activation is initiated by DNA double strand breaks (DSB) in the nucleus and can stimulate gene expression. NF-κB activation has been reported to occur in this manner in diverse tumor cell lines in an ataxia telangiectasia mutated (ATM) dependent pathway, mostly at doses above 3Gy. ATM signaling is also a well-known activator of p53. p53 target genes, however, are related mostly to cell cycle, apoptosis and DNA repair pathways (reviewed in (Criswell, Leskov et al. 2003)). While most p53 inducible genes are not typical immune relevant genes, apoptosis inducing death receptors could be important for immune cell mediated signals transmitted from death ligands on cytotoxic immune cell. Unfortunately, less than 50% of tumor cells retain functional p53.

Epigenetic changes play an important role in regulating the expression of many genes through histone modifications and DNA methylation, and radiation has recently been reported to influence these pathways. For example, radiation has been shown to inhibit the expression of histone deacetylase 1 (HDAC1) and HDAC2 [37]. HDAC enzymes remove acetyl groups from histones and often inhibit transcription of genes [Kadosh D; Wang Z; Glozak MA]. In several tumors, HDACs are up regulated (Ropero S, 2007 Mol Oncol 1(1): 19–25; Sharma N, 2010 Br J Urol Int; in press).[Barna-de-Zahonero B 2012, Marks P 2001]. Recent studies have shown that HDAC inhibition can have immune-modulatory properties, such as increasing expression of
HLA-DR, ICAM-1 and B7-2 in cancer cell lines (Takahiro Maeda, blood 2013). HDAC inhibitors (HDACi) have been shown to induce a potent antitumor response (Saunders N, Cancer Res. 1999; 59:399-404, Redner RL, Blood. 1999; 94:417-428).

DNA methylation also plays an important role in regulation of genes (Razin A, 1991) and IR has recently been reported to induce DNA hypomethylation and alter gene expression in CRC cells treated with 2-5Gy (Bae, Kim et al. 2015). DNA methyl transferase enzyme (DNMT1) adds a methyl group to cytosine residue and causes silencing of genes (Gal-Yam EN 2008). DNA hyper-methylation occurs at CpG islands and more than half of human promoter genes contain CpG islands (Yam, G., Annu. Rev. Med. 2008. 59:267–80). Aberrant methylation is found in colorectal cancer such as hyper methylation of mismatch repair gene (hMLH1) (Chiam, K. PLoS ONE September, 2011). In addition, promoter hyper-methylation and genome-wide hypo methylation alters genes expression in colorectal cancer (Cheng, Y.W., Clin Cancer Res 2008; 14:6005-6013). 5-Aza-2′-deoxycytidine (5Aza) is a DNA methyl-transferase inhibitor (DNMTi) that is incorporated into DNA and leads to rapid loss of DNA methyl transferase activity (Kane MF, Loda M, Gaida GM, et al. 1997). 5Aza has been reported to alter the expression of other immune relevant genes, such as MHC I, MHCII, CD80, CD86 and CD40 on chronic lymphocytic leukemia cells has also been reported (Dubovsky, J.A., Clin Cancer Res 2009; 15:3406-3415). However, it is remains unclear if radiation modulates expression of immune-relevant genes via epigenetic mechanisms. This could be important for combination radiation-immunotherapy approaches, as epigenetic changes can be maintained for quite some time within the cell population. **Thus, a major gap in our knowledge is how radiation modulates expression of immune relevant genes in tumor cells that survive radiation.**
III. The impact of RT on CD4+ T\textsubscript{REG} cells versus CD4+ non-T\textsubscript{REG} cells

Relatively few and contradictory data exist in the literature regarding the effect of irradiation on T\textsubscript{REGS} and there are many gaps in our knowledge about the impact of RT directly on T\textsubscript{REGS}. The absolute number and frequency of T\textsubscript{REGS} was reported to be decreased in naïve mice receiving low dose total body irradiation (Liu, Xiong et al. 2010). However, studies by others suggest that regulatory T cells are more resistant to radiation than non-T\textsubscript{REGS} (Billiard, Buard et al. 2011, Balogh, Persa et al. 2013). Thus it is not clear how hypofractionated doses of IR (2-10Gy) directly impact human T\textsubscript{REGS}. Further, it is unclear if sub-lethal irradiation of tumors can indirectly impact human T\textsubscript{REG} number and function through modulation of tumor cells as these reports were made in non-tumor bearing mice. For example, do irradiated tumor cells secrete cytokines or chemokine’s that could decrease conversation and/or recruitment of T\textsubscript{REGS} within tumors? Do irradiated tumor cells modulate the expression of ligands that can alter the biology of T\textsubscript{REGS} and repress T\textsubscript{REG} suppressive function?
Human colorectal carcinoma cells surviving radiation modulate expression of both OX40L and 41BBL, and promote CTL activation, survival and tumoricidal effector function.

2.1 ABSTRACT:

Sub-lethal doses of ionizing radiation (IR) can alter the phenotype of target tissue by modulating genes that make tumor cells more susceptible to T-cell–mediated immune attack. Previous studies indicate that colorectal cancer cells responded to radiation by up-regulating surface expression of CTL relevant proteins including death receptors, cell adhesion molecules and tumor-associated antigens (TAA). The aim of this study is to investigate the effect of noncytolytic doses of ionizing radiation on co-stimulatory molecule expression on tumor cells and the impact of their modulation on effector T-cell biology and tumoricidal activity. Here, several human tumor cell lines were exposed to various doses of radiation (0-10Gy) and TAA-specific T-cell tumoricidal activity and changes in the expression of effector CTL co-stimulatory molecules were evaluated. One to seven days post-IR, changes in expression of OX40L and 41BBL, as well as expression of other molecules stimulatory to effector T-cell activity (ICOSL and CD70), was examined by flow cytometry. We found OX40L and 41BBL to be the most consistently upregulated proteins post-IR, and increased transcripts for both genes were detected 24 and 48hr post-IR by quantitative RT-PCR. Furthermore, we saw enhanced survival and activation of human CD8+ T-cells exposed to irradiated tumor cells. Importantly, expression of these gene products correlated with enhanced killing of irradiated tumor cells by TAA-specific CTLs in cytotoxicity assays. Blocking OX40L and 41BBL reversed radiation-enhanced T-cell
killing of tumor targets as well as T-cell activation. Overall, results of this study suggest that human tumor cells surviving radiation are more amenable to immune attack by modulating expression of molecules that are co-stimulatory to effector T cells.

2.2 INTRODUCTION:

In addition to surgery and chemotherapy, radiotherapy (RT) is a common modality in cancer treatment. Many cancer patients undergo RT during their course of illness, but as a definitive therapy RT can fail to control local tumor growth as tumor cells acquire mutations during development that inhibit cell death by radiation (Baskar, Lee et al. 2012). RT also fails to control systemic disease, such as undetectable metastasis, and many cancer patients experience disease recurrence and death (Hodge, Guha et al. 2008). Moreover, radiation kills both malignant cells and non-malignant cells, resulting in significant dose limiting toxicities when used as a single agent (Yeoh 2008, Kumar, Juresic et al. 2010). It has becoming increasingly clear that no single modality will be effective against advanced stage cancers. Cancer immunotherapy (CIT) is emerging as an attractive therapeutic option and many standard cancer therapies, such as chemotherapy and radiation, rely on induction of functional immune cells for efficacy (Demaria, Kawashima et al. 2005, Zitvogel, Apetoh et al. 2008). Indeed, combination CIT and RT is more effective in treating metastatic and reoccurring cancers than either of the therapies alone (Hillman, Xu et al. 2003, Chakraborty, Abrams et al. 2004, Chi, Liu et al. 2005, Kaufman and Divgi 2005, Newcomb, Demaria et al. 2006, Yokouchi, Chamoto et al. 2007, Lechleider, Arlen et al. 2008, Ferrara, Hodge et al. 2009, Gough, Crittenden et al. 2010). Disappointingly, synergy has yet to be achieved in many combination CIT and RT clinical studies. The reasons for this unexpected lack of clinical response have yet to be resolved and the
relevant molecular details of combining RT with CIT are just beginning to be revealed. Fully understanding the role of RT in tumor immunity will have a major impact on the treatment of cancers combining these modalities.

It is well established that the most effective cancer immunotherapy (CIT) strategies generate tumor-associated antigen (TAA)-specific CD8+ CTLs capable of killing tumor cells (Blattman and Greenberg 2004, Dunn, Old et al. 2004). To elicit an effective immune response against tumors, the immune system needs to recognize TAA presented to the TCR within MHC Class-I molecules, in conjunction with appropriate co-stimulation (Kroczek, Mages et al. 2004, Jensen, Maston et al. 2010). Interestingly, anti-tumor immune cells can be detected in most cancer patients, and therapies that further induce large numbers of anti-tumor CTLs do not consistently translate into objective clinical tumor responses. Several possibilities for this include weak immunogenicity of TAA, low expression of co-stimulatory molecules on tumor cells and APCs, and/or secretion of suppressive molecules or cells. Thus, it takes more than increasing tumor-specific CTL numbers to control malignant cells and eliminate cancer.

Local tumor irradiation has been shown to generate tumor-specific CTL and enhance anti-tumor immune responses (Friedman 2002, Chakraborty, Abrams et al. 2003, Chakravarty, Guha et al. 2006, Formenti and Demaria 2008, Takeshima, Chamoto et al. 2010). Sub-lethal doses of ionizing radiation (IR), for example, have been reported to up-regulate expression of immune-stimulatory proteins in various tissue types both in vitro and in vivo (Friedman 2002, Garnett, Palena et al. 2004). We previously reported that exposure of human carcinoma cell lines to sub-lethal radiation results in enhanced susceptibility to lysis by tumor specific cytotoxic T cells (CTLs) (Garnett, Palena et al. 2004, Gelbard, Garnett et al. 2006). Significantly enhanced killing by CEA-specific CD8+ CTLs was observed in five of five colorectal carcinoma
(CRC) cell lines exposed to a single dose of 10Gy radiation. T-cell killing was MHC-restricted, as irradiated CEA+/HLA-A2- colon cells were not killed. Furthermore, enhanced attack by CTLs in head and neck squamous cell carcinoma (Gelbard, Garnett et al. 2006) and prostate carcinoma (Chakraborty, Wansley et al. 2008) suggests the functional enhancement is not limited to a single antigen-specificity or cancer type. More recently we reported that irradiation of human tumor cells imparts enhanced and sustained susceptibility to multiple death receptor signaling pathways (Ifeadi and Garnett-Benson 2012); however, the differences in magnitude of lysis among the cell lines does not correlate with altered expression of death receptors, nor altered surface expression of MHC-I, ICAM-1 or TAAs (Garnett, Palena et al. 2004). Thus, the mechanism of enhanced CTL killing against human carcinoma cells is unclear and surprisingly few studies focus on understanding the effect of radiation-induced changes in tumor cells on CTL effector activity.

As self-antigens, tumor derived antigens often induce insufficient co-stimulation, resulting in tolerance. Antigen presentation in a toleragenic or immunosuppressive environment where robust costimulation is not present leads to sub-optimal immune responses such as T-cell anergy. T-cell co-stimulatory agonists can program T cells encountering these non-immunogenic antigens to expand and develop anti-tumor effector activities (Adler and Vella 2013). As a result, strategies for improving positive co-stimulation and reversing negative regulation are currently very attractive therapeutic approaches for cancer therapy. In this regard, the co-stimulatory TNFSF members 41BB ligand (41BBL/TNFSF9/CD137L) and OX40 ligand (OX40L/TNFSF4/CD134L/CD252) represent important regulators of effector CTL activity, and lack of signaling through these molecules results in sub-optimal CTL activity (Kroczek, Mages et al. 2004, Watts 2005, Mescher, Curtsinger et al. 2006, Kober, Leitner et al. 2008). In tumor bearing mice, intratumoral OX40 activation increases CD40 expression on T cells and increases
the effector memory T cells (T_{EM}) subset (Burocchi, Pittoni et al. 2011). 41BBL (TNFRSF9/CD137) costimulation of tumor-specific T cells is important for T-cell proliferation, cytokine production and activation. 41BBL transfected DCs elicit more effective responses and enhanced CTL killing of tumor cells, due to increased expression of perforin and IFN-γ (Song, Guo et al. 2012). In recognition of the importance of these pathways to generating effective antitumor immunity, clinical studies have started to evaluate the effectiveness of humanized agonist antibodies to both OX40 and 41BB (Ascierto, Simeone et al. 2010, Garber 2011, Kohrt, Houot et al. 2011, Weinberg, Morris et al. 2011). Engagement of OX40 and 41BB by agonist (activating) antibodies increases tumor immunity, resulting in long-term survival in a number of murine tumor models (Melero, Shuford et al. 1997, Lee, Myers et al. 2004, Watts 2005, Murata, Ladle et al. 2006). These costimulatory signals may be particularly important for effective responses against self-antigen such as those expressed by many tumor cells. In the absence of these costimulatory signals anti-tumor effector T-cells may be rendered anergic.

Our previous studies suggest that sub-lethal doses of radiation cause altered expression of genes within tumor cells, making tumor cells more susceptible to CTL-mediated lysis (Garnett, Palena et al. 2004). Subsequently, we have found that radiation-enhanced CTL killing of tumor cells is independent of death receptor pathways (Ifedai and Garnett-Benson 2012). The present study was designed to test the hypothesis that enhanced killing of human colorectal carcinoma cells by CTLs is due to prolonged survival and activation of CD8 T cells that is imparted by increased expression of co-stimulatory molecules on tumor cells after radiation treatment. To our knowledge, this is the first study to a) demonstrate that doses as low as 5Gy can enhance lysis of human CRC tumor cells by CTL of diverse antigen specificity, b) show that T cells co-cultured with irradiated CRC cells have increased expression of activation...
markers and enhanced survival as compared to T cells cultured with non-irradiated tumor cells, c) measure the expression of several effector CTL co-stimulatory molecules including OX40L, 41BBL, CD70 and ICOSL on human tumor cells and find OX40L and 41BBL expression upregulated in five of six CRC tumor cell lines post-IR, d) find that CTL killing of irradiated tumor cells was abolished in the presence of a neutralizing antibody against OX40L and silenced 41BBL expression. Overall, the results of this study suggest that sub-lethal doses of radiation can be used to make human tumors more amenable to immune attack, even in the absence of innate immune responses to “danger” from dying cells.

2.3 MATERIALS AND METHODS:

Cell lines: Human colorectal carcinoma cell lines HCT116, Caco-2 and WiDr were obtained from the laboratory of tumor immunology and biology, LTIB, NCI, NIH. HT-29, LS174T and Colo205 cells were purchased from ATCC. The cell line SW620 was kindly provided by Zhi-Ren Liu from Georgia State University, Department of Biology. All cells were cultured as recommended by ATCC and tested periodically to ensure absence of Mycoplasma. Cells were incubated at 37°C incubator with 5% CO2.

Irradiation: A RS-2000 biological X-ray irradiator (Rad source technology, Suwanee, GA) was used to irradiate tumor cells. Cells were irradiated at a dose rate of 2Gy/min at voltage and current of 160kV and 25mA, respectively. Cells were maintained in suspension and kept on ice during irradiation. Immediately after irradiation, cells were centrifuged and cells were plated in tissue culture plates in fresh media.

RNA isolation: At 24 or 48hr post-IR, RNA was extracted from tumor cells using RNeasy mini kit (Qiagen Inc. Valencia, CA) according to manufacturer’s instructions. Purified
RNA was DNase-treated by Rnase-free DNase (Qiagen Inc. Valencia, CA) following manufacturer’s instructions.

**Quantitative real time PCR:** Expression of OX40L and 41BBL mRNA was determined using real time qRT-PCR. cDNA was synthesized using 500ng of mRNA. Amplification of cDNA was done using DyNaAmo cDNA synthesis kit (Finnzymes. Vantaa, Finland). Quantitative RT-PCR was conducted using TaqMan gene expression assay (Applied Biosystems; OX40L; Hs00967195, 41BBL; Hs00169409, and HPRT; Hs99999909) according to manufacturer’s protocol. PCR thermal cycling condition was 50°C for 2min, 95°C for 10min, 40 cycles of 95°C for 15sec and 60°C for 1min in a total volume of 20 μl/reaction. Data were collected using a 7500 Real Time PCR System. All samples were run in duplicate. The expression level of each gene was compared between irradiated and non-irradiated samples 24 or 48hr post-IR. Hypoxanthine phosphoribosyltransferase (HPRT) was used as an endogenous house-keeping control gene and samples were normalized to expression of this gene, which was unchanged by radiation. Data were analyzed using the comparative ΔΔCt method (Livak and Schmittgen 2001).

**Flow cytometry:** Cells were stained with primary labeled mAb [CD137L (41BBL)-PE, CD252 (OX40L)-PE, CD70-FITC, ICOSL-PE, CD8α-FITC, CD25-APC, CD69-PE, CD66-PE, CD227 (MUC-1)-FITC] purchased from BioLegend or BD biosciences (San Diego, CA). Surface staining was done in cell staining buffer for 30min on ice. Annexin-V-PE and 7AAD dye were obtained from BD biosciences (San Diego, CA) and used according to manufacturers instructions to measure cell death. Intracellular staining of active caspase-3 was done according to manufacturer’s instruction. Flow cytometry data were acquired on BD Fortessa and analyzed with FlowJo software (TreeStar, version 9.6). The live cells population was gated on the FSC
and SSC scatter plots for analysis of surface proteins. No live cells gate was used for cell death analysis samples. Samples were stained with the appropriate isotype control antibodies and gates were set to less than 5% in all isotype control samples. Expression was considered increased if the absolute percent positive population increased by 10% or greater.

**T-cell activation and survival assay:** $1 \times 10^3$ colorectal tumor cells were irradiated with 5, 10, or 15Gy and plated in 96-well plate for 48hr. $1\times10^4$ human CEA specific CD8+ T cells were subsequently added and co-cultured with the irradiated colorectal tumor cells for 24-48hr. The percent of T cells expressing CD69 or CD25 was measured by flow-cytometry. In parallel experiments, Annexin-V and 7AAD were used to measure T-cell death. Cell stimulation cocktail (1X) of PMA and ionomycin (eBioscience) was used as a positive control for activation of TAA-specific T-cells. In some experiments, activation of CTLs was determined after additional co-incubation of T-cells and tumor cells for 48h (beyond the 4h cytotoxicity assay).

**Generation TAA-specific cytotoxic T-lymphocytes:** PBMCs from HLA-A2+ donors were purchased from Hemacare (Van Nuys, CA) for the generation of antigen specific CTLs as described elsewhere (Tsang, Zaremba et al. 1995, Tsang, Zhu et al. 1997, Gelbard, Garnett et al. 2006). Briefly, PMBCs were allowed to adhere to T150 flask for 2hr in AIM-V media. After 2hr, non-adherent cells were removed for lymphocyte isolation. Adherent cells were cultured for seven days in the presence of 100ng/ml of human granulocyte colony stimulating factor (GM-CSF) and 20ng/ml of IL-4 (Miltenyi Biotec, Inc. Auburn, CA) in AIM-V media and 500ng/ml CD40L (Millipore corporation, Temecula, CA) was added on day five to mature the DCs. On day seven DCs were collected and pulsed with 40μg/mL of HLA-A2 binding CEA peptide (YLSGANLNL (CAP-1; (Tsang, Zhu et al. 1997)), 40μg/mL of MUC (ALWGQDVTSV) or peptide or 40μg/mL of Melan-A/MART-1 (AAGIGILTV) peptide (Rivoltini, Barracchini et al.
1995) for 4hr in a 37°C 5% CO₂ incubator. Unused DCs were frozen and stored in liquid nitrogen for subsequent restitulations. DCs loaded with peptide were subsequently irradiated with 50Gy. Immunomagnetic beads (Miltenyi Biotec Inc. Auburn, CA) were used to isolate CD8+ T cells from the non-adherent cells, following manufacturer instructions. Subsequently, isolated CD8+ T cells were co-cultured with peptide pulsed DCs. IL-7 (Millipore, Temecula, CA) at 10ng/ml and IL2 (Millipore, Temecula, CA) at 30U/ml were added to each well on the first and third day, respectively. T-cells were restimulated in this manner weekly using mature autologous DCs. Restimulated T cells were isolated over ficoll on day four or five of culture, and used in a standard cytotoxic killing assay.

**Cytotoxicity assay:** CTL lysis of HCT116, SW620, Colo205 (HLA-A2+) and LS174T (HLA-A2-) tumor cells was measured using the DELFIA cell cytotoxicity kit (Perkin Elmer). 72hr after irradiation, viable and proliferating tumor cells (2x10^6/2ml) were incubated with 5μl of BATDA (bis (acetoxymethyl) 2,2′:6′,2″- terpyridine- 6,6″- dicarboxylate; PerkinElmer, Boston, MA) for 20min at 37°C. After incubation, cells were washed four times with PBS. 5x10^3 cells were added in triplicate to a 96-well U-bottom plate, and either CEA or MUC specific CD8+ T-cell were added to the wells (E:T ratios between 10:1 and 30:1) and incubated for 4-5hr at 37°C. After incubation, the plate was centrifuged (500Xg for 5min) and 20μl of supernatant were transferred into a flat bottom plate. 200μl of Europium solution was added and incubated for 15min at room temperature on plate shaker (Bohlen, Manzke et al. 1994). Lysis was measured on a time resolved Victor3 plate reader fluorometer. The percentage of tumor lysis was calculated as follows: % tumor lysis=experimental release (counts) -spontaneous release (counts))/ maximum release (counts)- spontaneous release (counts) x 100. T-cells stimulated using Melan-A peptide were used as a control for T-cell antigen specificity.
**Expression knockdown and blocking:** 41BBL gene expression was knocked down using a gene specific siRNA. Briefly, tumor cells were plated in a 6-well dish at 1x 10⁵ cells/well one day prior to transfection, with 50-70% confluence on the day of transfection. In some experiments 2 x 10⁴ cells were plated in 24-well plates. 41BBL Flexi Tube siRNA (Qiagen Inc. Valencia, CA) was diluted in optiMEM medium (Invitrogen) and transfected using Hyperfect (Qiagen Inc. Valencia, CA). Twenty-four hours post transfection; cells were irradiated with 10Gy or mock-irradiated. The cells were harvested 24 to 48hr post irradiation and 41BBL mRNA expression was measured. A negative control siRNA that was not specific to 41BBL was also transfected into cells and 41BBL mRNA similarly evaluated. To block OX40L activity, we used a Goat anti-human OX40L-neutralizing antibody (R&D system, Minneapolis, MN) to block OX40 ligand and receptor interaction (cat #: AF1054). In the indicated groups, 500ng/ml of anti-human OX40L neutralizing antibody was added to Eu-labeled tumor cells for 15min prior to adding TAA-specific CTLs. The anti–human 41BB monoclonal blocking antibody BBK-2 (Lee, Park et al. 2002) was added 20μg/ml 15min before T-cells were added. Isotype matched antibodies were added to the other groups as a negative control.

**Statistical Analysis:** For graphed flow cytometry data, the mean of three to four independent experiments were calculated and an un-paired one-tailed student T-test was performed using Graphpad by Prism. Statistical differences between groups in the cytolysis assays were calculated using un-paired two-tailed student T-test.

### 2.4 RESULTS

Sub-lethal irradiation increases CEA and MUC-specific cytotoxic T-cell mediated killing of HCT116 and SW620 cells, but not of Colo205 cells.
We previously demonstrated that treatment of tumor cells with 10Gy of radiation enhanced tumor cell sensitivity to carcinoembryonic antigen (CEA) specific CTL mediated killing in an 18hr cytolysis assay (Garnett, Palena et al. 2004). We wanted to further evaluate if this enhanced killing occurred if a lower dose of radiation was used. Human colorectal tumor cell lines HCT116, SW620 and Colo205 were irradiated with a single dose of 0, 5 or 10Gy radiation. Following tumor cell irradiation only adherent and proliferating cells were harvested. We have previously demonstrated that tumor cells remain viable and continue to proliferate using this method (Ifeadi and Garnett-Benson 2012). At 72hr post-IR, tumor cells were evaluated in a 4hr Europium-release cell cytotoxicity assay (Bohlen, Manzke et al. 1994) with CEA-specific CTLs. Similar to our previous observations, 10Gy irradiated SW620 and HCT116 tumor cells were killed significantly better by CEA-specific CTLs when compared to non-irradiated tumor cells (Fig. 2.1A). Tumor cell lysis by CTLs could also be observed in tumor cells receiving as low as 5Gy of radiation (17.9% lysis of SW620 and 5% lysis of HCT116). We also wanted to evaluate if CTL that were specific to another TAA expressed in these colorectal tumor cells would demonstrate enhanced killing of irradiated tumor targets. For this, tumor cells were evaluated 72hr post-IR in a cytotoxicity assay using mucin-1 (MUC1) specific cytotoxic T cells. MUC-specific CTLs did not lyse non-irradiated SW620 cells, however lysis increased to 32.7% and 44.1% if tumor cells received 5Gy (P=0.0278) or 10Gy (P=0.0013) of radiation, respectively (Fig. 2.1B). Here, both HCT116 cells and SW620 tumor cells displayed significantly enhanced killing by MUC specific CTLs after irradiation with 10Gy (p=0.015 HCT116), and again killing could be observed when 5Gy of radiation was used. Thus, these data suggest that tumor cells surviving irradiation are more susceptible to cytotoxic T-cell killing by T cells of diverse antigen specificity and at doses lower than the previously reported 10Gy. In contrast to the killing of
SW620 and HCT116 tumor cells, the percent lysis by both CEA-specific and MUC-specific T-cells was below 10% after irradiation of Colo205 cells and untreated cells were also not killed (Fig. 2.1C). Furthermore, Lysis of MHC-mismatched colorectal tumor cells LS174T (CEA+MUC+/HLA-A2-) was less than 5% at all doses of radiation in the same assay even though both CEA and MUC expression was increased (Fig. 2.1D).

It is unclear what causes the variable magnitude of cytolysis among the cell lines examined, however these differences do not appear to correlate with altered surface expression patterns of MHC-I, or TAA. Specifically, HCT116 cells are killed post-IR while Colo205 cells are not (Fig. 2.1A & 2.1C). This occurs despite the fact that Colo205 cells express more MHC molecules per cell than HCT116 cells, as determined by both mean fluorescence intensity (MFI) (255 vs 226 respectively) and percent positive cells (74.6% vs 99.5%) (Fig.2.1E). SW620 and HCT116 cells demonstrate similarly enhanced cytolysis by CEA-specific CTLs post-IR (Fig. 2.1A). SW620 cells express more MHC-I (MFI 302) than HCT116, however, they actually decrease the amount of surface CEA (93% vs 85.3%). Antigen processing and presentation in MHC-I was not directly assessed here, however, given the levels of TAA and HLA-A2 among the cells lines it is difficult to imagine SW620 and HCT116, but not Colo205 cells, enhance antigen processing and presentation of CEA and MUC-1 HLA-A2 restricted peptides. Particularly when Colo205 cells and HCT116 cells express comparable amounts of CEA post-IR, and HCT116 cells actually decrease the frequency of HLA-A2 cells (Table 2.1E). Though the impact of radiation on antigen processing and presentation remains under study, it is likely that altered antigen presentation would work in concert with other tumor changes. Increased antigen presentation in a toleragenic or immunosuppressive environment where robust costimulation is not present could still lead to sub-optimal immune responses such as T-cell anergy. All four cell lines were greater
than 90% positive for Pan MHC class I detected using and HLA-ABC antibody (data not shown), however LS174T were less than 5% positive for HLA-A2.

A. HCT116 and SW620 cells treated in vitro with 0Gy (white bar), 5Gy (gray bar) or 10Gy (black bar) of ionizing radiation were used as targets in a 4hr CTL cytolysis assay. At 72hr post-IR, HLA-A2 restricted CEA-specific T cells were used as effector cells at an E:T of 25:1. B. Irradiated (5Gy and 10Gy) and non-irradiated (0Gy) HCT116 and SW620 cells were used in a 4hr lysis assays with MUC-specific T cells. At 72hr post-IR, MUC-specific T cells were used as effector cells at an E:T of 25:1 (HCT116) or 12:1 (SW620). C. Colo205 cells were used in a cytolysis assay with either CEA-specific or MUC-specific T cells at an E:T of 30:1. D. HLA-A2 negative LS174T cells were used in a cytolysis assay with either CEA-specific or MUC-specific T cells at an E:T of 30:1. * indicates P value <0.05. Error bars indicate variability in technical replicates. Experiments were repeated at least three times with similar results. E. Irradiated (10Gy) and non-irradiated (0Gy) CRC cells were cultured and subsequently stained with PE-labeled antibodies for flow cytometry to measure surface expression of HLA-A2 and TAA proteins on the surface of colorectal tumor cells. Isotype control staining of irradiated cells was less than 5% positive. Numbers indicate % of cells positive and those in parenthesis indicate mean fluorescence intensity (MFI) of cells expressing molecule on the cell surface 72h post-irradiation. (-) indicates level of detection below background. CEA= Carcinoembryonic antigen. MUC=Mucin-1.

Figure 2.1: Sensitivity to CEA- and MUC-specific T-cell mediated cytolysis in irradiated colorectal tumor cells.
Interaction with irradiated tumor cells enhances activation of T cells.

We observed increased lytic activity against irradiated tumor cells of diverse antigen specificity. In this in vitro assay, the increased T-cell activity is occurring in the absence of accessory cells, such as antigen presenting cells, suggesting that the increased activity was a result of direct T-cell and tumor cell interactions. To further define the impact that irradiated tumor cells were having on T cell biology, we evaluated changes in the expression of two markers of activation, CD69 and CD25, on the surface of T cells. Irradiated and non-treated SW620 and HCT116 tumor cells were cultured and human TAA-specific T cells were subsequently added to the cultures. T cells were also cultured alone, and 37.4% of these control T cells expressed CD25 on their surface (Fig. 2.2A). When these T cells were co-cultured with non-irradiated SW620 tumor cells for 48hr, the frequency detected was 8.35%. Interestingly, when these T cells were incubated with SW620 cells irradiated with 5, 10 or 15Gy, CD25+CD8+ T-cell frequency increased to 14.4%, 19.5% and 30.1%, respectively. This same increase in CD25+CD8+ T cells was observed following exposure of T cells to irradiated HCT116 cells in a dose dependent manner (Fig. 2.2B). More CD25+CD8+ T cells were detected if T cells were incubated with both 5Gy (black bar) or 10Gy (gray bar) treated HCT116 cells, than if they were incubated with untreated cells (white bar). In contrast, T cells exposed to Colo205 cells did not display increased expression of CD25 on CD8+ cells. In fact, expression appeared to decrease from 10.4% (non-irradiated) to 6.22% (5Gy) and 5.81% (10Gy). Moreover, Colo205 cells were also not killed better by CTLs post-IR (Fig. 2.1C). In these analysis the total lymphocyte population was analyzed based on scatter profile gating, and the total number of CD8+ cells (including both CD25-positive and CD25-negative cells) also appears to increase when T cells
are incubated with irradiated tumor cells (Fig. 2.2A). We next gated on the CD8+ cell population only, and found that the frequency of CD25+ cells within the CD8+ T-cell population similarly increased in T cells exposed to irradiated SW620 tumor cells (24.1% in 10Gy tumor cells) when compared to the frequency detected in non-irradiated tumor cells (17.1%) (Fig. 2.2C). Moreover the loss of CD25 expression when T-cells are incubated with non-irradiated tumor cells remains apparent (26% expression reduced to 17.1%).

While the expression of CD69 was higher in our T cells cultured alone, we were still able to observe an increase in expression following incubation with irradiated tumor cells in both SW620 cells and HCT116 cells. T cells co-cultured with non-irradiated SW620 tumor cells contained 11.9% CD69+CD8+ (Fig. 2.2D) and this increased to 19.6% (5Gy), 26.3% (10Gy) and 40.5% (15Gy) following co-incubation with irradiated tumor cells. 40.4% of control T cells cultured alone were CD69+CD8+. While T cells co-cultured with HCT116 cells displayed a similar increase in CD69 expression (Fig. 2.2E), those cultured with Colo205 cells again displayed a decrease in CD69+CD8+ cells. When only the CD8+ cells within the co-culture were gated and evaluated, CD69 expression increased significantly from 36.8% up to 47.2% (Fig. 2.2F). There was no change in the expression of activation markers on MART-1 specific T-cells incubated with either irradiated HCT116 or SW620 cells suggesting that antigen recognition is required (data not shown).
Figure 2.2: T-cell activation is increased following exposure to irradiated colorectal tumor cells.

1 x 10^3 colorectal tumor cells were irradiated with 5, 10, or 15 Gy and plated in a 96-well plate for 48 hr. 1 x 10^4 human CEA-specific CD8+ T cells were subsequently cultured alone or co-cultured with the irradiated colorectal tumors for 48 hr. A. Flow cytometry plots showing the frequency of CD8+ CD25+ T-cell co-incubated with SW620 cells. B. The frequency of CD8+ CD25+ T-cell was determined by flow cytometry in HCT116 and Colo205 cell cultures. * =
Statistically significant relative to untreated cells by K-S. C. Flow cytometry plots showing the frequency of CD25+ cells within the CD8+ cell population following co-incubated with SW620 cells. Isotype control staining is shown in gray filled histogram. CD25-APC positive cells are shown in solid black line histogram. D. Flow cytometry plots showing the frequency of CD8+ CD69+ T-cell co-incubated with SW620 cells. E. The frequency of CD8+ CD69+ T-cell was determined by flow cytometry in HCT116 and Colo205 cell cultures. As a control, T cells incubated alone were also evaluated (striped bar). * = statistically significant relative to untreated cells by K-S. F. Flow cytometry plots showing the frequency of CD25+ cells within the CD8+ cell population following co-incubated with SW620 cells. Isotype control staining is shown in gray filled histogram. CD69-PE positive cells are shown in solid black line histogram. 

Experiments repeated three times with similar results.

**Interaction with irradiated tumor cells decreases cell death of T cells.**

We observed a significant increase in frequency of activated CD8+ T cells as well as total CD8+ T-cell population following incubation with irradiated tumor cells and sought to determine if increased T-cell survival could be responsible for this. For this, we measured apoptosis by 7AAD staining of TAA-specific T cells following 24hr incubation with tumor cells. Irradiation reduced the number of dead T cells when compared to co-incubation with non-irradiated counterparts, and 31.1% of T cells incubated with SW620 are dead, while only 11.4% of T cells incubated with SW620 cells irradiated with 10Gy are dead (Fig. 2.3A). Very few dead cells were detected when T cells were incubated alone (3.4%) (Fig. 2.3B; checked bar), while there was a robust increase in T-cell death upon incubation with untreated HCT116 tumor cells (20.1%)(Fig 2.3B; white bar). HCT116 cells also demonstrated reduced death of T cells co-incubated with 5Gy (14.5%) and 10Gy (7.76%) irradiated tumor cells (Fig. 2.3B). These findings suggest that irradiated tumor cells not only enhance the frequency of activated T cells, but also increase the survival of T cells. Irradiated Colo205 tumor cells showed a reduced ability to promote T-cell survival (0Gy-25.4%, 5Gy-24%, 10Gy-19.4%) when compared to both SW620 and HCT116 cells. The levels of active caspase-3 as a marker of T cells undergoing
early apoptosis following co-incubation with tumor cells was also evaluated. Active caspase-3 expression in CD8+ T cells was measured after 4h (Fig. 2.3C) and 18h (Fig. 2.3D) of co-culture with irradiated and non-irradiated tumor cells. Tumor irradiation significantly decreased the number of early apoptotic (active caspase-3 positive) T cells when evaluated after 18h co-incubation (Fig. 2.3D, P<0.01), but was not significant after 4h.

Figure 2.3: T-cell death is reduced following exposure to irradiated colorectal tumor cells.

1 x 10^3 colorectal tumor cells were irradiated with 5Gy or 10Gy and plated in a 96-well plate for 48hr. 1 x 10^4 human CEA-specific T cells were subsequently added and co-cultured with the irradiated colorectal tumors for 24hr. A. Flow cytometry plots showing the frequency of 7AAD-positive CD3+ T-cells co-incubated with SW620 cells. B. The frequency of 7AAD-positive T cells (CD3+) was determined by flow cytometry in HCT116 and Colo205 cell cultures. As a control, T cells incubated alone were also evaluated (checked bar). Experiment repeated two times with similar results. C. The frequency of T cells expressing active caspase-3 was determined by flow cytometry after 4h co-culture SW620 and HCT116 cell cultures. D. The frequency of T cells expressing active caspase-3 was determined by flow cytometry after 18h co-culture SW620 and HCT116 cell cultures. * = statistical significance relative to untreated cells as determined by Probability Binning (Chi(T)) test. * P<0.01. Experiments repeated three times with similar results.
Sub-lethal irradiation of colorectal carcinoma cell lines enhances OX40L and 41BBL expression.

There are a number of proteins that, when expressed by target cells, can contribute to enhanced local activity of CD8+ cytolytic T cells through increased activation/costimulation or survival. Signals transduced by proteins such as 41BB, OX40, CD27 and ICOS are regarded as especially important for survival, expansion and effector function of T cells that have initially received activating signals via the CD28 receptor (Watts 2005, Kober, Leitner et al. 2008). To further investigate the mechanisms underlying enhanced T-cell activation and survival, we examined changes in CTL effector co-stimulatory molecules, such as OX40L, 41BBL, CD70 (CD27L/TNFSF7) and ICOSL (CD275/B7-H2) on irradiated tumor cells. A panel of colorectal tumor cells (HCT116, SW620, HT-29, Caco-2, Colo205 and WiDr) were irradiated, and the surface expression of these proteins was evaluated by flow cytometry after 48-72hr. We observed 18.8% of non-irradiated WiDr cells expressed OX40L, and this increased to 20.9% following 5Gy, and 60.9% following 10Gy (Fig. 2A). The average of three replicate experiments in WiDr cells revealed an average increase in expression of 44% following radiation from 17% in cells receiving no radiation (P=0.0295). There was a significant increase in OX40L expression in both HCT116 and SW620 (Fig. 2B). Though radiation increased the expression of OX40L in HT-29 and Caco-2 cells repeatedly, it was not significant based on the average of replicate experiments and there was no increase in expression observed in Colo205. Staining of cells with isotype control antibody was below 5% in all cells evaluated (data not shown). We next evaluated the surface expression of 41BBL in the same colorectal tumor cell lines. Figure 2.5A shows the level of 41BBL protein on the surface of untreated WiDr cells (1.75%). The level of 41BBL increases following both 5Gy (13.6%) and 10Gy (37.5%) treatment. The average of three replicate experiments revealed an average increase in expression from 4% (0Gy)
up to 33% following 10Gy irradiation of WiDr cells, and this increase was statistically significant (P=0.0013). Expression of 41BBL also increased in four of the five other tumor cell lines tested (Fig. 2.5B) and this increase was significant in SW620, HCT116 and Caco-2 cells. Again, Colo205 cells were the exception and radiation did not increase the expression of 41BBL. We also evaluated the longevity of increased OX40L and 41BBL and found that the elevated expression of 41BBL protein could still be seen seven days post-IR in WiDr, HCT116 and SW620 cells, and the elevated expression of OX40L was maintained in WiDr cells but not in SW620 cells (data not shown). These data suggest that the modulation of these proteins in tumor cells by radiation can occur and be sustained. Furthermore, we detected significantly increased expression of both OX40L and 41BBL mRNA in all colorectal tumor cell lines evaluated (data not shown), suggesting that increased gene transcription or transcript stability is responsible for surface protein changes.
Figure 2.4: Tumor cells modulate OX40L protein expression after treatment with radiation.

A. Irradiated (5Gy and 10Gy) and non-irradiated (0Gy) WiDr colorectal carcinoma cells were stained with PE-labeled antibody to human OX40L. Isotype control staining in irradiated cells was all less than 5% positive in all samples. B. OX40L expression in five additional tumor cell lines receiving 10Gy (black bar) or 0Gy (gray bar). * indicates P value <0.05. Data graphed are the mean of two (Caco2), three (HCT116, Colo205, HT-29) or four (SW620) experimental repeats and error bars represent the SEM across the independent experiments.
Figure 2.5: Tumor cells modulate 41BBL protein expression after treatment with radiation.

A. Irradiated (5 and 10Gy) and non-irradiated (0Gy) WiDr colorectal carcinoma cells were cultured and subsequently stained with PE-labeled antibody to 41BBL. Isotype control staining of irradiated cells was less than 5% positive. B. 41BBL expression in five additional colorectal tumor cells lines receiving 10Gy (black bar) or 0Gy (gray bar). * indicates P value <0.05. Data graphed are the mean of three (Colo205, HT-29, Caco2) or four (HCT116, SW620) experimental repeats and error bars represent the SEM across the independent experiments.

We next wanted to evaluate if the expression of other co-stimulators of CD8+ effector cells was also changed in irradiated tumor cells. For this we extended our evaluation to another TNFSF member, CD70, and to a B7-related protein family member, ICOSL. No increase in either CD70 (Fig. 2.6A) or ICOSL (Fig. 2.6B) was detected in HCT116 cells treated with 10Gy of radiation. This is in contrast to the increase in 41BBL detected in the same cells post-IR (Fig. 2.6C). We also did not detect an increase in CD70 in any other colorectal tumor cell lines evaluated (Fig. 2.6D).
Furthermore, while we saw a mild increase in ICOSL expression in SW620 cells (0.2%-0Gy versus 14.10%-10Gy), we were unable to see a change in ICOSL expression in any of the other tumor cell lines. In addition, no increase in the expression of B7-1 (CD80) was observed following irradiation in any of the cell lines evaluated (data not shown).

Figure 2.6 Expression of CD70 and ICOSL following tumor cell irradiation.

A. CD70 expression (black line). B. ICOSL expression (black line). C. 41BBL expression (black line) 48 post-IR in HCT116 cells. Isotype control stained cells were all less than 5% positive (filled grey histogram). D. CD70 and ICOSL expression in treated and untreated SW620, WiDr, HT-29 and Colo205 cells. Experiments repeated two and three times with similar results.
41BBL and OX40L dual co-stimulation is required for radiation-enhanced sensitivity to CTL killing.

In addition to delivering anti-apoptotic signals to T cells, Salih et al., (2000) demonstrated that 41BBL expression on carcinoma cells induced the production of IFN-γ in T-cell and tumor cell cocultures (Salih, Kosowski et al. 2000). Moreover, OX40L and 41BBL have been reported to program effector function in T cells (Croft 2003, Walch, Rampini et al. 2009, Chacon, Wu et al. 2013), and result in effective anti-tumor immunity. These data indicate that T-cell effector function could be enhanced through co-stimulation of these pathways. Indeed, in the present study we detected no change in the expression of OX40L or 41BBL in Colo205 cells (Fig. 2.4B & 2.5B) and, interestingly, these cells also showed no enhancement of CTL killing post-IR (Fig. 2.1C). These results suggest that the enhanced CTL killing of irradiated tumor cells may be due to the enhanced expression of the co-stimulatory molecules OX40L and 41BBL. To further investigate whether OX40L and 41BBL are involved in enhanced CTL killing of irradiated colorectal tumor cells, we performed CTL cytotoxicity assays after blocking and/or inhibiting these molecules. Knocking down two genes in a single cell simultaneously using siRNA is technically difficult, so we chose to use a combination of siRNA gene knockdown and a functional blocking antibody to allow us to evaluate tumor cells in which both pathways were blocked. For these experiments, the ligand-receptor interaction of OX40/OX40L was blocked using neutralizing antibody against human OX40L, and radiation-induced 41BBL was knocked down in tumor cells using homologous siRNA. We confirmed siRNA knockdown in our cells by quantifying mRNA in several tumor cells. We observed elimination of the radiation-induced
increase in 41BBL mRNA in HCT116 cells transfected with 41BBL-specific siRNA but not negative control siRNA (Fig 2.7A). Non-irradiated HCT116 cells had limited lysis by TAA specific CTLs and this was enhanced to 29.3% (black bar) after irradiation with 10Gy (Fig. 2.7B). Irradiated tumor cells knocked down for 41BBL were lysed less than irradiated tumor cells expressing endogenous levels of 41BBL (17.2%; data not shown); however, this lysis was completely lost when both 41BBL was knocked down and the OX40L blocking antibody was used in combination (P=0.067). Thus, radiation-enhanced killing of tumor cells was completely reversed when both effector co-stimulatory molecules were inhibited (Fig. 2.7B). To determine if OX-40L and 4-1BBL expressions could reverse radiation-enhanced T-cell survival we evaluated active Caspase-3 expression in T-cells co-incubated with HCT116 tumor cells for 5h. The flow cytometry data (Fig. 2.7C) are representative of 3 independent experiments with similar results and suggest that dual blockade on irradiated tumor cells increases the amount of T-cell death. Overall, our study reveals that radiation-enhanced tumoricidal activity of CTLs could be due, in part, to enhanced expression of both OX40L and 41BBL.
Radiation-enhanced sensitivity to T-cell mediated lysis is reduced in the absence of OX40L and 41BBL. A. 4-1BBL was knocked down in tumor cells as described in Materials and Methods. Briefly, 1x10^5 HCT 116 cells were and transfected the following day with 4-1BBL siRNA or a control siRNA. 24hr post-transfection, the cells were irradiated with 10Gy. 24hr post-IR, cells were harvested and 4-1BBL mRNA was quantified. * indicates P value <0.05 and **<0.0001.

Data graphed are the mean of two experimental repeats and error bars represent the SEM across the independent experiments. B. 2x10^4 HCT 116 cells were plated in 24-well plates and transfected with 4-1BBL siRNA or a control siRNA. After transfection, the cells were irradiated with 10Gy and used the next day in a Eu-release cytotoxicity assay as described in the material and methods. In the indicated group, neutralizing antibody to human OX-40L was added to tumor cells used in the cytolysis assay. Error bars indicate variability in technical replicates. Experiments repeated at least two times with similar results. C. Flow cytometry plots showing active Caspase-3 expression in T-cells incubated alone or with treated HCT116 cells for 5h. * = statistical significance relative to indicated group by K-S. Experiments repeated three times with similar results.
We performed a similar evaluation of SW620 cells to determine if this effect could be observed in more than one colorectal tumor cell line. SW620 cells displayed a mild reduction in CTL killing when either 41BBL (P=0.0724) or OX40L (P=0.0379) signals were blocked and this was further reduced to levels similar to untreated cells when both signals were blocked (P=0.0316) (Fig. 2.8B). CTL cytolysis of irradiated SW620 cells was also inhibited if 41BB signals were blocked using a neutralizing antibody to 41BB (on the T-cell) in combination with the OX40L neutralizing antibody (data not shown). Furthermore, radiation-induced activation, as determined by CD25 expression, of CD8+ CTLs was reversed when dual blockade was performed (Fig. 2.8C). 48h after cytolysis assay set-up 48.6% of CD8+ cells expressed CD25 when incubated with SW620 cells irradiated with 10Gy as compared to 40% of CD8+ cells following interaction with non-irradiated SW620 cells. When both 41BBL and OX40L signals were absent 42.9% of CD8+ cells expressed CD25. As a positive control, CD25 expression was detected on 84% of T-cells stimulated with phorbol myristate acetate (PMA) and ionomycin (data not shown).
Figure 2.8: Radiation-enhanced sensitivity to T-cell mediated lysis and T-cell activation is reduced in the absence of OX40L and 41BBL.

A. 41BBL was knocked down in tumor cells as described in Materials and Methods. Briefly, 1x10⁵ SW620 cells were and transfected the following day with 41BBL siRNA or a control siRNA. 24hr post-transfection, the cells were irradiated with 10Gy. 48hr post-IR, cells were harvested and 41BBL mRNA was quantified. B. 2x10⁴ SW620 cells were plated in 24-well plates and transfected with 41BBL siRNA or a control siRNA. After transfection, the cells were irradiated with 10Gy. 48h post-IR cells were used in a Eu-release cytotoxicity assay using CEA-specific T-cells at an E:T ratio of 30:1. In the indicated groups, neutralizing antibody to human OX40L was added to SW620 cells used in the cytolysis assay. C. Supernatants were collected and analyzed for cytolysis at 4h. Incubation of T-cells and tumor cells was allowed to continue for an additional 48h. 48h after cytolysis assay set-up, cells were harvested and stained for markers of T-cell activation. Flow cytometry plots showing the frequency of CD25+ cells within the CD8+ cell population after incubation with irradiated SW620 cells. * indicates P value <0.05. Error bars indicate variability in technical replicates. *Experiments repeated at least two times with similar results.*
2.5 DISCUSSION

There is a wide array of CIT strategies under clinical investigation in combination with RT for the treatment of advanced cancers. With the exception of studies using RT for lymphodepletion prior to adoptive cell transfer (Dudley, Yang et al. 2008), most clinical investigations utilize RT as an adjuvant to immune-based therapies (Chi, Liu et al. 2005, Kaufman and Divgi 2005, Lechleider, Arlen et al. 2008). These studies demonstrate enhanced immune responses, including expanded numbers of circulating anti-tumor CTLs and antibodies in treated patients. Unfortunately, these increased immune responses have not translated to a significant reduction in tumor burden often enough (Gulley, Arlen et al. 2005, Nesslinger, Sahota et al. 2007, Lechleider, Arlen et al. 2008) and the reasons for this unexpected lack of clinical response have yet to be resolved. More recently, modulation of tumor cells by RT has come into the spotlight as it has become clear that radiation survivors likely have an altered phenotype that can be exploited by CIT approaches (Makinde, John-Aryankalayil et al. 2013). Detailed investigation into the molecular mechanism that results in the ability of IR to enhance anti-tumor immune responses will be required to capitalize on these biological changes and allow for additional opportunities for eliminating advanced stage cancers. To our knowledge, our study reports for the first time that human colorectal carcinoma cells surviving radiation modulate expression of both OX40L and 41BBL, and that irradiated tumor cells promote CTL activation, survival and tumoricidal activity related to these changes.

Our previous CTL lysis assays were done using CTLs against single antigen specificity, CEA. Here we expanded this evaluation to MUC-1 specific CTLs. Killing activity of both CEA-specific and MUC-specific T cells was enhanced, suggesting that radiation-enhanced lysis is not limited to a single antigen specificity or tumor cell line (Fig. 2.1). We also detected
enhanced susceptibility to CTL killing in tumor cells treated with a lower dose of radiation (5Gy) than previously reported. Overall, these in vitro studies highlight the ability of irradiated tumor cells to directly enhance effector T-cell activity in the absence of tumor cell death (and subsequent induction of antigen presentation by APCs). We have recently reported on such “immunogenic modulation” (occurring in the absence of “immunogenic cell death”) following treatment of human tumor cells with docetaxel chemotherapy (Hodge, Garnett et al. 2013). Utilization of such direct tumor cell to T-cell mechanisms from phenotypically altered tumor cells that do not die post-IR, in addition to enhanced “danger” signals from dying cells, should allow for synergy resulting in a more robust anti-tumor immune attack. Thus it seems plausible that both of these mechanisms could be rationally applied to current combination CIT and RT therapy designs.

We observed enhanced CTL activity against tumor cells treated with radiation and wondered if other aspects of T-cell biology were also altered. We reasoned that T cells capable of surviving longer after interacting with tumor cells would be more likely to have productive interactions with tumor cells resulting in enhanced ability to kill tumor cells. Very few dead T cells were detected in cultures of T cells alone; however, there was an increase in T-cell death upon incubation with untreated tumor cells (Fig 2.3). Death of T-cells following interaction with tumor cells has been reported by others, and is thought to be caused by tumor expressed PDL1, FasL and/or activation induced cell death (AICD) (Zaks, Chappell et al. 1999, Chiou, Sheu et al. 2005, Prado-Garcia, Romero-Garcia et al. 2012). Surprisingly, we detected a decrease in the number of dead T cells if the tumor cells had been treated with radiation as compared to non-irradiated cells. We next measured T-cell activation by evaluating changes in the expression of two general markers of T-cell activation, CD25 and CD69. We found that the frequency of
CD8+CD25+ and CD8+CD69+ T cells was increased following exposure to irradiated SW620 and HCT116 cells, as compared to non-irradiated tumor cells (Fig 2.2).

Signaling through OX-40 (TNFRSF4/CD134) promotes T-cell survival and expansion (Garber 2011). The expression of IL2 receptor (CD25), IL7 and IL12 receptors is also up-regulated on the surface of T cells by OX-40/OX-40L engagement (Mousavi, Soroosh et al. 2008). 4-1BB engagements with its ligand promote survival of CD8+ T cells by up regulating expression of Bcl-xL and Bfl-1 (Lee, Park et al. 2002). In addition to promoting T-cell proliferation and survival, 4-1BBL reactivates anergic T cells (Habib-Agahi, Jaberipour et al. 2009). As both OX-40L and 4-1BBL have been reported to enhance T-cell survival and T-cell activation, we next evaluated their expression in our tumor cells. We found that irradiating five of six CRC tumor cell lines increased surface expression of both OX-40L (Fig. 2.4), and 4-1BBL protein (Fig. 2.5) after treatment with 10Gy of ionizing radiation. Furthermore, increased mRNA of both genes was also detected (Fig. 2.6), suggesting that the increase in protein expression was a result of increased transcription (or stability of transcripts). Though non-treated tumor cells expressed variable amounts of both OX-40L and 4-1BBL on their cells surface, this rarely exceeded 20% in our experiments. Previous reports by Salih et al. (2000) measured 4-1BBL expression on carcinoma cells and found that HCT116 cells expressed higher levels than HT-29 cells, which we also saw in our evaluation of untreated tumor cells (Fig 2.5B; gray bars)

The mechanism of selective gene expression is currently under investigation, and other data from our lab suggests that radiation is epigenetically regulating expression of 4-1BBL and OX-40L (Kumari, Cacan et al. 2013).

We were surprised to see that, in contrast to the lysis of irradiated HCT116 cells and SW620 cells, we did not observe enhanced lysis of irradiated Colo205 cells (Fig 2.1C). It is
interesting that this cell line also had the lowest level of 4-1BBL expression among all the cell lines, and its expression was unchanged by radiation (Fig 2.5B). The expression of OX-40L protein was also unchanged by radiation in these cells (Fig 2.4B). Furthermore, the number of activated CD8+ T cells (expressing CD25 or CD69) was not increased when T cells were co-cultured with irradiated Colo205 cells (Fig 2.2B and 2.2D), though a modest increase of T-cell survival was seen (Fig. 2.3B). These data support the hypothesis that increased expression of OX-40L and 4-1BBL on HCT116 and SW620 tumor cells results in enhanced T-cell activation and killing activity. Other studies from our lab suggest that these genes can be increased by IR in other tumor cell types including both breast and prostate (Bernstein, Garnett et al. 2014).

Antigen processing and presentation in MHC-I was not directly assessed here, however, given the levels of TAA and HLA-A2 among the cells lines it is difficult to imagine SW620 and HCT116 enhancing antigen processing and presentation of CEA and MUC-1 HLA-A2 restricted peptides, but not Colo205 cells. Both HCT116 and Colo205 cells increase expression of CEA, MUC and HLA-A2. However, HCT116 cells are killed post-IR while Colo205 cells are not. This occurs despite the fact that Colo205 cells express more MHC molecules per cell (255) than HCT116 (226) cells as determined by both MFI and percent positive (Fig 2.1E). SW620 cells express more MHC-I than HCT116 and were killed slightly better than HCT116 cells by CEA-specific T-cells, however, they actually decrease the amount of surface CEA. HLA-A2 negative cells (LS174T) are also not killed even though they express CEA and MUC and upregulate both OX-40L and 4-1BBL (data not shown). While the impact of radiation on antigen processing and presentation in human tumor cells remains under investigation, it remains likely that altered antigen presentation would work in concert with other tumor changes. Increased antigen presentation in a toleragenic or immunosuppressive
environment, where robust costimulation is not present, would still lead to sub-optimal immune responses.

To determine if radiation-induced expression of OX-40L and 4-1BB-L plays a direct role in enhanced tumor cell susceptibility to lysis by CTLs, we conducted CTL cytolysis experiments in combination with molecular inhibition. We found that neither knocking down 4-1BB-L in tumor cells, nor inhibiting OX-40L signaling independently, completely reversed radiation-enhanced sensitivity to cytolysis (Fig. 2.8B). However, when both molecules were inhibited there was a more prominent loss of the radiation-enhanced killing of both HCT116 and SW620 cells by T cells (Fig. 2.8C & 2.9B), and a reduction in expression of CD25 (Fig 2.8C) and CTL survival (Fig. 2.9C). Both 4-1BB-L and OX-40L signals have been reported by others to increase the production of effector molecules such as perforin and granzyme in stimulated CTLs (Walch, Rampini et al. 2009, Chacon, Wu et al. 2013). Whether irradiated tumors are impacting production or release of effector molecules from CTLs is currently under investigation. Ongoing mechanistic studies are evaluating which mechanism (increased survival or increased production/release of effector molecules or both) is primarily responsible for enhanced tumoricidal activity, as well as the relative contribution of each co-stimulatory molecule following both in vivo and in vitro tumor cell irradiation.

The aims of this study were meant to provide data supporting the growing use of RT in combination with CIT. If IR-modulated expression of 4-1BB-L and OX-40L is shown to play a significant role in the ability of RT to enhance effector CTL killing, this could be an alternative therapeutic approach to enhancing these important T-cell signals. This approach is particularly relevant given the severe toxicity that can occur when using agonistic antibodies. Furthermore, the use of agonist antibodies is not limited to tumor-specific T-cells and, as a result, non-tumor
specific T cells can become activated and induce off-target effects. Using RT to induce these molecules specifically on a focused target represents a refinement in the approach by triggering these pathways in anti-tumor CTLs infiltrating irradiated tumors. Ongoing investigations include determining the impact of RT on tumor modulation in vivo to assess the specificity of changes on tumor cells versus other cells in the microenvironment. Ultimately, if IR is shown to have a profound and consistent effect on CTL activity this would provide support for using IR, along with CIT strategies, specifically to enhance signals to these cells and optimize anti-tumor CTL responses.
3 Turning T cells on: Epigenetically enhanced expression of effector T cells costimulatory molecules on irradiated human tumor cells.

**PUBLICATION:** ANITA KUMARI, ERCAN CACAN, SUSANNA F GREER AND CHARLIE GARNETT-BENSON*

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3.1 ABSTRACT:

Sub-lethal doses of radiation can alter the phenotype of target tissue by modulating gene expression and making tumor cells more susceptible to T-cell-mediated immune attack. We have previously shown that sub-lethal tumor cell irradiation enhances killing of colorectal carcinoma cells by tumor-specific cytotoxic T cells by unknown mechanisms. Recent data from our lab indicates that irradiation of tumor cells results in the upregulation of OX40L and 41BBL, and that T cells incubated with irradiated tumor cells displayed improved CTL survival, activation and effector activity. The objective of this current study was to determine the mechanism of enhanced OX40L and 41BBL expression in human colorectal tumor cells. **Methods:** Two colorectal carcinoma cell lines, HCT116 and SW620, were examined for changes in the expression of 41BBL and OX40L in response to inhibition of histone deacetylases (using TSA) and DNA methyltransferases (using 5-Aza-2’-deoxycytidine) to evaluate if epigenetic mechanisms of gene expression can modulate these genes. Tumor cells were treated with radiation, TSA, or 5-Aza-dC, and subsequently evaluated for changes in gene expression using RT-qPCR and flow cytometry. Moreover, we assessed levels of histone acetylation at the 41BBL promoter using chromatin immunoprecipitation assays in irradiated HCT116 cells. **Results:** Our data indicate that expression of 41BBL and OX40L can indeed be epigenetically regulated, as inhibition of histone deacetylases and of DNA methyltransferases results in increased OX40L and 41BBL mRNA and protein expression. Treatment of tumor cells with TSA enhanced the expression of these genes more than treatment with 5-Aza-dC, and co-incubation of T cells with TSA-treated tumor cells enhanced T-cell survival and activation, similar to radiation. Furthermore, chromatin immunoprecipitation experiments revealed significantly increased
histone H3 acetylation of 41BBL promoters specifically following irradiation. **Conclusions:** Full understanding of specific mechanisms of immunogenic modulation (altered expression of immune relevant genes) of irradiated tumor cells will be required to determine how to best utilize radiation as a tool to enhance cancer immunotherapy approaches. Overall, our results suggest that radiation can be used to make human tumors more immunogenic through epigenetic modulation of genes stimulatory to effector T-cells.

### 3.2 INTRODUCTION:

Previous reports by others and us demonstrate that sub-lethal doses of radiation alter the expression of genes within tumor cells (Friedman 2002, Chakraborty, Abrams et al. 2003, Demaria, Kawashima et al. 2005). Furthermore, it has been directly demonstrated that tumor irradiation, as well as treatment with some chemotherapy drugs, results in increased susceptibility to killing of tumor cells by cytotoxic T cells (CTLs) (Chakraborty, Abrams et al. 2003, Garnett, Palena et al. 2004, Gelbard, Garnett et al. 2006). Notably, many genes that are important for T-cell anti-tumor effector activity are up-regulated following treatment with sub-lethal doses of radiation (Friedman 2002, Garnett, Palena et al. 2004, Ifeadi and Garnett-Benson 2012). However, the mechanisms of radiation-mediated changes in the expression of such immune stimulatory genes are poorly understood.

It is clear that human cells respond to DNA-damage from ionizing radiation (IR) by inducing the expression of a number of genes at the transcriptional level (Garnett, Palena et al. 2004, Brzoska and Szumiel 2009, Makinde, John-Aryankalayil et al. 2013). Induction of altered gene expression can be due to direct cellular radiation effects or to radiation-induced changes in cellular milieu. Direct cellular effects appear to be regulated through parallel signaling pathways
that originate from the nucleus following DNA damage, as well as signaling pathways that originate in the cytoplasm via reactive oxygen species production (Janssens and Tschopp 2006, Brzoska and Szumiel 2009). These pathways induce NF-kB activation and nuclear translocation (Schreck, Albermann et al. 1992, Li and Karin 1998). As would be expected, DNA damage by IR can induce cellular stress responses, which result in activation of DNA damage repair pathways and apoptotic pathways (Amundson, Do et al. 1999, Ifeadi and Garnett-Benson 2012).

Interestingly, regulation of the expression of a variety of genes, not related to known or typical DNA repair or apoptotic pathways, also occur (Sreekumar, Nyati et al. 2001, Friedman 2002, Gasser and Raulet 2006). Indeed, we previously examined 23 human carcinoma cell lines for their phenotypic response to sub-lethal doses of IR (Garnett, Palena et al. 2004), and found that RT increased the expression of several genes commonly down-regulated by tumors to escape immune recognition and elimination (Kojima, Shinohara et al. 1994, Zamai, Rana et al. 1994, French and Tschopp 2002, Bubenik 2003, Modrak, Gold et al. 2003, Slavin-Chiorini, Catalfamo et al. 2004), including Fas (CD95), Intercellular adhesion molecule-1 (ICAM-1/CD54), tumor associated antigens (TAA) and major histocompatibility (MHC)-Class I. Most recently we found that radiation enhances the expression of OX40 ligand (OX40L/TNFSF4/CD134L/CD252) and 41BB ligand (41BBL/TNFSF9/CD137L), important co-stimulators of effector CTLs on tumor cells (submitted manuscript).

To elicit an effective immune response against tumors, T cells need to recognize tumor antigens presented by MHC in conjunction with appropriate co-stimulation (Kroczek, Mages et al. 2004, Jensen, Maston et al. 2010). In the absence of proper co-stimulation, these anti-tumor T cells become anergic. Proteins such as 41BBL and OX40L represent important co-stimulators of effector CTL activity (Curtsinger, Lins et al. 2003, Watts 2005, Mescher, Curtsinger et al. 2006,
Kober, Leitner et al. 2008), and we have seen sub-lethal doses of radiation increase their expression in human tumor cells; however, the mechanisms regulating radiation-enhanced modulation of the expression of these two genes remain unclear. OX40 (TNFRSF4/CD134) was originally characterized as a transiently expressed co-stimulatory molecule regulating CD4 and CD8 immunity (al-Shamkhani, Birkeland et al. 1996), and signaling through OX40 promotes T-cell survival and expansion (Garber 2011). 41BBL co-stimulation of 41BB (TNFRSF9/CD137) on tumor-specific T cells is important for T-cell proliferation (Waller, McKinney et al. 2007, Habib-Agahi, Jaberipour et al. 2009), cytokine production and activation (Curran, Kim et al. 2011). Engagement of OX40 and 41BB by agonist antibodies increases immunity against tumors, resulting in long-term survival (Pan, Zang et al. 2002) in a number of murine tumor models (Melero, Shuford et al. 1997, Murata, Ladle et al. 2006). Recent evidence indicates that the expression of 41BBL is transcriptionally activated by HDAC inhibitors in leukemia cell lines (Vire, de Walque et al. 2009), and that HDAC11 plays an essential role in regulating OX40L expression (Buglio, Khaskhely et al. 2011). Interestingly, radiation has been shown to inhibit the expression of HDAC1 and HDAC2 (Han, Zhang et al. 2012), and we have seen enhanced cytolysis by T-cells following tumor irradiation. Thus, epigenetic mechanisms may be at work during radiation-enhanced susceptibility to T-cell killing.

Epigenetic changes such as histone modifications and DNA methylation play important roles in regulating gene expression. DNA methyltransferase enzyme (DNMT1) adds methyl group to cytosine residue (Gal-Yam, Saito et al. 2008). DNA hypermethylation of CpG dinucleotides accumulates in promoter regions of genes and contributes to their loss through epigenetic silencing. Promoter hypermethylation and genome-wide hypomethylation alters genes expression in colorectal cancer (Cheng, Pincas et al. 2008). It has been found that genes having
hypermethylation also exhibit altered acetylation and methylation of histones (Kouzarides 2007). Histone acetylation via histone acetyltransferases (HATs) is another major epigenetic mechanism controlling gene expression (Roth, Denu et al. 2001, Seo, McNamara et al. 2001, Eberharter and Becker 2002). Gains in histone acetylation neutralize the positive charge on lysine residues and contribute to disrupted nucleosome structure, allowing unfolding of DNA, increased transcription factor access and enhanced gene expression (Richon, Sandhoff et al. 2000, Dion, Altschuler et al. 2005, Choudhary, Kumar et al. 2009). HDACs remove acetyl groups from histones and return DNA to a less accessible conformation, thereby decreasing transcription (Kadosh and Struhl 1997, Glozak and Seto 2007, Wang, Zang et al. 2009). Alterations in HAT and HDAC activity have been identified in many human cancers (Marks, Rifkind et al. 2001, Barneda-Zahonero and Parra 2012). HDAC inhibitors (HDACi) therefore promote hyperacetylation of histones, which in turn leads to chromatin relaxation and selective expression of genes.

The roles of DNA methylation and histone acetylation in the expression of OX40L and 41BBL in response to radiation have not been investigated. Hence, we designed the present study to test the hypothesis that irradiation leads to increased expression of OX40L and 41BBL in colorectal tumor cells via epigenetic regulation. We measured the expression of effector CTL co-stimulatory molecules OX40L and 41BBL on human colorectal tumor cells lines after treatment with trichostatin (TSA) and 5-Aza-2’-deoxycytidine (5-Aza-dC). Ours is the first study to report that a) OX40L and 41BBL expression increases in CRC cells when DNMTs are inhibited, b) expression of OX40L and 41BBL increases in human CRC cells when HDACs are inhibited, c) HDAC inhibition in CRC cells can increase the activation and survival of T cells, and d) radiation treatment of tumor cells results in epigenetic modification of the histones in the
promoter of the costimulatory gene 41BBL. The use of ionizing radiation to specifically enhance cancer immunotherapy (CIT) strategies through epigenetic modulation of genes stimulatory to CTLs will have a broad impact on cancer therapy approaches and will extend the use of radiation into new directions.

### 3.3 METHODS:

**Cell lines:** Human colorectal carcinoma cell lines HCT116 cells were obtained from the laboratory of tumor immunology and biology, NCI, NIH. The cell line SW620 was kindly provided by Zhi-Ren Liu (Wang, Gao et al. 2013) from Georgia State University, Department of Biology. All cells were cultured as recommended by ATCC and tested periodically to ensure absence of Mycoplasma. Cells were incubated at 37°C incubator with 5% CO2.

**Reagents:** 5-Aza-2′-deoxycytidine (5-Aza-dC) and Trichostatin A (TSA) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies recognizing histone H3 and acetylated histone H3 were from Millipore (Lake Placid, NY).

**Irradiation:** A RS-2000 biological X-ray irradiator (Rad source technology, Suwanee, GA) was used to irradiate tumor cells. Cells were irradiated at a dose rate of 2Gy/min by setting irradiator voltage and current at 160kV and 25mA, respectively. Cells were maintained in suspension and kept on ice during irradiation. Immediately after irradiation, the culture media was replaced with the fresh media.

**Quantitative real time PCR:** RNA was extracted from tumor cells using RNeasy mini kit (Qiagen Inc. Valencia, CA) according to manufacturer’s instructions. Purified RNA was DNase- treated by Rnase-free DNase (Qiagen Inc. Valencia, CA) following manufacturer’s instructions. Expression of OX40L and 41BBL mRNA was determined using real time RT-PCR.
cDNA was synthesized using 500ng of mRNA. Amplification of cDNA was done using DyNaAmo cDNA synthesis kit (Finnzymes. Vantaa, Finland). Quantitative RT-PCR was conducted using TaqMan gene expression assay (Applied Biosystems; OX40L; Hs00967195, 41BBL; Hs00169409, and HPRT; Hs99999909) according to manufacturer’s protocol. PCR thermal cycling condition was 50°C for 2min, 95°C for 10min, 40 cycles of 95°C for 15sec and 60°C for 1min in a total volume of 20 μl/reaction. Data were collected using a 7500 Real Time PCR System. All samples were run in duplicate. The expression level of each gene was normalized over non-irradiated samples. HPRT was used as an endogenous gene whose expression was unaltered by treatment. Data were analyzed using the comparative Ct method (Livak and Schmittgen 2001).

**Flow cytometry:** Cells were stained with primary labeled mAb CD137L (41BBL)-PE, and CD252 (OX40L)-PE purchased from BioLegend (Sen Diego, CA). Surface staining was done in cell staining buffer for 30min on ice. Flow cytometry data were acquired on BD Fortessa and analyzed with FlowJo software (TreeStar, version 9.6). Isotype control was kept less than 5% in all the samples. Expression was considered increased if the absolute percent positive population increased by 10% or greater.

**Chromatin Immunoprecipitation (ChIP) Assay:** ChIP assays were performed as previously described (Ali, Cacan et al. 2013). Briefly, 48h after irradiation (10Gy) and TSA (250nM) treatment cells were seeded at a density of 2.0 x 106 and crosslinked with 1% formaldehyde. The crosslinking reaction was stopped by the addition of 0.125 M glycine. Cell nuclei were isolated and concentrated by lysing in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, plus protease inhibitors) on ice followed by flash freezing in liquid nitrogen. Cell nuclei were sonicated using a Bioruptor to generate an average of 500 bp of sheared DNA;
DNA shearing was confirmed by subjecting lysates to agarose gel electrophoresis. Sonicated lysates were then precleared with salmon-sperm/agarose beads (Upstate) and 5% of the total lysate was stored as input for normalization. Half of the remaining lysate was immunoprecipitated with control antibody, and the other half was immunoprecipitated with 5μg of indicated antibody overnight at 4°C. Following an additional two hour immunoprecipitation with salmon-sperm/agarose beads, all samples were washed with each of the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl), LiCl buffer (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH 8.0), and 1xTE buffer. DNA was eluted with SDS elution buffer (1% SDS, 0.1M NaHCO3) and then cross-links were reversed overnight with 5M NaCl at 65°C and immunoprecipitated DNA was isolated using phenol:chloroform:isopropanol mix (Invitrogen) as per the manufacturer’s instructions. Isolated DNA was quantified by real time PCR on an ABI prism 7900 (Applied Biosystems, Foster City, CA) using the following primers and probe for 4-1BBL: forward, 5’- GCA CGC ATA GAC ATA AAT TGG C-3’, reverse, 5’-TCT GTG TCT CCC CGT TAA C-3’ and probe, 5’-TCC ACC CAC TGC AGA GGC AAT CAA-3’; for GAPDH: forward, 5’-AAT GAA TGG GCC GTT A-3’, reverse, 5’-TAG CCT CGC TCC ACC TGA CT-3’ and probe, 5’-CCT GCC GGT GAC TAA CCC TGC GCT CCT-3’; and for CIITA: forward, 5’-CAG TTG GGA TGC CAC TTC TGA-3’, reverse, 5’-TGG AGC AAC CAA GCA CCT ACT-3’ and probe, 5’-AAG CAC GTG GTG GTG GC-3’. Values generated from real time PCR reactions were calculated based on standard curves generated, were run in triplicate reactions and were analyzed using the SDS 2.0 program.

**Generation TAA-specific cytotoxic T-lymphocytes:** PBMCs from HLA-A2+ donors
were purchased from Hemacare (Van Nuys, CA) for the generation of antigen specific CTLs as described elsewhere (Tsang, Zaremba et al. 1995, Tsang, Zhu et al. 1997, Garnett, Palena et al. 2004). These leukopheresis samples, derived from HLA-A2+ patients, were obtained from Hemacare with appropriate informed consent. The use of these de-identified and commercially purchased tissues is under a human investigation protocol approved by the GSU IRB (exempt approval #H13305). Briefly, PMBCs were allowed to adhere to T150 flask for 2hr in AIM-V media. After 2hr, non-adherent cells were removed for lymphocyte isolation. Adherent cells were cultured for seven days in the presence of 100ng/ml of human granulocyte colony stimulating factor (GM-CSF) and 20ng/ml of IL-4 (Miltenyi Biotec, Inc. Auburn, CA) in AIM-V media and 500ng/ml CD40L (Millipore corporation, Temecula, CA) was added on day five to mature the DCs. On day seven DCs were collected and pulsed with 40μg/mL of HLA-A2 binding CEA peptide (YLSGANLNL (CAP-1; (Tsang, Zhu et al. 1997)),) peptide for 4hr in a 37°C 5% CO2 incubator. Unused DCs were frozen and stored in liquid nitrogen for subsequent restimulations. DCs loaded with peptide were subsequently irradiated with 50Gy. Immunomagnetic beads (Miltenyi Biotec Inc. Auburn, CA) were used to isolate CD8+ T cells from the non-adherent cells, following manufacturer instructions. Subsequently, isolated CD8+ T cells were co-cultured with peptide pulsed DCs. IL-7 (Millipore, Temecula, CA) at 10ng/ml and IL2 (Millipore, Temecula, CA) at 30U/ml were added to each well on the first and third day, respectively. T-cells were restimulated in this manner weekly using mature autologous DCs. Restimulated T cells were isolated over ficoll on day seven of culture, and used in a T-cell activation and survival assays.

**T-cell activation and survival assay:** 1 × 10³ colorectal tumor cells were irradiated or treated with TSA and plated in 96-well plate for 48hr. 1×10⁴ human CEA specific CD8+ T cells
were subsequently added and co-cultured with the colorectal tumor cells for 48hr. The percent of CD8+ T cells expressing CD69 or CD25 was measured by flow-cytometry. In parallel experiments, 7AAD was used to measure T-cell death. Flow cytometry data were acquired on BD Fortessa and analyzed with FlowJo software (TreeStar, version 9.6). The live cells population was gated on the FSC and SSC scatter plots for analysis of surface proteins. No live cells gate was used for cell death analysis samples. T cell stimulation for 24h using a (1X) cocktail of PMA and ionomycin (eBioscience) was used as a positive control for activation of TAA-specific T-cells. **Statistical Analysis:** Statistical differences between groups were calculated using un-paired two-tailed student T-test and calculated at 95% confidence using Graphpad by Prism. P-values less that 0.05 were considered statistically significant.

### 3.4 RESULTS:

**OX40L and 41BBL transcripts increase when DNMTs and HDACs are inhibited.**

Exposure of human carcinoma cell lines to sub-lethal radiation results in enhanced susceptibility to lysis by tumor specific cytotoxic T cells (CTLs) (Garnett, Palena et al. 2004, Gelbard, Garnett et al. 2006), and co-stimulatory proteins such as 41BBL and OX40L represent important regulators of effector CTL activity (Kroczek, Mages et al. 2004, Mescher, Curtsinger et al. 2006). These ligands for OX40 (OX40L/CD134L) and 41BB (41BBL/CD137L) are normally expressed on antigen presenting cells and activated endothelial cells (Liang, Gonzales et al. 2002, Dubovsky, McNeel et al. 2009). However, we have recently demonstrated expression of both proteins on tumor cells following treatment with radiation (submitted manuscript). Others have reported that changes in DNA methylation can upregulate the expression of costimulatory proteins in human tumor cells. The dynamics of the induction of DNA methylation
in irradiated tissue is currently unknown, and the role of methylation in expression of co-stimulatory molecules in response to radiation has not been investigated. We began our investigation by treating cells with 5-Aza-2’-deoxycytidine (5-Aza-dC) to inhibit DNA methylation in order to determine if this would alter expression of 41BBL or OX40L in human CRC cells. 5-Aza-dC is a DNA methyltransferase inhibitor (DNMTi) and is incorporated into DNA resulting in the rapid loss of DNA methyl transferase activity (Kane, Loda et al. 1997). The human colorectal cell line HCT116 was treated with 5-Aza-dC for 48 or 72hr, and OX40L and 41BBL mRNA was quantified. OX40L mRNA increased 1.4-fold (Fig. 3.1A) and 41BBL mRNA increased approximately 2-fold (Fig. 3.1B) at both 48 and 72hr post-treatment with 5-Aza-dC. OX40L mRNA increased over time in tumor cells treated with radiation, as there was a 2.3-fold increase at 48hr and a 3.6-fold increase at 72hr (Fig. 3.1A). Radiation induced a similar increase in 41BBL transcript levels. Interestingly, this temporal increase was not observed in tumor cells treated with 5-Aza-dC as relatively equal levels of both OX40L and 41BBL mRNA were detected after 48hr (gray bar) and 72hr (black bar) drug treatment. Moreover, the level of OX40L mRNA in cells treated 5-Aza-dC never exceeded those observed 72h post-IR. HDACs enzymes remove acetyl groups from histones and suppress gene transcription. Recent studies have shown that HDAC inhibitors also have immune-modulatory properties, such as increasing expression of HLA-DR, ICAM-1 and B7-2 in acute myeloid leukemia cell lines (Maeda, Towatari et al. 2000). We next asked if inhibition of HDACs would result in increased expression of OX40L and 41BBL similar to the increase seen in radiation-treated cells. For these experiments we used Trichostatin A (TSA), an inhibitor of the class I and class II family of HDAC enzymes, and evaluated OX40L and 41BBL mRNA expression.
HCT116 cells treated with TSA for 48hr (gray bar) contained more OX40L (Fig. 3.1A) and 41BBL mRNA (Fig. 3.1B) as compared to cells treated with 5-Aza-dC for 48 or 72hr. Messenger RNA levels decreased after 72hr (gray bar) of TSA treatment; we note that these cells were sensitive to TSA toxicity and began dying after 48hr TSA treatment though this loss of viability did not reach significance (Fig. 3.1C). It is likely that mRNA expression at 48 and 72h is not representative of early radiation events. As changes in promoter activation are often an early event we next evaluated cells at 8 and 24h post- treatment. We found no significant increase in OX40L mRNA. Surprisingly, while radiation did not induce a significant increase in 41BBL RNA at 8 or 24h, TSA did at both time points (Fig. 3.1). Indeed the increase in 41BBL mRNA at 24h (4-fold) exceeded levels observed after 48h treatment (Fig. 3.1B). 5-Aza-dC began to increase 41BBL as early as 24h after treatment by slightly greater that 2-fold (Fig. 3.1E) and this increase was maintained during 48 and 72h treatment (Fig. 3.1B). However, both radiation and TSA induced more 41BBL mRNA than 5-Aza-dC at their respective times of maximum induction. Overall, inhibition of both HDACs and DNMTs increased the levels of OX40L and 41BBL mRNA in HCT116 cells.
Figure 3.1 5AZA and TSA up-regulate the expression of OX40L and 41BBL mRNA in HCT116 cells.

(A) OX40L, and (B) 41BBL mRNA level was quantified using real time qPCR as described in Materials and Methods. Cells were plated and treated with 5AZA-dC (20uM), TSA (500nM) or 10Gy radiation (IR). Untreated control cells were cultured with the equivalent amount of DMSO present in drug treated samples. Adherent cell were collected and relative levels of OX40L and 41BBL transcripts were normalized to expression of an endogenous unchanging housekeeping gene. 48 (gray bar) and 72hr (black bar) mRNA values were compared to the level of gene expression see in untreated control samples (white bar), which was set to 1. The mean of data is graphed with SEM and the p-values are based on technical replicates. Experiments were repeated at least three times with similar results. (C) Cells were plated and untreated (DMSO) or treated with 5AZA-dC (20uM), TSA (500nM, 250nM or 125nM) or 10Gy radiation (IR). Both floating and adherent cells were collected after 48h of treatment and tumor cell viability was determining by trypan blue dye uptake. The mean of three separate experiments is graphed with SEM and the p-values are based on combined data from separate experiments. (D) OX40L and (E) 41BBL mRNA level was quantified. Eight (gray bar) and 24hr (black bar) mRNA values were compared to the level of gene expression see in untreated control samples (white bar). The mean of data is graphed with SEM and the p-values are based on technical replicates. Experiments were repeated at least three times with similar results. (F) Cells were plated and untreated (DMSO) or treated with 5AZA-dC (20uM), TSA (500nM, 250nM or 125nM) or 10Gy radiation (IR). Adherent cells were collected after 24, 48, and 72h of treatment and live tumor cell number was determining using a cell counter. The mean of three separate experiments is graphed with SEM and the p-values are based on combined data from separate experiments. Significant P-value shown in the indicated groups was determined at 48h. *indicates P value of <0.05, **indicates P value of <0.001.
To determine if epigenetic regulation of these genes was a common mechanism observable in carcinoma cells, we evaluated a second human CRC cell line, SW620. Again, SW620 cells were treated with 5-Aza-dC and TSA for 48 or 72hr and mRNA expression was measured by qRT-PCR. Overall, SW620 cells were more responsive to these treatments than HCT116 cells. 5-Aza-dC upregulated the expression of OX40L by 5.3 fold (Fig. 3.2A) and 41BBL by 3.5 fold (Fig. 3.2B) in SW620 cells treated for 72hr (gray bar). HDAC inhibition by TSA robustly altered the expression of 41BBL mRNA resulting in a 25-fold increase (Fig. 3.2B), and again resulted in a more modest upregulation of OX40L by 1.8-fold in SW620 cells treated for 72hr (Fig. 3.2A). Interestingly, these cells were more sensitive to TSA toxicity (Fig. 3.2C) and displayed significantly reduced cell numbers following 48 and 72h treatment with TSA concentrations ranging from 500nM to 125nM (Fig. 3.2F). Viable cell numbers decreased with TSA treatment time and dose (Fig. 3.2C), however, RNA was isolated and analyzed from the adherent and viable cells remaining in the culture (Fig. 3.2F) for our experiments (Fig. 3.2A & 3.B). Moreover, we observed similar cell numbers remaining between the treatment groups after 24h treatment with TSA and next evaluated changes in gene expression after 8 and 24h treatment. Increased message for OX40L could be detected as early as 24h in cells treated with radiation and 5-Aza-dC (Fig. 3.2D) and was further increased after 48 and 72h (Fig. 3.2A). The largest increase in OX40L in response to TSA treatment in SW620 cells was detected following treatment for 8h (2.7-fold) and was reduced slightly thereafter (2.1-fold). We also evaluated 41BBL expression after 8 and 24h treatment. No significant change in 41BBL mRNA was observed at either of the earlier time points in cells treated with 5-Aza-dC or radiation. In
contrast, a significant and robust increase in 41BBL expression could be detected after both 8 and 24 hr TSA treatment (20-fold) (Fig. 3.2E) that was further increased after 72hr treatment (Fig. 3.2B). We noted that the relative level of 41BBL mRNA in untreated control cells appeared to be higher than OX40L mRNA levels in both cell lines evaluated. Overall, the largest increases in mRNA were detected for 41BBL mRNA following treatment of CRC cells with TSA. We also found that TSA induced robust mRNA changes at earlier times of treatment (8h and 24h) while radiation-induced changes took longer and were greatest at later times of treatment (48h and 72h).

Following tumor cell irradiation only adherent and proliferating cells were harvested for analysis. We have previously demonstrated that irradiated tumor cells continue to proliferate and remain viable using this method (Ifeadi and Garnett-Benson 2012) (Fig. 3.1C & 3.2C). HCT116 cells appear to be less sensitive to TSA than SW620 cells as significantly reduced proliferation of treated HCT116 cells was detected only when the highest dose of TSA (500nM) was used (Fig. 3.1F). In contrast to TSA, there was very little impact of 5-Aza-dC on viability of tumor cells 48h after treatment in either cell line (Fig. 3.1C & 3.2C). Though cell numbers were slightly reduced following 5-Aza-dC treatment of SW620 cells this was not significant (Fig. 3.2F).
Figure 3.2 5AZA and TSA up-regulate the expression of OX40L and 41BBL mRNA in SW620 cells.

(A) OX40L, and (B) 41BBL mRNA level was quantified in SW620 tumor cells. Cells were plated and treated with 5AZA-dC (20uM), TSA (250nM) or 10Gy radiation (IR). Untreated control cells were cultured with the equivalent amount of DMSO present in drug treated samples. Adherent cell were collected and relative levels of OX40L and 41BBL transcripts were normalized to expression of an endogenous unchanging housekeeping gene. 48 (gray bar) and 72hr (black bar) mRNA values were compared to the level of gene expression see in untreated control samples (white bar), which was set to 1. The mean of data is graphed with SEM and the p-values are based on technical replicates. Experiments were repeated at least three times with similar results. (C) Cells were plated and untreated (DMSO) or treated with 5AZA-dC (20uM), TSA (500nM, 250nM or 125nM) or 10Gy radiation (IR). Both floating and adherent cells were collected after 48h of treatment and tumor cell viability was determining by trypan blue dye uptake. The mean of three separate experiments is graphed with SEM and the p-values are based on combined data from separate experiments. (D) OX40L and (E) 41BBL mRNA level was quantified. Eight (gray bar) and 24hr (black bar) mRNA values were compared to the level of gene expression see in untreated control samples (white bar). The mean of data is graphed with SEM and the p-values are based on technical replicates. Experiments were repeated at least three times with similar results. (F) Cells were plated and untreated (DMSO) or treated with 5AZA-dC (20uM), TSA (500nM, 250nM or 125nM) or 10Gy radiation (IR). Adherent cells were collected after 24, 48, and 72h of treatment and live tumor cell number was determining using a cell counter. The mean of three separate experiments is graphed with SEM and the p-values are based
on combined data from separate experiments. Significant P-value shown in the indicated TSA-treatment groups was determined at 48h. *indicates P value of <0.05, **indicates P value of <0.001.

**Surface expression of OX40L and 41BBL protein increases when DNMTs and HDACs are inhibited.**

The largest increase in mRNA was detected in SW620 cells treated with 5-Aza-dC (OX40L, Fig. 3.2A) or TSA (41BBL, Fig. 3.2B), and we wanted to determine if increased protein expression also occurred. There was no significant difference in the total cell number (Fig. 3.2F) or the viability (data not shown) of SW620 cells following 24h hour treatment with 125nM TSA. As such, we evaluated surface expression of 41BBL protein by flow cytometry after 24hr treatment with either TSA (125nM) or 5-Aza-dC. Untreated SW620 cells expressed modest amounts of 41BBL on the surface (38.4%), and as expected radiation increased the frequency to 60.4% (Fig. 3.3A). Treatment with 5-Aza-dC had less of an impact on protein expression and 48% of cells expressed 41BBL after treatment with the drug (Fig. 3.3C). In contrast, TSA had a much larger impact on protein expression and, similar to radiation-induced expression, 61% of TSA-treated SW620 cells expressed 41BBL (Fig. 3.3D) (66% in cells treated with 500nM). Thus, relative changes in 41BBL protein expression (Fig. 3.3A) and 41BBL mRNA quantities (Fig. 3.2B) were similar in this cell line. We next evaluated OX40L protein expression. SW620 tumor cells increased surface OX40L following exposure to 10Gy of radiation (IR; 46.4%), as compared to untreated cells (DMSO; 23.1%) (Fig. 3.3E). TSA increased protein expression of OX40L to a similar magnitude (46.7%) as irradiated cells. Again, as seen with 41BBL, there was a smaller increase in surface OX40L detected (31.1%) following treatment with 5-Aza-dC. This was surprisingly low given the 3- to 5-fold increase in OX40L mRNA seen in these cells upon 5-Aza
dC treatment (Fig. 3.2A & 3.2D). Thus, mRNA modulation of the two genes (Fig. 3.2) was similar to protein changes by TSA and radiation (Fig. 3.3), but not 5-Aza-dC. Furthermore, the modulation of OX40L protein was less robust than that observed for 41BBL protein in SW620 cells (Fig. 3.3B-3.3D & 3.3F-H).

Overall, our results show that TSA-treated cells demonstrated the largest increase in protein expression, and the increase was at least as good as that observed following treatment with radiation (Fig. 3.3). As such, we focused our subsequent experiments on the impact of TSA HDAC inhibition on co-stimulatory molecule expression. Our data reveal increased expression of OX40L (53.2%) 48hr after irradiation of HCT116 cells as compared to untreated (0Gy) cells (30.7%) (Fig. 3.4A-B & 3.4E). Expression of OX40L is detected on the surface of 56.6% TSA-treated HCT116 cells (Fig. 3.4F) as compared to expression in control (DMSO) cells (38.2%). Expression of 41BBL was also enhanced to much greater levels following treatment with both IR (43.6% 10Gy) (Fig. 3.4C-D & 3.4E) and TSA (58.6%-250nM TSA versus 23%-untreated) at 48hr (Fig. 3.4G). The relative change in 41BBL surface expression compared to untreated cells was larger that the change in OX40L following TSA treatment in HCT116 cells (Fig. 3.4H). Elevated levels of these co-stimulatory proteins could still be detected after 3- to 4-days of TSA treatment and radiation-induced changes where greater after 72h (data not shown). Overall, both HCT116 and SW620 cells showed a more robust increase in expression of 41BBL as compared to OX40L protein expression upon TSA treatment.
Figure 3.3 TSA and ionizing radiation increase surface expression of co-stimulatory molecules in SW620 cells more than 5AZA.

(A-D) 41BBL, and (E-H) OX40L protein expression on the surface of SW620 cells was evaluated by flow cytometry. Cells were either untreated (DMSO), or treated with 5-Aza-dC (20uM), TSA (125nM) or 10Gy radiation (IR). Adherent cells were harvested 24hr post treatment, and stained with PE-labeled antibody to human OX40L or 41BBL. Isotype control stained cells were analyzed for each treatment group individually and set to 5% positive. Isotype control staining is shown as the gray filled histogram and protein specific staining is shown as black line histogram for the FACS plot data graphed in A and E. Experiments were repeated twice with similar results.
Figure 3.4 TSA and ionizing radiation treated HCT116 cells increase surface levels of 41BBL protein more than OX40L protein.

(A, B and F) OX40L, and (C, D and G) 41BBL protein expression on the surface of HCT116 cells was evaluated by flow cytometry. (E) Cells were untreated (0Gy) or treated with 10Gy radiation (IR). (H) In separate experiments HCT116 cells were untreated (DMSO) or treated with either TSA (250nM). After 48hr, cells were collected and stained with PE-labeled antibody to either OX40L and 41BBL. Control cell staining with a non-specific isotype control antibody was less than 5% positive. Isotype control staining is shown as the gray filled histogram and protein specific staining is shown as black line histogram for the FACS plot data graphed in E and H. Dotted line histogram in F and G indicates specific protein expression in untreated cells. Values shown on histograms are for treated cells and untreated and treated values are displayed graphically in H for comparison. 

Experiments were repeated three times with similar results.
Radiation increases Histone H3 acetylation at the 41BBL promoter.

Our data indicates that 41BBL and OX40L are epigenetically regulated and radiation increases expression of these genes in CRC cell lines. Histone acetylation facilitates transcription initiation by loosening interactions between the histones and DNA. Whereas, HDACs remove these acetyl groups from histones which reduces transcription. We observed that inhibition of HDACs by TSA increased 41BBL mRNA expression and surface protein levels in tumor cells. We observed that radiation increase 41BBL gene expression in a similar manner but was more robust at later times during treatment. As radiation has been reported to inhibit HDACs (Han, Zhang et al. 2012), we next wanted to determine if radiation could be increasing 41BBL expression by promoting increased promoter histone acetylation. To explore whether histone modifications are regulated in part by radiation, we assessed levels of histone acetylation at the 41BBL promoters using chromatin immunoprecipitation (ChIP) assays in both non-radiated and irradiated HCT116 cells. We evaluated promoter acetylation at 48h post-IR when radiation-induced changes in mRNA levels were robust (Fig. 3.1). TSA-treated HCT116 cells were used as a positive control for 41BBL promoter acetylation. As TSA inhibits HDAC activity, we expect to see robust increases in histone acetylation status following TSA treatment. As expected, Figure 3.5A shows increased acetylation at the 41BBL promoter following TSA treatment (gray bar) as compared to untreated control cells (white bar). Surprisingly, acetylated H3 histone levels were significantly higher at 41BBL promoters in irradiated cells (black bar). In contrast, similar levels of acetylated histone H3 were associated with the GAPDH promoter in both untreated and irradiated HCT116 cells (Fig. 3.5B). Moreover, total levels of histone H3 were similar at 41BBL and GAPDH promoters revealing that there was no global change in overall histone levels (data not shown). These data indicate that radiation increases 41BBL expression by increasing histone
acetylation. To determine if radiation non-specifically increases histone acetylation levels at other genes, histone H3 ChIP assays were performed on the Class II Transactivator (CIITA) promoter IV. Histone H3 acetylation levels were similar for non-irradiated, irradiated and TSA treated cells at CIITA promoter IV (Fig. 3.5C), which suggests gene-specificity for radiation-induced 41BBL promoter acetylation, likely via HDAC inhibition.

Figure 3.5 Histone acetylation at 41BBL promoters in non-radiated and irradiated cells.

ChIP assays were carried out in non-irradiated, irradiated (10 Gy), and TSA-treated HCT116 cells. Following 48h of TSA treatment (250nM), lysates were immunoprecipitated with control antibody or with anti-acetyl histone H3. Associated DNA was isolated and analyzed via real time PCR using primers spanning the 41BBL, GAPDH and CIITA promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Input values represent 5% of the total cell lysate. Values represent mean ± SEM of three independent experiments. **P<0.005. A. Global levels of Histone H3 acetylation associated with the 41BBL promoter. B. Global levels of Histone H3 acetylation associated with the GAPDH promoter. C. Global levels of acetylated Histone H3 associated with the CIITA promoter.
Figure 3.6: Enhanced survival of T-cells co-incubated with CRC tumor cells treated with TSA.

SW620 and HCT116 cells were treated with DMSO, TSA (250uM), or radiation (10Gy). After 48hr, tumor cell were harvested and co-cultured with human CEA-specific CD8+ T-cells for another 48hr. Cell death was also measured in CD8+ T-cells cultured alone. Data were gated first gated on CD8+ population and the percent of 7AAD-positive within the CD8+ cell population is shown in (A-D) as zebra plots. (E) Representative graphs showing percentage of dead CD8+ T-cells. Experiments were repeated twice with similar results.

Treatment of CRC cells with TSA enhances T-cell survival and activation similar to co-incubation with irradiated tumor cells.

To investigate the impact of HDAC inhibition in tumor cells on T-cell survival, we measured T-cell death by 7AAD staining after 48hr co-incubation with tumor cells. 7AAD+ staining determined cell death of 8.96% of CD8+ T cells incubated alone (Fig. 3.6A). The frequency of dead CD8+ T cells increased to 24.8% following co-incubation with untreated
SW620 cells (Fig. 3.6B). Death of T-cells following interaction with tumor cells has been reported by others, and is thought to be caused by tumor expressed PDL1, FasL and/or activation induced cell death (AICD) (Zaks, Chappell et al. 1999, Chiou, Sheu et al. 2005, Prado-Garcia, Romero-Garcia et al. 2012). Incubation of T-cells with SW620 cells, that had been treated with TSA for 48hr, reduced the percentage of dead T cells to 17.6% (Fig. 3.6D) similar to incubation with irradiated tumor cells (16.6%). A reduction in T-cell death (18%) was also observed when T-cells were co-incubated with TSA-treated HCT116 cells as compared to untreated tumor cells (26%) (Fig. 3.6E). These data indicate that HDAC inhibition by TSA treatment of tumor cells increases the survival of CD8+ T cells following co-incubation with tumor cells. CD25 and CD69 are surface markers expressed on activated T cells (Zola 2000). Data from our lab supports the hypothesis that changes in the expression of tumor-expressed 41BBL and OX40L contribute to increased killing of irradiated tumor cells by CTLs (submitted manuscript). We have also observed increased expression of CD25 and CD69 on T cells following co-incubation with irradiated tumor cells compared to non-irradiated tumor cells. Lastly, we have observed increased viability of T cells cultured with irradiated tumor cells. We next determined if tumor cells treated with HDACi induced similar changes in T cell activation. Non-treated, irradiated or TSA treated tumor cells were co-cultured with CD8+ T cells, and after 48hr the expression of CD25 on T cells was measured by flow cytometry. We found that 29.5% of CD8+ T cells incubated with untreated tumor cells expressed CD25 (Fig. 3.7A), and this frequency was reduced compared to activation of T cells incubated alone (34.1%) (Fig. 3.7D). This reduction is not surprising as reduced activity and activation of T-cells following interaction with tumor cells has been described by others (Zaks, Chappell et al. 1999, Chiou, Sheu et al. 2005, Prado-Garcia, Romero-Garcia et al. 2012). The frequency of CD25+ within the CD8+T cell population
increased following co-incubation with either radiation-treated (Fig. 3.7B) or TSA-treated tumor cells to 35.3% (Fig. 3.7C). In fact, the frequency of activated T cells following co-incubation with TSA-treated cells was equal to T cells not co-incubated with tumor cells (34.1%). CD25 expression in T-cells activated with PMA and ionomycin are shown as a positive control (Fig. 3.7E). We evaluated a second CRC cell line and found that TSA-treated HCT116 cells also increased the frequency of CD8+CD25+ cells to 41%, as compared to the frequency activated in the presence of untreated HCT116 cells (36.6%) (Fig. 3.7F). Irradiated tumor cells also increased CD25+ expression to 36.4% and the dynamics of T-cell activated were similar in repeat experiments. We observed a similar increase in the frequency of CD69+ T cells following co- incubated with TSA-treated or irradiated tumor cells (data not shown). These data suggest that T cells exposed to TSA treated tumor cells have improved activation. As a component of the IL-2 receptor, CD25 it has been linked to increased survival in studies by others and thus could be a contributor to the increased survival we observe following TSA treatment (Fig. 3.6).
Figure 3.7 TSA-treated CRC tumor cells induce enhanced activation of CD8+ T-cells.

SW620 cells were either (A) untreated, (B) irradiated or incubated with (C) 250uM TSA for 48hr or as previously described. Tumor cells were subsequently co-cultured with human CEA-specific CD8+T-cells for 48hr and the frequency of CD8+CD25+ T-cells was measured by flowcytometry. T-cell activation was also measured in (D) CD8+ T-cells cultured alone or (E) activated with PMA and ionomycin (P/I) for 24h. Data were gated first gated on CD8+ population and the percent of CD25+ cells within the CD8+ cell population is shown in (A-E) as histogram plots. (F) a summary graph of results showing percentage of CD8+ T-cells expressing CD25 in both SW620 and HCT116 cells. Experiments were repeated twice with similar results.
3.5 DISCUSSION:

Modulation of costimulatory molecules such as OX40L and 41BBL appear to be particularly important for maintaining effective immune responses against self-antigens presented by tumor cells. Here, we report that costimulatory molecule promoter histones can be acetylated in colorectal tumors in response to sub-lethal radiation (Fig. 3.5A). Most studies of radiation-induced gene expression have used large cytotoxic doses of radiation, and mechanisms of altered gene expression are much less explored in cells receiving low or sub-lethal doses of radiation. Results of this study suggest that radiation therapy may be useful to specifically modulate gene expression within tumor targets. This mechanism would be useful against radioresistant cancer cells, and could occur even in the absence of immunogenic cell death (cell death that invokes enhanced antigen processing and presentation) (Tesniere, Panaretakis et al. 2008). Full understanding of specific mechanisms of immunogenic modulation (altered expression of immune relevant genes) (Hodge, Garnett et al. 2013) of irradiated tumor cells will be required to determine how to best utilize radiation as a “tool” to enhance cancer immunotherapy approaches. Dramatic changes in DNA methylation are common in cancer, and manifest primarily as global DNA hypomethylation, paralleled by local hypermethylation at gene promoters resulting in loss of gene expression (Feinberg and Vogelstein 1983, Rhee, Bachman et al. 2002). Tumor cells down-regulate the expression of many genes needed for induction of effective anti-tumor immune activity (Zamai, Rana et al. 1994, French and Tschopp 2002, Bubenik 2003, Slavin-Chiorini, Catalfamo et al. 2004), and DNA methylation may be one mechanism employed to accomplish this. Our studies reveal that inhibition of DNMT in tumor cells using 5-Aza-dC could induce mRNA expression of both OX40L and 41BBL on two different CRC cell lines (Fig. 3.1 & 3.2). Although a greater than 5-fold induction of mRNA was
detected in SW620 cells treated with 5-Aza-dC, we did not observe a robust increase in protein expression upon 5-Aza-dC treatment of these cells (Fig. 3.3). These discordant results could simply be a result of the time of evaluation post-treatment. 41BBL mRNA was maximally increased 72hr post-treatment with 5-Aza-dC, while protein expression was evaluated after 24h of treatment to keep cell death low at time of evaluation. Current studies are underway to determine if 5-Aza-dC can indeed upregulate protein expression at later times post-treatment.

HDAC inhibition has been shown to be involved in modulating the expression of TNF family members (Sutheesophon, Nishimura et al. 2005, Earel, VanOosten et al. 2006). In this study we extended analysis to other TNF family members and found that both 41BBL and OX40L expression could also be modulated by inhibition of HDACs. We found that the expression of both OX40L and 41BBL was increased on the surface of tumor cells treated with TSA for 24hr (Fig. 3.3) or 48hr (Fig. 3.4). Interestingly, the impact of HDAC inhibition by TSA on 41BBL protein expression was much more robust than changes observed in the expression of OX40L protein following TSA treatment. Studies are currently underway to evaluate changes in histone acetylation at the OX40L promoter to determine how acetylation is impacted by TSA inhibition of HDACs. We also observed increased expression of co-stimulatory proteins as long as four days after TSA-treatment and irradiation. While many of the cellular stress response genes are acute response genes whose expression is altered transiently, other genes remain altered for prolonged periods of time (Chen, Quintans et al. 1995, Woloschak and Paunesku 1997, Fornace, Amundson et al. 2002). As such, altered gene expression following radiation treatment that is sustained is not unexpected.

The TNF family includes numerous costimulatory molecules known to play an important role in CD8+ T cell activation and survival. We found that inhibition of HDACs in tumor cells
resulted in enhanced T-cell survival (Fig. 3.6) and activation (Fig. 3.7). To our knowledge this is the first study to explore the impact of radiation-induced epigenetic changes in tumor cells on the quality of anti-tumor CTLs. We are currently investigating if, by promoting T-cell survival and activation, the altered expression of these specific genes by HDACi enhances the tumor cells’ susceptibility to T-cell-mediated immune attack in a manner similar to observations in irradiated tumor cells (submitted manuscript). Future studies seek to more fully investigate if increase signaling through CD25 is directly responsible for the increased survival of T-cells by evaluating T cells after shorter periods of co-incubation as well as investing intracellular regulators of T-cell apoptosis.

HDACs enzymes reverse the activity of HATs by removing acetyl group and thus suppressing gene transcription. In several tumors, the expression of HATs is down-regulated, whereas HDACs is upregulated (Ropero and Esteller 2007, Sharma, Groselj et al. 2013). As previously mentioned, alteration of HAT and HDAC activity has been observed in tumor cell lines. HDACi induce a potent anticancer response by inhibiting HDACs (Redner, Wang et al. 1999, Saunders, Dicker et al. 1999). HDACi have various biological effects, such as inhibition of cell cycle at G1/G2 phase, induction of differentiation and apoptosis of tumor cells (Rajgolikar, Chan et al. 1998, Kosugi, Towatari et al. 1999, Yoshida and Horinouchi 1999).

Our results reveal that radiation treatment changes the epigenetic landscape of the 41BBL gene via an increase in histone acetylation, displaying a marked increase in H3 acetylation at this specific promoter, as compared to our positive control of cells treated with the HDACi, TSA. We also observed that TSA induced robust 41BBL mRNA changes at earlier times of treatment (8h and 24h) while radiation-induced changes took longer and were greatest at later times of treatment (48h and 72h). These data, in combination with increase promoter
acetylation, suggest that radiation mediated effects take longer to modulate histone acetylation events than direct modulators such as TSA. This could be related to differences in modulation of HATs versus HDAC inhibitors. Current lab efforts are pursuing the mechanism for these epigenetic changes in primary carcinoma cells; specifically, does IR treatment change the activity of HATs, HDACs or both? If HDACs are involved, specific HDAC inhibitors will be utilized to identify which HDACs suppression(s) are vital for the upregulation of 41BBL expression. Also, how long can these epigenetic changes be maintained to promote increased effector T-cell function? Finally, we note that expression of OX40L and 41BBL varied with different concentrations of drug exposure. Our focus here is to describe a novel gene regulatory mechanism by epigenetic modification in response to irradiation. However, the application of clinically relevant doses of TSA and 5-Aza-dC, which might be combined with radiation, will also require a further investigation in a broad range of tumor cells. The current study was meant to enhance our ability to design cancer immunotherapy (CIT) approaches in combination with RT. A better understanding of how IR modulates the expression of 41BBL and OX40L will allow improvement in our ability to use RT to specifically enhance CTL killing. Epigenetic mechanisms of gene expression could be an alternative therapeutic approach to enhancing these important T-cell signals. This approach is particularly relevant given the toxicities associated with using agonistic antibodies to 41BB and anti-OX40 antibodies in the clinic (Garber 2011, Weinberg, Morris et al. 2011). Alternate ways of triggering these signal pathways would be widely applicable in current CIT approaches. Furthermore, if radiation is shown to have a profound and consistent effect on immune stimulatory gene expression, this would provide support for using IR in conjunction with CIT strategies to specifically enhance such signals to T-cells arriving at tumor sites and optimize anti-tumor CTL responses.
4 The impact of radiation directly on T\textsubscript{REG} viability and phenotype and indirectly via tumor modulation

4.1 ABSTRACT

CD4+CD25+ T regulatory (T\textsubscript{REG}) cells are critical in maintaining self-tolerance to prevent autoimmune disease. On the other hand, T\textsubscript{REG} infiltration into tumors is undesirable as it inhibits endogenous cytotoxic antitumor immune responses, thereby boosting cancer escape from immune-surveillance (Curiel 2007). As a result, T\textsubscript{REGS} hinder the success of cancer immunotherapies in patients. This preliminary and exploratory study was designed to investigate the effects of immunogenic modulation of tumor cells by sub-lethal irradiation on T\textsubscript{REG} frequency and viability. CD4+CD25+FoxP3+ TREGS were purified from human PBMC samples. T\textsubscript{REGS} were co-cultured with human colorectal cancer cells and exposed to radiation together and separately. We found that irradiated human tumor cells do not robustly upregulate expression of T\textsubscript{REG} stimulating/promoting molecules. In vivo, we found that local RT to tumor-bearing mice resulted in reduced frequencies of T\textsubscript{REG} cells within the tumor. In vitro, T\textsubscript{REGS} were less sensitive to cytotoxicity from IR as compared to CD4+ non- T\textsubscript{REGS}. Additionally, no significant difference was observed in T\textsubscript{REG} vs non- T\textsubscript{REG} viability after co-culture with irradiated tumor cells. Interestingly, we found that IR modulated the phenotype of T\textsubscript{REGS} and reduced expression of T\textsubscript{REG} associated markers including Fox-P3. Importantly, the loss of FOXP3-mediated suppressive functions may be linked to increased antitumor immune response. Overall, our findings suggest that sub-lethal doses of IR may reduce T\textsubscript{REG} detection within tumor tissues by altering their phenotype, and further, that it may reduce T\textsubscript{REG} mediated activities by reducing expression of genes required for their suppressive function.
4.2 INTRODUCTION

T\textsubscript{REGS} comprise 1-3% of total peripheral CD4+ T cells in human and 5-10% of mouse splenocytes. Regulatory T cells express high levels of CD25 (interleukin-2 receptor alpha chain) and forkhead box P3 (FOXP3); FOXP3 is recognized as a specific marker of T\textsubscript{REGS} and it controls transcription of several genes responsible for the suppressive function of T\textsubscript{REGS} (Baecher-Allan, Brown et al. 2001, Brunkow, Jeffery et al. 2001). T\textsubscript{REGS} are important cells of the immune system that inhibit and control unwanted immune responses against self-antigens, thus preventing the development of autoimmune diseases. Tumor cells secrete different chemokines such as CCL2, CCL-17 and CCL-22 which enhance trafficking of natural and induced T\textsubscript{REGS} (n/i T\textsubscript{REGS}) from peripheral circulation to tumor site (Gobert, Treilleux et al. 2009) (Facciabene, Motz et al. 2012). Recruited T\textsubscript{REGS} suppress the effector function of cytotoxic T cells present within the tumor by several mechanisms(Curiel 2007). In addition, transforming growth factor beta (TGF-\beta), released by tumor cells, increases expansion of preexisting T\textsubscript{REGS} within tumor (Yamagiwa, Gray et al. 2001, Ghiringhelli, Puig et al. 2005). Apart from recruitment and expansion of T\textsubscript{REGS} by tumor cells, naïve CD4+, memory CD4+ cells can be converted to T\textsubscript{REGS} in the presence of IL-10, TGF\beta, and programmed death ligand-1 (PD-L1) (Puccetti and Grohmann 2007, Zhou, Zhou et al. 2009, Pothoven, Kheradmand et al. 2010).

Increased number of T\textsubscript{REGS} has been found in several different cancers; Woo and colleagues have reported increased number of intra-tumoral T\textsubscript{REGS} in lung and ovarian cancers (Clarke, Betts et al. 2006, Wolf, Rumpold et al. 2006). In report by Ling and colleagues, both intra-tumor and systemic accumulation of T\textsubscript{REGS} has been reported in colorectal cancer patients. In addition, the proportion of T\textsubscript{REGS} in peripheral blood of CRC patients was four times higher than the control. Furthermore, an increased frequency of T\textsubscript{REGS} was reported within tumor (19.2)
than the control (normal colon tissue, 9%) (Ling, Pratap et al. 2007). Infiltration of \( \text{T}_{\text{REGS}} \) in gut under normal condition is desired, as it is a tolerogenic organ, however increased accumulation of \( \text{T}_{\text{REGS}} \) in tumor tissue can inhibit effective tumor control by CD8+ T cells. Therefore, intra-tumoral infiltration of \( \text{T}_{\text{REGS}} \) is a major obstacle to success of current cancer immunotherapies.

We previously reported that sub-lethal doses of radiation modulated tumors making them more immunogenic for CD8+ effector T cells. Surprisingly, relatively few (and contradictory data) exist in the literature regarding effect of irradiation on \( \text{T}_{\text{REGS}} \) directly, or the impact of irradiated tumors on \( \text{T}_{\text{REGS}} \). This is an important question because the cytolytic activity of activated CD8+ effector cells can be directly inhibited by the presence of regulatory T cells. It is unclear if immunogenic modulation of tumors by sub-lethal irradiation can influence \( \text{T}_{\text{REG}} \) number and function. In addition, there is little known about the effect of radiation on human \( \text{T}_{\text{REGS}} \) phenotype. The present study was designed to begin exploring the hypothesis that irradiated tumor cells undergo immunogenic modulation that decreases \( \text{T}_{\text{REGS}} \) number and activity.

### 4.3 MATERIALS AND METHODS

**Cell lines:** Human colorectal carcinoma cell lines HCT116 were obtained from the laboratory of tumor immunology and biology, LTIB, NCI, NIH. SW620, Colo205 and human breast carcinoma cells lines MDA231 and MDA468 were purchased from ATCC cells. All cells were cultured as recommended by ATCC and tested periodically to ensure absence of Mycoplasma. Cells were incubated at 37°C incubator with 5% CO2.

**Irradiation:** A RS-2000 biological X-ray irradiator (Rad source technology, Suwanee, GA) was used to irradiate tumor cells. Cells were irradiated at a dose rate of 2Gy/min at voltage
and current of 160kV and 25mA, respectively. Cells were maintained in suspension and kept on ice during irradiation. Immediately after irradiation, cells were centrifuged and cells were plated in tissue culture plates in fresh media.

**Generation and culture of T\textsubscript{REGS}:** PBMCs from HLA-A2+ donors were purchased from Hemacare (Van Nuys, CA) for the generation of T\textsubscript{REGS}. Peripheral blood mononuclear cells were separated by density gradient centrifugation using Ficoll-Hypaque. Tregs were purified using the Miltenyi Biotec’s CD4+CD25+ regulatory T cells isolation kit. The freshly isolated cells cultured in a medium containing 1000IU/ml of IL-2, and activated with CD3 and CD28 beads for 14 days (Hoffmann, Eder et al. 2004, Battaglia, Di Schino et al. 2005). Likewise CD4+ T cells were cultured under same condition. The purity of T\textsubscript{REGS} was determined by staining for CD4, CD25 and FOXP3 markers using Human regulatory T cells staining kit (ebioscience) by flow cytometry. To determine if changes are specific for T\textsubscript{REGS}, non- T\textsubscript{REGS} (CD4+ T cells) were cultured under same conditions and evaluated.

**RNA isolation:** At 24 or 48hr post-IR, mRNA was extracted from tumor cells using RNeasy mini kit (Qiagen Inc. Valencia, CA) according to manufacturer’s instructions. Purified RNA was DNase-treated by Rnase-free DNase (Qiagen Inc. Valencia, CA) following manufacturer’s instructions.

**Quantitative real time PCR for CCL22 and TGF\textbeta:** Expression of CCL22 and TGF-\textbeta was mRNA was determined using real time qRT-PCR. cDNA was synthesized using 500ng of mRNA. Amplification of cDNA was done using DyNaAmo cDNA synthesis kit (Finnzymes.
Vantaa, Finland). Quantitative RT-PCR was conducted using sybr green. PCR thermal cycling condition was 50°C for 2min, 95°C for 5min, 40 cycles of 95°C for 30sec and 50°C for 35sec in a total volume of 20 μl/reaction. Data were collected using a 7500 Real Time PCR System. All samples were run in duplicate. The expression level of each gene was compared between irradiated and non-irradiated samples 24 or 48hr post-IR. Hypoxanthine phosphoribosyltransferase (HPRT) was used as an endogenous house-keeping control gene and samples were normalized to expression of this gene, which was unchanged by radiation. Data were analyzed using the comparative ΔΔCt method.

**Flow cytometry:** Cells were stained with primary labeled mAb PDL1-PE from BD biosciences (San Diego, CA) and Human regulatory T cells staining kit (ebioscience). Surface staining was done in cell staining buffer for 30min on ice. Annexin-V-PE and 7AAD dye were purchased from BD biosciences (San Diego, CA) and used according to manufacturers instructions to measure cell death. Flow cytometry data were acquired on BD Fortessa and analyzed with FlowJo software (TreeStar, version 9.6). The live cells population was gated on the FSC and SSC scatter plots for analysis of surface proteins. Samples were stained with the appropriate isotype control antibodies and gates were set to less than 5% in all isotype control samples.

**Statistical Analysis:** For graphed flow cytometry data, the mean of three independent experiments was calculated and an un-paired one-tailed student T-test and one-way analysis of variance (ANOVA) were performed using Graph pad by Prism.
4.4 RESULTS

Radiation therapy decreases T\textsubscript{REG} frequencies within tumors in vivo

MC38 murine colon carcinoma cells were implanted s.c. into C57BL/6 mice. Local RT (8-10Gy) was given to the tumors 15 days after implant. The percentage of T\textsubscript{REGS} and CD4+ T cells was determined by flow-cytometry. We found that T\textsubscript{REG} numbers were reduced within tumors after 8Gy radiation as compared to CD4+ non- T\textsubscript{REGS} (Fig 4.1A). Moreover, the frequencies of both T\textsubscript{REGS} and CD4+ non-T\textsubscript{REGS} did not change after treatment in the spleen (data not shown). We also evaluated changes in intratumoral T\textsubscript{REG} numbers using fluorescent immunohistochemical approaches and observed a reduction in FoxP3+ cells on tumors treated with 10Gy radiation (Fig 4.1B). To determine if this was unique to the murine tumor cell line we selected we performed a similar analysis of a second murine tumor cell line, 4T1. 4T1 cells are derived from a murine mammary tumor cell line as thus also represent a different type of cancer. Tumor cells were implanted s.c. and treated with 8Gy between day 10 and 15 (to allow for the treatment of tumors of relatively equal size). Forty-eight hours after RT tumors were excised, sectioned and stained by immunofluorescence for Fox-P3 cells. RT resulted in a significant and robust reduction in the presence of FoxP3 cells present in the tumor tissue (Fig 4.2). These data suggest that the doses of radiation used in this study are able to reduce the frequency of T\textsubscript{REGS} in vivo and that this is observable in more than one murine tumor type.
Figure 4.1. The number of T\textsubscript{REG} cells following vivo RT of murine colorectal tumors.

A. Percentage of CD4+ T cells and CD4+CD25+Foxp3+ cells from \textit{in vivo} tumor samples determined by flow cytometry. Female C57BL/6 mice were s.c implanted with 5 x 10\textsuperscript{5} MC38 colorectal tumor cells. After 10-15 days tumors (200-250mm\textsuperscript{3}) were irradiated with 0 or 8 Gy. 48h later mice were euthanized and tumors excised. Tissue was homogenized into a single cell suspension, red blood cells lysed and samples stained for overall CD4+ cell numbers and CD4+CD25\textsuperscript{hi}Foxp3+ T\textsubscript{REG} cell numbers. 

B. The number of FoxP3+ cells per mm\textsuperscript{2} in tumor tissue. Irradiated (10 Gy) and non-irradiated (0 Gy) tumor tissue was excised 24-hrs post-IR. Tissue was fixed, sliced, and immunofluorescence performed to detect FoxP3 expression. Each dot represents the average FoxP3+ cells in individually treated mouse tumor (4-6 view regions were counted per tumor). Unpaired t Test (one-tailed) was performed to assess statistical significance; p=0.0911.

Figure 4.2. The number of FoxP3+ cells following vivo RT of murine breast tumors.

Irradiated (8 Gy) and non-irradiated (0 Gy) female Balb/c mice implanted s.c. with 8 x 10\textsuperscript{5} 4T1 breast cells were irradiated 10-15 day post-implant. 48-hrs post treatment tumor tissue was excised, fixed, sliced, and immunofluorescence performed to detect FoxP3 expression. A. Each dot represents the average FoxP3+ cells in individually treated mouse tumor (4-6 view regions were counted per tumor). An unpaired t Test (one-tailed) was performed to assess statistical
Irradiation increases TGF-β mRNA but does not change other TREG promoting molecules

We previously reported that IR could modulate tumor cells to express surface proteins that were stimulatory to CD8+ effector T cells (Chapter 1). On the contrary, both OX-40L and 4-1BB have been reported to reduce TREG function. This supports the notion that IR modulation of tumors favors promotion of effector T cell activity instead of TREG activities. This is in contrast to speculation by many who predict that RT will promote TREG accumulation based on the finding of a single study using high dose RT. Thus it is critical to investigate if radiation is capable of modulating important signals to TREGs. This could occur through change in the expression of TREG promoting molecules that could alter recruitment or maintenance of TREGs in tumor cells following RT. To determine whether irradiated tumor cells secrete or express molecules that could alter TREG numbers, we evaluated expression of TREG promoting cytokines, chemokines and ligands from irradiated tumors. We reasoned that reduced expression of such proteins following IR could be responsible for the reduced presences of TREGs in irradiated tumors. CCL22 is a chemokine, which recruits TREGs. CCL22 mRNA was measured in HCT116 cells 8-48h-post irradiation, and we observed no significant change in mRNA expression over the time (Fig 4.3A). Next we examined if surface expression of PDL1, which causes conversion of CD4 cells into TREGs, was reduced following irradiation of tumor cells. Surface expression was measured by flow cytometry following 10Gy tumor radiation (Fig 4.3B). The expression was not changed in irradiated cells versus untreated tumor cells 48hr post IR. In
contrast, a breast cancer cell line MDA231 a, expressed very high basal level expression of PDL1 and was used as a positive control since no change was observable in the CRC cell lines. Next, we examined TGF-β transcript level as this cytokine is known to enhance proliferation of T\textsubscript{REGS}. TGF-β was significantly increased in both HCT116 and SW620 at 48hr post-IR (Fig 4.3C-D). Further, increased expression could be detected as early at 24hr post-IR in SW620 cells (Fig 4.3D). Curiously, though TGF-β mRNA expression was increased in irradiated tumors, we did not detect an increase in the protein in the supernatants of irradiated tumor cells (data not shown). The biology of active TGF-β from its latent form is a regulated process (ref) and our findings suggest that the message could be subject to post-translational regulation that inhibits production of the functioning cytokine. Overall, these data suggest that irradiated tumor cells do not globally reduce the production of pro- T\textsubscript{REG} factors. Though there are other T\textsubscript{REG} promoting products it seems unlikely that modulation of tumors by radiation is reducing T\textsubscript{REG} numbers by reducing the expression of T\textsubscript{REG} promoting genes.
Figure 4.3. Radiation induced changes in T\textsubscript{REG} promoting factors in human tumor cells.

A. HCT116 and SW620 cells were irradiated with 10Gy, mRNA was harvested, and the expression of CCL22 was evaluated 8, 24 and 48h post-IR. B. HCT116 and Colo205 CRC cells were treated with 10Gy IR and surface expression of PD-L1 was evaluated 48 h later by flow cytometry. MDA231 cell were used as a positive control for detection. C. TGF-\beta mRNA expression was evaluated in irradiated HCT116 and SW620 cells 8, 24 and 48h post-IR.

Irradiated tumor cells do not promote death of T\textsubscript{REG}, and CD4+ T\textsubscript{REGS} are not more sensitive to radiation than CD4+ non- T\textsubscript{REG}-cells

As we did not observe a robust reduction in T\textsubscript{REG} promoting molecules in irradiated tumor cells we next evaluated if irradiated tumor cells had an impact on T\textsubscript{REG} viability. To test the influence of irradiated tumor on newly recruited T\textsubscript{REGS} from periphery in tumor microenvironment, tumor cells were irradiated alone and after 48hr T\textsubscript{REGS} were added (Fig
4.4A). The number of dead T\textsubscript{REG} was measured by Annexin-V and 7AAD staining 24hr post co-culture. Very few dead T\textsubscript{REGS} cells were detected in T\textsubscript{REGS} incubated with both irradiated and non-irradiated tumor cells (less than 20% dead cells). A similar trend was seen in CD4+ non-T\textsubscript{REG} cells. Next, to mimic \textit{in vivo} conditions, where both TREG and tumor cells receive radiation simultaneously, we irradiated T\textsubscript{REGS} and tumor cells during co-culture and evaluated T\textsubscript{REG} viability 48h later. We observed an increase in the number of dead cells in both the T\textsubscript{REG} and CD4+ non-T\textsubscript{REGS} cultures after 5Gy of RT however the difference was not statically significant (Fig 4.4B). Collectively, we saw no difference in the viability of T\textsubscript{REGS} as compared to CD4+ non- T\textsubscript{REGS} following irradiation in the presence of tumor cells or when added to cultures of previously irradiated tumor cells. We next tested the impact of radiation directly on T\textsubscript{REG} and non-T\textsubscript{REG} cell viability directly as others contradicting studies have reported that T\textsubscript{REGS} are both more and less sensitive to RT than non- T\textsubscript{REGS} (ref). For these experiments T\textsubscript{REGS} and CD4 non T\textsubscript{REG} cells were irradiated alone and cultured for 48 (Fig 4.4C) or 72h (Fig 4.4D). We detected significantly more dead CD4 non-T\textsubscript{REG} cells after direct radiation compared to the increase in dead T\textsubscript{REG} cells after radiation treatment. This suggests that T\textsubscript{REG} were more resistant to radiation than the non-T\textsubscript{REG} cells. To verify our results by another method, we measured viability by trypan blue. Again, CD4 T cells were more sensitive to radiation than T\textsubscript{REGS} (data not shown). The experiment was repeated three times with similar results. These data suggest that human CD4+ T\textsubscript{REGS} are more radioresistant than CD4+ non- T\textsubscript{REGS} at lower doses such as 5Gy.
Figure 4.4 The effects of irradiated tumor cells and direct RT on T\textsubscript{REG} and non-T\textsubscript{REG} cell viability.

A. HCT116 cells were irradiated with different doses of irradiation (2.5 or 5Gy). After 24hr, T\textsubscript{REGS} or non T\textsubscript{REGS} were added to the culture of irradiated tumor cells. After 72-hrs T\textsubscript{REGS}/ non T\textsubscript{REGS} were harvested, stained with Annexin-V and 7-AAD, and analyzed by flow cytometry to assess cell viability. B. HCT116 tumor cells and T\textsubscript{REGS}/ non T\textsubscript{REGS} were irradiated (2.5 and 5 Gy), co-cultured for 48-hrs and cell viability measured by flow cytometry. C. T\textsubscript{REGS} and CD4+ non-T\textsubscript{REGS} were irradiated alone (2.5 and 5 Gy) and cell viability measured 48-hrs post-IR. D. The viability of T\textsubscript{REGS}/ non T\textsubscript{REGS} was measured by Trypan blue staining 72-hrs post-IR after irradiation alone.

Radiation directly modulates the phenotype of T\textsubscript{REG} cells.

T\textsubscript{REGS} express CD4, high levels of CD25 on their surface and FOXP3 intracellularly. FOXP3 is an important transcription factor that regulates T\textsubscript{REGS} suppressive function (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003). Our results suggest that T\textsubscript{REGS} number is not significantly affected by tumor irradiation. Next, we examined if radiation altered phenotype of T\textsubscript{REGS} reasoning that if the expression of T\textsubscript{REGS} markers was reduced post-IR this could account
for the decreased frequencies of T\textsubscript{REGS} within irradiated tumor cells (Fig 4.1) CD4+CD25+ cells were isolated from human peripheral blood mononuclear cells (PBMC) and T\textsubscript{REGS} were irradiated in vitro and evaluated for expression of T\textsubscript{REG} specific markers. We found that less cells stained positive for both CD4 and CD25 after exposure to IR (Fig 4.5A). We also found that within the CD4+ population the frequency of cells that expressed high levels of CD25 was reduced (Fig 4.5A) which could explain why there is less change observed in CD4+ cells following in vivo RT of tumors as compared to the change in CD4+CD25hi cells post-IR (Fig 4.1). We next evaluated how the expression of FOXP3 was affected by radiation treatment of Treg cells. We found Foxp3 expression to be significantly reduced after 10Gy of radiation (48 or 72hr) (Fig 4.5B). Thus, it seems plausible that the reduction in T\textsubscript{REGS} number (\textit{in vivo}) is due to loss of T\textsubscript{REG} specific markers in T\textsubscript{REGS} after RT.
4.5 DISCUSSION:

Radiotherapy is integral part of cancer treatment. Traditionally, RT is thought to mediate its effect by direct killing of tumor cells. Interestingly, recent research indicates that radiation also affects systemic antitumor immune response (Formenti and Demaria 2013). There are many conflicting reports on the impact of radiation on CD4+ regulatory T cells (T\textsubscript{REGS}), which play a major role in immune tolerance by suppressing autoreactive T cells. In cancer patients, however, T\textsubscript{REGS} inhibit the generation of successful antitumor immune response and increased infiltration of adaptive T\textsubscript{REG} is often detected within the tumor microenvironment (Woo, Chu et al. 2001). Therefore, defining strategies that reduce T\textsubscript{REG} number and function in
cancer patients is important for augmenting the success of cancer immunotherapies. In this study we focused on both direct and indirect (via irradiation of tumor) effects of radiation on T\textsubscript{REG} cell biology and viability.

Human T\textsubscript{REGS} were treated under three different conditions. First, tumor cells were irradiated and T\textsubscript{REGS} were subsequently added to elucidate the effect of locally irradiated tumor on newly recruited T\textsubscript{REGS} to tumor site. There was no difference in viability of T\textsubscript{REGS} after coculturing T\textsubscript{REGS} with irradiated tumor vs non-treated (Fig 4.4). Second, tumor and T\textsubscript{REGS} were irradiated simultaneously during cocultured together to mimic what might occur \textit{in vivo} if T\textsubscript{REGS} were already present within the tumor. There was a dose dependent increase in T\textsubscript{REG} cell death as well as death of CD4+ non T\textsubscript{REGS} suggesting that there was no specific enhancement of T\textsubscript{REG} cell death in the presence of tumor cells. Lastly, T\textsubscript{REGS} and non- T\textsubscript{REGS} were irradiated alone to compare their individual sensitivity to radiation. We observed that T\textsubscript{REGS} are more resistant to radiation compared to non- T\textsubscript{REGS}. Overall, our data suggest that radiation of tumor cells does not alter the viability of T\textsubscript{REGS} but that T\textsubscript{REGS} are less sensitive to radiation-induced toxicity compared to CD4 cells. This finding is in contrast to a report by Cao et al that apoptosis was higher in T\textsubscript{REGS} than effector T cells. This difference could be a result of the different methods of isolation of human T\textsubscript{REGS} used or method for detection for cell death.

Because direct toxicity to T\textsubscript{REGS} did not seem to account for reduced number of T\textsubscript{REGS} in irradiated tumors we considered if tumor cells could be modulated in such a way that makes them less likely to recruit or induce T\textsubscript{REGS}. Tumor cells secrete different chemokines such as CCL-22 which enhance trafficking of natural and induced T\textsubscript{REGS} (n/i T\textsubscript{REGS}) from peripheral circulation to tumor site (Gobert, Treilleux et al. 2009) (Facciabene, Motz et al. 2012). In

Overall our results suggest that sub-lethal radiation of tumor cells does not change CCL22 and PDL1 (Fig 4.3). In contrast the expression of TGF-β mRNA was up regulated in both HCT (48hr) and SW620 cells after 10Gy. However, we saw a decrease in TGF-β protein in preliminary experiments (data not shown). Future experiments will need to further assess if there is a true increase in functionally active TGF-β. Apart from tumor cells, there are many other cells present within the tumor microenvironment such as stromal cells, myeloid and DC cells also capable of secreting different factors, which could be responsible for the decreased frequency of TREGs. Overall, our data do not argue strongly for modulation of tumors by radiation subsequently reducing intratumoral TREGs.

Radiation has, however, been shown by us and others to modulate tumors directly making them more immunogenic and stimulatory for CD8+ effector T cells. This activity occurs in the absence of antigen presenting cells and is distinct from the immunogenic cell death (ICD) mechanism reported by others. In this regard, investigations aimed at promoting ICD use high doses of radiation (24-30Gy) to induce some tumor cell death. Some, but not all, of these investigations have reported increased frequencies of TREG cells post-IR. In contrast, our current study utilizes lower dose of radiation (10Gy) and we found a reduction in TREG cells following RT of murine tumors (Fig 4.1). This loss appears to be specific for TREGs as there was no
reduction in CD4+ non T\textsubscript{REG} cells. This finding is in line with reports using total body radiation (TBI), which demonstrate a reduction in T\textsubscript{REG} numbers. TBI also uses lower doses of radiation (2Gy or less). However, as these studies were conducted in non-tumor bearing mice it was unclear what the effect of lower dose of radiation would be in tumor bearing mice. Because the tumor type evaluated could also play a role in whether RT promoted or inhibited T\textsubscript{REGS} we also evaluated mice implanted with a murine breast tumor (4T1). This model was selected because several of the studies demonstrating increased T\textsubscript{regs} (after 24-30Gy) utilized this tumor model system. Again, we found a reduction in intratumoral T\textsubscript{REG} cells following 8Gy RT of murine tumors in vivo (Fig 4.2). Our findings, suggest that dose is a major factor in the control of T\textsubscript{REG} cell frequencies within IR-treated tumors and that it can be effective in reducing T\textsubscript{REG} numbers in diverse tumor types.

Radiation did specifically induce death in T\textsubscript{REGS} as compared to non T\textsubscript{REGS} (Fig 4.1 and Fig 4.4), nor did irradiated tumor cells appear to modulate the expression of genes that would result in reduced frequencies of intratumoral T\textsubscript{REGS} (Fig 4.3). We next wanted to determine if radiation could perhaps be changing the phenotype of T\textsubscript{REG} cells. We observed that 10Gy radiation reduced the population of CD4+CD25\textsuperscript{hi} cells in in vitro purified human T\textsubscript{REGS} (Fig 4.5A). We also observed that T\textsubscript{REGS} lost intracellular FoxP3 expression after 10Gy (Fig 4.5B). Similarly, Cao et al reported that RT decreased Foxp3 expression but the radiation dose used in their study was very high (30Gy). Thus, it could be possible that the decreased number of T\textsubscript{REGS} we observe in our in vivo model, may be due to losses in the expression of phenotypic markers of T\textsubscript{REGS}. Foxp3 is a transcription factor that controls transcription of several immunosuppressive genes such as IL-10, TGFB, and CTLA-4 that mediate the suppressive function of T\textsubscript{REGS}. Thus, future studies will need to measure the expression of genes that are regulated by FOXP3 as well
as measure the suppressive activity of these T\textsubscript{REGS} post RT. Moreover, we also want to evaluate how FOXP3 expression is altered by radiation as HDACi with TSA has been reported to reduce FOXP3 expression. This is of interest as I have seen TSA alter gene expression in tumor cells in a manner similar to the changes occurring following TSA treatment (Chapter 3).

Overall, results of this exploratory study have found that human T\textsubscript{REGS} are not more sensitive to radiation than non- T\textsubscript{REGS} at doses of radiation within the hypofractionated range used clinically. Importantly, these doses of radiation do however reduce the frequency of T\textsubscript{REGS} present within the tumors of irradiated mice. This may be a consequence of radiations ability to alter the phenotype of T\textsubscript{REG} cells and could be responsible for reduced T\textsubscript{REG} function within tumors.

5 CONCLUSION:

RT is an integral component of cancer treatment. The standard therapies in advanced stages of CRC include surgery, chemotherapy, targeted therapy, and radiation therapy (RT). In rectal cancer, RT is usually given either before or after surgery. In addition, radiation can be given in colorectal patient to reduce pain symptoms. Traditionally, RT was thought to be immunosuppressive; however, current studies suggest that RT can enhance antitumor immune responses. More recent reports demonstrate that sub-lethal doses of radiation can cause
immunogenic modulation of tumors that involves radiation-induced molecular alteration of tumor cells and make tumor cells sensitive to immune cell mediated killing (Hodge, Kwilas et al. 2013). This activity of radiation is separate from its ability to induce immunogenic cell death (ICD) and induction of activated antigen presenting cell and cross priming of T cells. Thus, fully defining the molecular mechanisms that allow IR to enhance anti-tumor immune responses will provide additional opportunities for enhancing cancer immunotherapies via combination strategies.

Our previous studies demonstrate that sub-lethal doses of radiation alters gene expression within tumor cells, that make irradiated tumor cells more sensitive to CTL mediated killing compared to non-irradiated cells (Garnett, Palena et al. 2004). Another study from our lab reported that the enhanced killing was not dependent on death receptors (such as Fas) because human colorectal cells, which lack a functional Fas pathway (SW620), are still more sensitive to CTL mediated lysis after IR (Ifeadi and Garnett-Benson 2012) Moreover, the relative sensitivities to other death receptor pathways did not relate to the differential sensitivity to CTL killing. These studies suggest that alternate mechanisms could be responsible for enhanced killing.. T cell activation requires peptide bound on MHC molecules presented by antigen presenting cells (APC) to T cell receptor, in conjunction with appropriate co-stimulation to augment and amplify the immune response. Thus, in the second chapter we investigated the expression of costimulatory molecules such as OX40L, 41BBL, ICOSL and CD70, which are known to enhance effector activity of CTLs, on irradiated tumor cells. Both 41BBL and OX40L are required for perforin, granzyme, and IFNgamma production and thus promote T cell mediated killing. Single dose radiation (10Gy) significantly enhanced the surface expression of OX40L and 41BBL in panel of CRC cells (HCT116, SW620, WiDR). All cells lines increased
expression of at least one of these two proteins except for the cell line Colo205. Transcript levels correlated well with the increase in protein expression. In contrast no change in other costimulatory molecules such as CD70 or ICOSL nor of the co-inhibitory molecule (PD-L1) was observed on the surface of colorectal tumor cells post irradiation. Furthermore, our study demonstrates that both OX40L and 41BBL are required for enhanced killing of irradiated tumor cells mediated by T cells as blocking of these molecules reversed CTL killing. Apart from killing, cell death was reduced and activation (as measured by CD69 and IL-2 receptor expression) was increased in cytotoxic T cells following co-culture with irradiated tumor cells compared to T cells cultured with untreated cells. Thus, it is plausible that T cells survive better which would result in better productive interaction between T cells and tumor cells and subsequently results in enhanced killing. Based on our findings we can now add increased expression of effector T cell co-stimulatory molecules to the immunogenic changes induced in tumor cells surviving radiation (Fig 5.1).
Figure 5.1 Immunogenic Modulation (IM) of Tumor Cells by IR.

*PD-L1 has been reported to be both increased, and decreased, by IR. Tumors have been reported to be modulated in several ways, which could directly enhance the function, activity or recruitment of CD8+ T cells, as well as the function of NK and dendritic cells. Increased TAA could result in increased presentation on MHC-I to effector CTLs but could also make more antigen available for uptake by APCs. There have also been limited reports of modulation of tumors in a manner that could negatively impact CD8+ T-cell activities. Note: IR-induced ICD mechanisms that enhance DC activities are not depicted in this figure. Ag-antigen (Figure from Kumari et al, Future Oncology, in press)

Overall, radiation appears to be overwhelming positive and in favor or enhanced effector T cells responses at the doses utilized in our studies (Fig 5.1). These are important findings given the fact that there are many patients where T cells of known specificity for tumors already exist (or are even transferred in) but are not effectively attacking the tumor (Chakraborty,
Abrams et al. 2003). In these situations radiation is not needed to “prime” an initial T cell response to the TAA, via ICD, because large numbers of effector cells are already present. Local RT can be quite useful in this situation to greatly enhanced activity of T cells by providing these existing T cells with different or better signals from the irradiated tumor (Cao, Daniel et al. 2002, Lugade, Moran et al. 2005). Thus, it seems likely that T cells within the tumor which are primed but anergic could benefit from the increased co-stimulation.

Currently, the “holy grail” of radiation therapy from the CIT perspective is to find the mechanisms which induce the abscopal effect. The abscopal response describes the phenomenon of radiation delivered to a primary tumor inducing a response or regression of a distant non-irradiated tumor. Unfortunately, abscopal responses are a rare unexplained phenomenon and the exact mechanisms remain unknown. Recent reports have achieved abscopal responses after combining radiation with immunotherapy (CTLA4 blockade) against melanoma and non-small lung carcinoma (Postow, Callahan et al. 2012, Golden, Demaria et al. 2013). Currently, various preclinical and clinical labs are investigating the underlying mechanism behind the abscopal effect so as to extend the effect to large number of patients and to achieve maximal benefit from combination of RT and CITs. Indeed, it seems likely that costimulatory molecules such as OX40L and 41BBL, changed after radiation could mediate such responses because these positive co-stimulators not only enhance local killing by CTLs but also enhance effector activity of CTLs, which are leaving the irradiated tumor.

In Chapter 3, we investigated the molecular mechanism by which radiation induced expression of co-stimulatory molecules. Previously several mechanisms have been reported for
RT-induced changes in gene expression in tumor cells. Radiation causes DNA double strand break that activate ATM, which further activates its target genes such as NFκb and p53. NFκb is known to increase some immune relevant genes such as MHC-I, ICAM-1 and some inflammatory cytokines. In contrast, p53 alters the expression of genes, which are involved in cell cycle, apoptosis or DNA repair pathways. These pathways typically induce short duration changes, however we have observed that expression OX40L and 41BBL is sustained for up to 7 days after single dose radiation. Others have found that these genes, as well as many other genes related to oncogenic pathways, could be controlled by epigenetic changes, which could mediate such long lasting changes in expression. For example, 41BBL expression was transcriptionally activated by HDAC inhibitors in leukemia cell lines (Vire, de Walque et al. 2009) and HDAC11 was shown to play an essential role in regulating OX40L expression (Buglio, Khaskhely et al. 2011). Importantly, radiation has been shown to inhibit the expression of HDAC1 and HDAC2 (Han, Zhang et al. 2012). Epigenetic changes such as histone modifications and DNA methylation play important roles in regulating gene expression. We found that both HDAC and DNMT inhibitors enhanced expression of OX40L and 41BBL mRNA in HCT116 and SW620 cells post irradiation. Surface expression of OX40L and 41BBL was also increased on tumor cells after 24hr treatment with HDACi (TSA) but not induced by DNMTi. We reasoned that DNMTi usually takes longer to induce gene expression, thus future experiments we will look at later times post treatment for the impact of DNMTi. In addition, HDACi (TSA) results in enhanced survival and activation of T cells, which are co-cultured with TSA, treated tumor cells. A finding very similar to the impact of radiation treated tumor cells on T cells. We determined that radiation induced expression of 41BBL by increased histone H3 acetylation at the 41BBL promoter. To further determine if radiation was globally increasing histone acetylation at other
genes promoter we evaluated the histone acetylation level at the CIITA gene promoter. The acetylation level was similar between untreated and irradiated tumor cells. Thus our study suggests that radiation is increasing histone acetylation specifically at the 41BBL promoter. These findings initiated a subsequent study by Cacan et al (Cacan IJO 2015) that demonstrated that radiation (5Gy) similarly increases histone acetylation at both the Fas and DR5 promoters in addition to the 4-1BBL promoter. This study also found less HDAC2, HDAC3, and DNMT1 were bound to the promoter regions of both 4-1BBL and Fas, but not other genes following IR of human tumor cells (Cacan, Greer et al. 2015). Overall, these findings allow for expansion of the known pathway through which RT can modulate gene expression in surviving tumor cells (Fig 5.2). Interestingly, these data also suggest that the therapeutic use of epigenetic drugs in cancer may also be working through modulating these positive signals to T cells. In our future experiments we will determine how HDACs are being recruited to specific promoter sites following radiation, as well as determine how long these epigenetic changes can be sustained on tumor cells.
Figure 5.2 Diverse Mechanisms of Immunogenic Modulation (IM) Reported in Tumor Cells Surviving Radiation.

*NFkB has been reported to be both inhibited and activated at lower radiation doses depending upon tissue and length of exposure. (Figure from Kumari et al, Future Oncology, in press)

There are many conflicting reports on the impact of radiation on CD4+ regulatory T cells (T\textsubscript{REGS}), which inhibit the generation of successful antitumor immune responses. In chapter 4, we began to explore the direct and indirect (via irradiation of tumor) effect of radiation on T\textsubscript{REG} biology and viability. Surprisingly we found that irradiated human tumor cells do not robustly alter expression of T\textsubscript{REG} stimulating/promoting molecules. On the contrary, both OX-40L and 4-
1BBL (shown to be modulated in Ch1) have been reported to reduce T_{REG} function. This supports the notion that IR modulation of tumors favors promotion of effector T cell activity instead of T_{REG} activities. In vivo, we found that local RT to tumor-bearing mice resulted in reduced frequency of T_{REG} cells within the tumor and increased expression of both OX40L and 41BBL could be detected in vivo (data not shown). The reduction in T_{REGS} could have been the result of increased cell death of T_{REGS} by IR directly, however, we found that human T_{REGS} are actually less sensitive to cytotoxicity from IR in vitro as compared to CD4+ non-T_{REGS}. Intriguingly, we found that IR modulated the phenotype of T_{REGS} and reduced expression of T_{REG} associated markers including the transcription factor FoxP3. Overall, our findings suggest that sub-lethal doses of IR may reduce T_{REG} detection within tumor tissues by altering their phenotype and further that it may reduce T_{REG} mediated activities by reducing expression of genes required for their suppressive function. Future experiments will determine if expression of OX40L and 4-1BBL could contribute additional mechanisms of T_{REG} inhibition within irradiated tumors, as well as if IR-mediated reduction in FoxP3 expression also reduces T_{REG} suppressive function.

The main goal of cancer immunotherapy is to trigger the host’s own immune response against tumor cells. Immunotherapy approaches include checkpoint blockade antibodies, therapeutic vaccines, positive costimulatory agonist antibodies, ACT, and TLR agonists. Given the evidences that radiotherapy can do more than induce immunogenic death of tumor cells, but can also modulate the phenotype of tumor cells in way that can stimulate cytolytic immune cells (Figure 5.1), it is time to fully take advantage of all the immune stimulating actions of radiation. The success of the combination of immunotherapies and radiation depends on immunologic
phenotype of the tumor. Tumors have multiple immunoevasive and immunosuppressive strategies that can hinder the success of immune therapy. The first barrier is tolerogenic dendritic cells which often accumulate within the tumor that suppress T cell priming and activation and in this scenario RT alone is not sufficient to activate T cells. Thus, in this condition, immunotherapies such as local administration of Toll-like receptor (TLR) agonist including TLR9 and TLR7 (TLR expressed on dendritic cells and recognize pathogen associated molecular patterns) are needed to activate or recover the function of DCs (Adams 2009). The second barrier is that tumors often express low MHC-1 and costimulatory molecules and escape from immune-recognition. Thus, radiation can be used after TLR agonist to enhance the expression of costimulatory molecules (such as OX40L and 4IBBL as I have shown in this dissertation) on tumor cells which would transduce positive signals to CTLs and enhance their killing and also survival and activation of effector T cells (chapter 2). In addition, based on the work I have shown here, it now seems likely that these positive signals can also be induced by epigenetic drugs such as HDAC inhibitor (chapter 3). Thus, combination of HDAC inhibitor with immunotherapies are another area of potential combination in future cancer therapy. Finally, in some patients despite the presence of activated T cells within tumors, programmed death ligand (PD-L1) causes T cells exhaustion. This protein is upregulated by colorectal tumors post irradiation (Dovedi, Adlard et al. 2014). So far it is unclear whether radiation induced positive signals can override the negative signal (PDL1) to a T cell but adding a checkpoint blocking antibody with radiation has been shown to enhance the generation of productive antitumor immune responses. Thus, combining an immunocheck point inhibitor (PDL1 or PD1 blockade) with radiation is required to overcome T cell inhibition. Overall, localized RT has emerged an adjuvant in combination with immunotherapies.
IM-induced phenotypic changes in tumor cells have been studied extensively (Fig 5.1). However, given the differential radio-sensitivities of immune cell subsets, it seems imperative to more fully elucidate the direct effects of radiation doses within the hypofractionated dose range on immune cell phenotype and function. Particularly given the changes we have observed within irradiated T\textsubscript{REG} cells. It will also be important to determine which phenotypic changes occur most broadly in surviving tumor cells, and can thus be capitalized on across diverse human tumor types. Regarding the TME, how other cells besides tumor cells are modulated at these doses needs to be elucidated. Given our evidence that IM of some genes is occurring via epigenetic mechanisms, determining how long these changes are retained in tumor cells as well as what other immune relevant genes are regulated this way will be helpful for defining the therapeutic usefulness of RT-induced IM. It is perhaps most important to determine which changes occur in instances where abscopal responses are seen so that we can induce this type of response more consistently. Overall, such data will allow for determination of which CITs should routinely incorporate RT for its IM activities allowing for the rationale incorporation of RT into CIT approaches.
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