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Dynamic regulation of CD4+ regulatory T cells by radiation treatment

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DYNAMIC REGULATION OF CD4+ REGULATORY T CELLS BY RADIATION

TREATMENT

by

SAMANTHA S. SIMON

Under the Direction of Charlie Garnett-Benson, PhD

ABSTRACT

Radiotherapy remains effective at treating primary, early-stage tumors, however it produces nominal results in late-stage and metastatic tumors. This has led to a shift towards more targeted immune-based therapies. Yet the use of most approved cancer immunotherapies is limited to only a few cancer types and in the absence of effective anti-tumor immunity tumors can successfully evade immune surveillance. Tumors employ multiple mechanisms for avoiding immune elimination including down-regulation of positive signals to tumor specific CD8+ cytotoxic T cells (CTLs) and the accumulation of CD4+ regulatory T (T_{REG}) cells which can suppress the anti-tumor activity of effector CTLs. Radiation has been reported to enhance anti-tumor immunity through such mechanisms as tumor cell death or phenotypic modulation of tumor cells, however the impact of radiotherapy on T_{REG} cells is less clear.

The goal of this dissertation was to investigate the direct effect of radiation on the phenotypic characteristics and functional activity of induced T_{REG} cells and to examine the indirect effect of radiation on T_{REG} frequency. We found that exposure to sub-lethal radiation decreased the expression of Foxp3 in T_{REG} cells and differentially modulated the expression of several T_{REG} signature molecules. This loss of Foxp3 and modulation of several T_{REG} associated molecules resulted in a reduction of suppressive activity. Radiation has previously been shown to modulate the expression of genes in tumor cells that can impact T cell activity such as OX40L and 4-1BBL. Thus, a secondary goal of the research was to assess the effect of radiation-induced expression of tumoral OX40L and 4-1BBL on T_{REG} number in two commonly used tumor models, 4T1 and MC38. Additionally, we examined 4T1 and MC38 tumors for changes in immune cell composition post-treatment. We found that radiation differentially modulated OX40L and 4-1BBL expression in our tumor models, as well as reduced T_{REG} frequency. However, induced expression of OX40L did not correlate with the observed decrease in T_{REG} frequency. Further, we found that radiotherapy differentially modulated the immune cell profile of 4T1 and MC38 tumors. These findings could support the design for rationale combinations of cancer immunotherapies with radiation treatment.

INDEX WORDS: T_{REG}, Radiation therapy, Co-stimulatory molecules, Cancer immunotherapy, Breast cancer, Colorectal cancer

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SAMANTHA S. SIMON

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

Doctor of Philosophy

in the College of Arts and Sciences

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Georgia State University

December 2019

DEDICATION

This work is dedicated to my amazing mother Carole who has always supported and believed in me even when I didn't believe in myself. To my husband William, thank you for your constant love and support, I couldn't have done this without you. To my kids Landen and Carli, you made me stronger than I ever thought I could be; everything I do is for the two of you. To my sister Jessica and my many friends, I love you all so much. Thank you for supporting me through this journey.

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LIST OF ABBREVIATIONS

7-AAD: 7-Aminoactinomycin D

ATRA: All-trans retinoic acid

CD: Cluster of differentiation

CFSE: Carboxyfluorescein succinimidyl ester

CTL: Cytotoxic T lymphocyte

CTLA-4: Cytotoxic T lymphocyte associated protein 4

DC: Dendritic cell

EGR-2: Early growth response gene 2

EIF1: Eukaryotic translation initiation factor 1

Foxp3: Forkhead box p3

GATA3: GATA binding protein 3

GITR: Glucocorticoid- induced tumor necrosis factor receptor

Gy: Gray

ICB: Immune checkpoint block(ing)

ICD: Immunogenic cell death

IHC: Immunohistochemistry

IFN-γ: Interferon-γ

IM: Immunogenic modulation

LAG-3: Lymphocyte activation gene 3

MACS: Magnetic activated cell sorting

MFI: Median fluorescence intensity

PBMCs: Peripheral blood mononuclear cells

PD-L1: Programmed death ligand 1

S.C.: Subcutaneous

T-bet: T-box transcription factor

T_{CONV}: T conventional cell

T_H: T helper cell

T_{REG}: Regulatory T cell

TGF-β1: Transforming growth factor beta 1

TNFSF: Tumor necrosis factor superfamily

WT: Wild-type

1 INTRODUCTION

Immense research has been done to combat the increasing incidence of cancer diagnoses, however it remains a public health concern. Ranking 2nd in disease-related deaths in the United States cancer is estimated to cause the death of more than half a million people in 2019 (American Cancer Society 2019). Standard therapies, such as radiation and chemotherapy, effectively treat primary and early-stage tumors, however metastatic and late-stage tumors offer few therapeutic options thus shifting the field towards immune-based therapies, which aim to stimulate a patients' own immune system to better attack cancer cells.

1.1 Cancer immunoediting

Cancer immunoediting is a novel theory that describes the effect of the immune system on tumor development. The initial stage of cancer elimination, or cancer immunosurveillance, is a process by which immune cells eliminate continuously arising transformed cells (Dunn, Old, and Schreiber 2004). During this stage tumor elimination is reportedly achieved by interferon-γ (IFN-γ) production and lymphocyte effector function. The production of endogenous IFN-γ was found to protect mice from transplanted tumor growth and spontaneous tumor formation (Kaplan et al. 1998; Street et al. 2002). Further, IFN-γ was shown to specifically target tumor cells by enhancing tumor immunogenicity by upregulating components of the MHC class I pathway (Shankaran et al. 2018). Cellular effectors required for cancer immunosurveillance include components of both the innate and adaptive immune system. Mice lacking $\alpha\beta$ T cells and $\gamma\delta$ T cells showed an increased incidence of tumor development compared to control mice (Girardi et al. 2003). Additionally, increased tumor formation was also observed in mice depleted of NK or NKT cells (Smyth et al. 2000).

The second stage of cancer immunoediting is equilibrium. This phase describes the dynamic balance where IFN-γ production and lymphocyte effector function relentlessly attack tumor cells thereby prohibiting tumor growth but are unable to eradicate transformed cells (Dunn, Old, and Schreiber 2004). As the longest phase, equilibrium allows for the development of tumor heterogeneity and genetic instability in cells that survive elimination. The final stage of immunoediting is escape. Tumor cell variants that were selected for during equilibrium are now able to grow unchecked even in the presence of a competent immune system (Dunn, Old, and Schreiber 2004).

1.1.1 *Immunosuppression and tumor escape*

Tumor cells are highly effective at inducing a variety of immunosuppressive mechanisms to aid in tumor escape. The secretion of immunosuppressive molecules such as transforming growth factor β1 (TGF-β1) by tumor cells can inhibit T cell responses (Y. Liu and Cao 2016). Additionally, TGF-β1, along with several chemokines, recruit suppressive cells to the tumor microenvironment. Suppressive cell types that play a crucial role in tumor escape and progression include MDSCs, TAMs, and CD4+ regulatory T (T_{REG}) cells (Y. Liu and Cao 2016).

T_{REG} cells are a specialized subset of CD4+ T cells. Naturally derived in the thymus, T_{REG} cells are characterized by their expression of the high affinity IL-2 receptor, CD25, and the transcription factor forkhead box P3 (Foxp3) (Shimon Sakaguchi et al. 2008). Important for immune tolerance and homeostasis, these cells function by suppressing the activity of auto-reactive and pro-inflammatory effector T cells (S Sakaguchi et al. 1995; Shimon Sakaguchi et al. 2009; Erdman and Poutahidis 2010). In addition to naturally derived T_{REGS} (nT_{REGS}), T_{REGS} can be induced (iT_{REG}) in the periphery such as within the tumor microenvironment (Tsai et al.

2014). Naïve CD4+CD25- T conventional cells can be induced to express a T_{REG} phenotype following T cell receptor stimulation in the presence of IL-2 and TGF-β1 (Facciabene, Motz, and Coukos 2012; Povoleri et al. 2013). Though identical in their suppressive function, these cells differ in their stability of Foxp3 (Zou 2006; Floess et al. 2007). Foxp3 is a master regulator in T_{REG} cells and is essential for their development and suppressive function (Maruyama et al. 2011). In nT_{REGS}, Foxp3 expression is highly stable and constitutively expressed whereas in iT_{REGS}, such as those induced at tumor sites, Foxp3 expression is unstable (Povoleri et al. 2013; Maruyama et al. 2011). This instability is linked to partial CpG demethylation of the Foxp3 promoter (Haiqi, Yong, and Yi 2011).

Unfortunately, in cancerous conditions the suppressive activity of T_{REGS} is intended to induce and maintain a suppressive tumor microenvironment thereby enabling immune evasion and unrestricted cell growth (Zou 2006). It is therefore unsurprising that an increase in T_{REG} frequency has been reported in several cancer types, including colorectal and breast (Watanabe et al. 2010; Hua et al. 2016; Hanke et al. 2015; Plitas et al. 2016). Though numerous cancer types exhibit elevated T_{REG} number, the prognostic implications are wholly cancer dependent. While T_{REG} cell accumulation correlates with a poor prognosis in such cancers as breast, ovarian, lung, and melanoma the opposite trend has been observed in colorectal and Hodgkin's lymphoma where high T_{REG} infiltration correlates with improved patient prognosis indicating the need to better understand T_{REG} cell biology and its role in tumor progression (Chaudhary and Elkord 2016).

1.2 Radiation and cancer treatment

More than half of cancer patients are treated with radiotherapy. Traditionally, radiation has been used to directly kill tumor cells. High dose radiotherapy, administered in fractionated

doses, induces DNA double-strand breaks. This leads to apoptosis and necrosis of tumor cells mediated by WT p53 (Fei and El-Deiry 2003; J. song Wang, Wang, and Qian 2018). However, several cancers contain mutated versions of p53 which has been shown to reduce sensitivity of tumor cells to radiation-induced cell death (Hollstein et al. 1991; J. M. Lee and Bernstein 1993).

Aside from the direct killing of tumor cells, radiation can be used therapeutically to induce immunogenic cell death (ICD) at single doses of <2 Gy or when given as hypofractionated doses of >2 Gy to <25 Gy (Kumari et al. 2016). ICD is defined as cell death that elicits a robust immune response. Two major factors that are needed to produce an effective immune response are antigenicity and adjuvanticity (Galluzzi et al. 2017). Radiation can induce antigenicity by increasing the peptide repertoire (Reits et al. 2006). Further, radiation induced ICD depends on danger signals such as ATP secretion, type I IFN signaling, and UPR-dependent exposure of CALR which induce adjuvant-like effects. ICD exposes CALR, an ER chaperone, on the cell membrane which serves as an "eat me" signal promoting phagocytosis and antigen uptake by antigen presenting cells (Galluzzi et al. 2017). Additionally, the secretion of ATP serves as a chemotactic agent leading to the recruitment of dendritic cells (DCs). Lastly, radiation stimulates the secretion of type I IFNs, notably IFN-β, by tumor cells leading to chemokine secretion and DC recruitment (Galluzzi et al. 2017; Vanpouille-Box et al. 2017). Overall, ICD of tumor cells induces the release of tumor-specific antigens that leads to enhanced DC activation and an effective antitumor immune response.

In addition to ICD, radiation has been shown to induce immunogenic modulation (IM), as reviewed in (Kumari et al. 2016). IM describes the radiation-induced alterations to tumor cell phenotype that enhances CTL killing (Fig. 1.1). Radiation has been shown to induce the expression of major histocompatibility complex class I (MHC class I), MICA/B, and multiple

death receptors (Fas, DR4, DR5) on tumor cells (Reits et al. 2006; J. Y. Kim et al. 2006; Ifeadi and Garnett-Benson 2012). In addition, radiation induces T cell infiltration by increasing the expression of vascular cell adhesion molecule (VCAM-1) and chemokines such as CXCL16 (Lugade et al. 2005; Matsumura and Demaria 2010). Thus, these findings highlight the ability of radiation to alter the phenotype of tumor cells, thereby inducing a more immunogenic microenvironment.

An intriguing effect of radiation treatment is the abscopal response. A rare event, the abscopal response is an immune-mediated response to local tumor irradiation that induces a systemic antitumor immune response (Y. Liu et al. 2018). It is not fully understood how local tumor irradiation can generate a systemic antitumor immune response though ICD plays an important role. Radiation monotherapy has resulted in abscopal responses in a number of case studies, however these results are not easily reproducible (Azami et al. 2018). It is now believed that combination radiation and immunotherapy treatment can enhance the abscopal effect as combination therapy has been shown to further enhance the antitumor immune response when compared to radiation or immunotherapy alone (Dewan et al. 2009; Deng et al. 2014).

While radiation remains a standard method of care for several cancer types, singular use fails to eradicate some advanced-stage tumors (Jarosz-Biej et al. 2019). Interestingly, research has demonstrated the effectiveness of radiation as a partner for immune based cancer immunotherapies (Demaria, Coleman, and Formenti 2017). Radiation has been shown to up-regulate the expression of immunomodulatory molecules such as MHC class 1 and Fas death receptors in various tumor types, proteins capable of inducing an antitumor immune response at radiation doses ≤10 Gy (Garnett et al. 2004; Ifeadi and Garnett-Benson 2012). Previous studies from our lab, and others, have shown an increase in tumor-specific CTL killing following

exposure to radiation (Kumari and Garnett-Benson 2016; Bernstein et al. 2014). This enhanced

sensitivity to killing was mediated by the radiation-induced expression of the co-stimulatory

molecules OX40L and 4-1BBL on tumor cells, which we found to be epigenetically regulated

(Kumari et al. 2013). Additionally, several studies have demonstrated the importance of CTLs in

effective radiation and immunotherapy treatment in murine tumor models (Deng et al. 2014; K.

J. Kim et al. 2017).
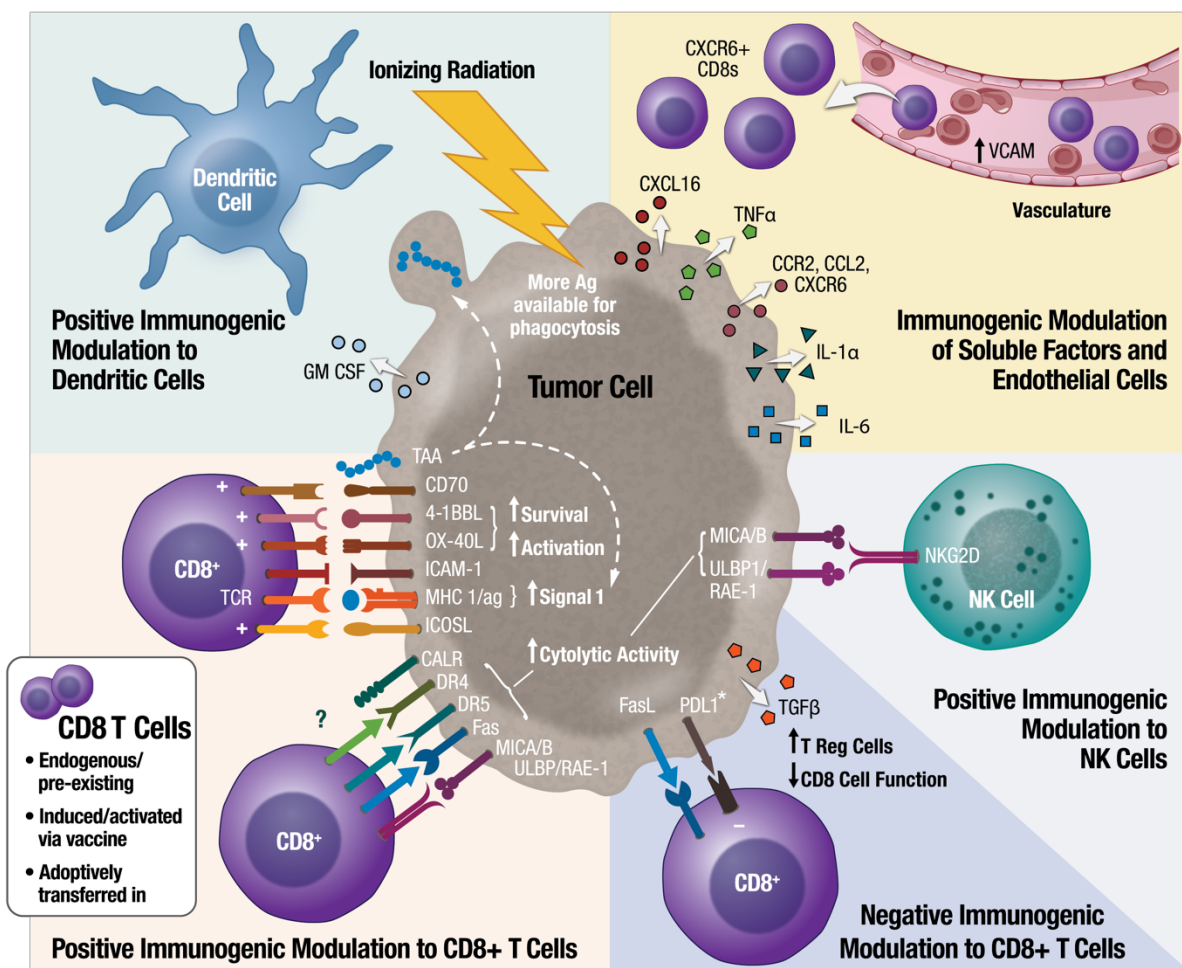
Figure 1.1 Immunogenic modulation of tumor cells by radiation
Generated from "Future Oncology" (Kumari et al. 2016)

1.2.1   Cancer immunotherapy strategies and ways to overcome resistance

It has been well documented that the effectiveness of cancer immunotherapy is dependent on its ability to generate antigen specific CTLs capable of killing tumor cells (Tsai et al. 2014)(Ellmark et al. 2017). However, the accumulation of suppressive immune cells (T$_{REGS}$, MDSCs, TAMs) and expression of inhibitory molecules (CTLA-4, PD-1, PD-L1) within the tumor microenvironment inhibits the cytolytic activity of CTLs. The use of immune checkpoint blocking (ICB) antibodies are intended to block the binding of inhibitory molecules and boost the antitumor immune response. Currently there are several FDA approved ICB antibodies on the market against CTLA-4 (Ipilimumab), PD-1 (Pembrolizumab, Nivolumab, and Cemiplimab), and PD-L1 (Atezolizumab, Avelumab, and Durvalumab).

Despite major advances in immunotherapy, the clinical use of ICB antibodies is limited to a small number of cancer types (Lee Ventola 2017). Currently, ICB antibodies are approved for use in several cancer types, including melanoma, non-small cell lung cancer, and bladder cancer. Treatment with ICB antibodies has been shown to increase patient survival (Hodi et al. 2010; Topalian et al. 2019). However, most eligible patients are non-responsive, while some that initially responded well to treatment later acquire resistance (Pitt et al. 2016; Koyama et al. 2016).

Acquired resistance to ICB antibodies has revealed the need for additional treatment approaches. One such method is the development and use of co-stimulatory agonist antibodies. Several clinical trial studies are currently underway to evaluate the efficacy of OX40 and 4-1BB agonist antibodies. OX40 and 4-1BB are co-stimulators belonging to the tumor necrosis factor superfamily that bind to OX40L and 4-1BBL, respectively (Croft 2009). The ligands OX40L (CD252) and 4-1BBL (CD137L) are typically expressed on antigen presenting cells (APCs) and

endothelial cells and send positive signals to T cells. Inducibly expressed on activated CD4+ and CD8+ T cells, OX40 and 4-1BB are involved in the promotion of effector T cell clonal expansion, survival, and cytokine induction (Croft et al. 2009; Vinay and Kwon 2012). Conversely, both OX40 and 4-1BB signaling have been shown to inhibit the conversion of naive CD4+ T cells into T_{REGS} and send negative signals into existing T_{REGS} (Vu et al. 2007; Piconese, Valzasina, and Colombo 2008; Bulliard et al. 2014; Smith, Hoeizinger, and Dominguez 2011). These are important signals for promoting tumor immunity. Treatment of tumor-bearing mice with agonist antibodies to OX40 and 4-1BB have been reported to induce effective anti-tumor immune responses (Vinay and Kwon 2012; Gough et al. 2008; Curran et al. 2011; Barsoumian, Yolcu, and Shirwan 2016).

1.3 Current gaps in knowledge

1.3.1 Direct impact of radiation on T_{REGS}

Despite extensive research, the impact of radiation on T_{REG} cells remains controversial. Several studies have shown that T_{REGS} are more radio-resistant compared to other lymphocyte populations, however it is unclear what effect it imparts on their functionality (Baba et al. 2012; Qu, Jin, et al. 2010). Studies by Qu *et al.* found no difference in the suppressive function of T_{REGS} from radiation treated mice compared to control mice, though research by Balogh *et al.* and Billiard *et al.* observed decreased functional activity in irradiated T_{REGS} (Qu, Zhang, et al. 2010; Balogh et al. 2013; Billiard et al. 2011). In addition, studies by Muroyama *et al.* and Kachikwu *et al.* reported increased T_{REG} number in locally irradiated tumors compared to control, *in vivo* (Muroyama et al. 2017; Kachikwu et al. 2011), while Cao *et al.* and Liu *et al.* observed a decrease in *in vitro* and whole-body tumor irradiated studies, respectively (Cao et al. 2009; R. Liu et al. 2010). The reason for such discrepancies is unclear. It is possible that the time

of data acquisition post-radiation may impact the frequency of T_{REGS} observed, as well as experimental design and tumor model examined. Additionally, the composition of natural versus induced T_{REGS} within the research model may also play a factor. As such, it is unclear how radiation directly effects T_{REG} phenotype and suppressive function, thus understanding its role could lead to a more targeted use of radiation in combination with cancer immunotherapy.

1.3.2   *How radiation-induced tumor modulation regulates T_{REG} frequency*

Radiation has been shown by our lab, and others, to alter the phenotype of colorectal and prostate cancer cells by enhancing their expression of the co-stimulatory molecules OX40L and 4-1BBL (Bernstein et al. 2014; Kumari and Garnett-Benson 2016). Tumor-induced expression of these proteins was found to enhance CTL effector function in a synergistic manner. Interestingly, signaling through OX40 or 4-1BB was found to abrogate Foxp3 expression and inhibit the suppressive function of T_{REG} cells (Smith, Hoeizinger, and Dominguez 2011; Vu et al. 2007; Piconese, Valzasina, and Colombo 2008). Thus, part of this study was to determine whether radiation-induced modulation of co-stimulatory and co-inhibitory molecules, on tumor cells, correlates with reduced T_{REG} cell frequency and if phenotypic changes occurring in common murine models impact the post-radiation tumor microenvironment.

2 IONIZING RADIATION MODULATES THE PHENOTYPE AND FUNCTION OF HUMAN CD4+ INDUCED REGULATORY T CELLS

Samantha S. Beauford, Anita Kumari and Charlie Garnett-Benson

Submitted to BMC Immunology

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2.1 Abstract

Background: The use of immunotherapy strategies for the treatment of advanced cancer is rapidly increasing. Most immunotherapies rely on induction of CD8+ tumor-specific cytotoxic T cells that are capable of directly killing cancer cells. Tumors, however, utilize a variety of mechanisms that can suppress anti-tumor immunity. CD4+ regulatory T cells can directly inhibit cytotoxic T cell activity and these cells can be recruited, or induced, by cancer cells allowing them to escape from immune attack. The use of radiation as a treatment for cancer has been reported to enhance anti-tumor immunity by several mechanisms involving tumor cell death or phenotypic modulation of tumor cells. Less is known regarding the impact of radiation directly on suppressive regulatory T cells. In this study we investigate the direct effect of radiation on human T_{REG} viability, phenotype, and suppressive activity.

Results: Both natural and TGF-β1-induced T_{REG} cells exhibited increased resistance to radiation (5-10 Gy) as compared to CD4+ conventional T cells. Radiation, however, decreased Foxp3 expression in natural and induced T_{REG} cells though the reduction was more robust in induced T_{REGS}. Treatment differentially modulated the expression of signature iT_{REG} molecules, inducing

increased expression of LAG-3 and CD73 and decreased expression of CD25 and CTLA-4. Irradiated iT$_{REGS}$ exhibited a reduced capacity to suppress the proliferation of CD8+ T cells.

Conclusions: Our data demonstrates that while human T$_{REG}$ cells may be more resistant to radiation-induced death it can cause downregulation of Foxp3 expression, as well as modulate the expression of CD25, CTLA-4, LAG-3, and CD73 signature molecules. Lastly, irradiated TGF-β1-induced T$_{REGS}$ were less effective at inhibiting CD8+ T cell proliferation. These data suggest that radiotherapy could be utilized to effectively target and reduce T$_{REG}$ activity particularly when combined with anti-tumor immunotherapies.

2.2   Introduction

Currently, a variety of immunotherapeutic agents are being used to treat advanced malignancies, most notably CTLA-4 and PD-1/PD-L1 checkpoint blocking antibodies. Efficient tumor control by immunotherapies relies heavily on CD8+ cytotoxic T lymphocyte (CTL) activity (Fransen et al. 2013; Redmond, Linch, and Kasiewicz 2014; Deng et al. 2014). While the most effective immunotherapies generate tumor-specific CTLs, tumors are often able to induce an immunosuppressive microenvironment thereby evading immune cell killing (A. A. Wu et al. 2015). A major strategy of tumor-induced immunosuppression is through the recruitment and induction of CD4+ regulatory T (T$_{REG}$) cells in the tumor microenvironment (Su et al. 2017; Wiedemann et al. 2016).

T$_{REGS}$ are a suppressive subset of CD4+ T cells important for preventing autoimmunity (Smigiel et al. 2014). These cells are characterized by expression of the high affinity IL-2 receptor, CD25, and the transcription factor forkhead box p3 (Foxp3) (Hori, Nomura, and Sakaguchi 2003). T$_{REGS}$ can be naturally derived in the thymus (nT$_{REG}$), or they can be induced in the periphery from naïve CD4+ precursors (iT$_{REG}$) (Su et al. 2017; Valzasina et al. 2006; G.

Zhou and Levitsky 2007). Several cancer types are known to contain high levels of T_{REGS} that facilitate escape from immune surveillance (Viguier et al. 2004; Miller et al. 2006; Mizukami et al. 2008). To maintain an immunosuppressive microenvironment tumor cells have been reported to recruit peripheral T_{REGS} as well as induce conversion of CD4+CD25- T cells into T_{REGS} within the tumor (Mizukami et al. 2008; Ward et al. 2015; Kuehnemuth et al. 2018; V. C. Liu et al. 2007; Mittal et al. 2008). Though nT_{REG} and iT_{REG} cells both have suppressive function iT_{REGS} reportedly have unstable Foxp3 expression due to partial demethylation of CpG motifs within the *foxp3* locus (Floess et al. 2007). Functionally, T_{REGS} are capable of inhibiting the proliferation and killing activity of CTLs through several mechanisms: [a] secretion of transforming growth factor-β1(TGF-β1) and IL-10, [b] metabolic disruption through CD39 and CD73 (Deaglio et al. 2007), or [c] contact-dependent inhibition via cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), and programmed death ligand 1 (PD-L1) signaling (Huang et al. 2004; S. P. Wu et al. 2018).

Ionizing radiation remains a common treatment modality for several cancer types and is often used in combination with immunotherapy based strategies as radiation alone is insufficient to eradicate tumor burden in advanced disease (Jarosz-Biej et al. 2019). Interestingly, radiation has been shown to promote and enhance antitumor immune responses. Research in our lab, and others, has shown that tumor cells exposed to low doses of radiation can increase the expression of several cell surface receptors on tumor cells including MHC class I, death receptors , and effector costimulatory molecules such as OX40L and 4-1BBL (Garnett et al. 2004; Ifeadi and Garnett-Benson 2012; Kumari and Garnett-Benson 2016; Spary et al. 2014). Induced expression of these molecules subsequently promotes increased sensitivity to killing by CTLs (Y. Lee et al. 2009; Filatenkov et al. 2015). In addition to local tumor control, radiation treatment has also

been reported to drive antitumor abscopal effects when used in combination with immunotherapy (Dewan et al. 2009; Niknam et al. 2018).

Though T_{REGS} are known to suppress the killing activity of CTLs, the impact of radiation treatment on T_{REG} frequency, phenotype, and suppressive function is less clear. While several murine studies have shown that T_{REGS} are more radioresistant compared to other lymphocyte populations it is less clear what effect it has on the phenotype and function of human T_{REGS} (Qu, Jin, et al. 2010; Baba et al. 2012). Moreover, functional studies in mice have been contradictory. Studies by Qu *et al* found no difference in the suppressive function of T_{REGS} from radiation treated mice compared to control mice, in contrast to Balogh *et al* and Billiard *et al* who reported decreased functional activity in irradiated T_{REGS} (Qu, Zhang, et al. 2010; Balogh et al. 2013; Billiard et al. 2011). In addition, studies by Muroyama *et al* and Kachikwu *et al* reported increased T_{REG} number in locally irradiated tumors compared to control mice, *in vivo* (Muroyama et al. 2017; Kachikwu et al. 2011), while Cao *et al (2009)* and Liu *et al* observed a decrease in human *in vitro* and murine whole-body tumor irradiated studies, respectively (Cao et al. 2009; R. Liu et al. 2010). It seems plausible that some of the disparities in observations may be due to differences between natural versus tumor-induced T_{REGS}.

Here, we assessed the direct effect of radiation on viability and Foxp3 expression in natural and induced human T_{REG} cells. We also sought to determine the impact of radiation on the suppressive function of induced T_{REGS} as well as the expression of molecules associated with functional T_{REG} activity: CD25, CTLA-4, LAG-3, CD39, CD73, and PD-L1. Our data revealed that radiation induces less death in human T_{REG} cells as compared to conventional CD4+ T cells and that radiation decreases expression of Foxp3 in both types of T_{REG} cells. Additionally, we

showed that iT$_{REG}$ cell phenotype is modulated by radiation and that cells are functionally less suppressive following radiotherapy.

2.3   Materials & Methods

2.3.1   *Human T cell isolation*

Commercially available human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors [HemaCare and ATCC]. PBMCs were purified from buffy coats by gradient centrifugation using Lymphocyte Separation Medium [Corning]. PBMCs were rested overnight in RPMI medium containing 10% FBS and 1% Penicillin/Streptomycin prior to T cell isolation by magnetic activated cell sorting (MACS). The CD4+ T cell fraction was isolated by negative depletion from total PBMCs using the human CD4+CD25+ Regulatory T Cell Isolation Kit [Miltenyi Biotec] according to manufacturer's instructions. CD25+ natural T$_{REGS}$ (nT$_{REGS}$) were subsequently positively selected for and separated from the CD4+CD25- naïve T cell population. Cell purity was assessed by flow cytometry staining. Cells were cultured in 37°C incubator with 5% CO$_2$ in TexMACS medium [Miltenyi Biotec]. nT$_{REG}$ and iT$_{REG}$ cells were supplemented with 500 U/mL and 100 U/mL of human recombinant IL-2 [Millipore], respectively.

2.3.2   *iT$_{REG}$ differentiation*

iT$_{REG}$ differentiation was performed as previously described (Schmidt et al. 2016). Briefly, following MACS isolation, naïve T cells were rested for 2-8 hours before plating under iT$_{REG}$ differentiation conditions at 1.1 to 1.5 x 10^5 cells/well in a 96U well plate. Cells were stimulated with 5 μg/mL plate-bound anti-CD3 antibody [OKT3, NA/LE], 1 μg/mL soluble anti-CD28 antibody [CD28.2, NA/LE; BD Biosciences] and 100 U/mL IL-2. Cells stimulated with only these reagents served as "mock" control cells. For iT$_{REG}$ differentiation, 5 ng/mL TGF-β1 [R&D Systems] and 10 nM all-trans retinoic acid [Sigma-Aldrich] were additionally added. On day 3,

100 μL of medium was removed and 100 μL of fresh medium plus growth supplements was added. Cells were then incubated for an additional 3 days.

2.3.3 Irradiation

A RS-2000 biological X-ray irradiator [Rad Source Technology] was used to irradiate cells. Irradiation was performed at a dose of 2 Gy/min at voltage 160 kV and 25 mA current. On day 6, cells were washed and resuspended in fresh TexMACS medium without cytokines. Cells were kept on ice and irradiated (5 Gy or 10 Gy) or mock-irradiated (0 Gy). Immediately following irradiation, the culture medium was replaced with fresh medium plus growth supplements minus anti-CD3 and anti-CD28.

2.3.4 Flow cytometry

Anti-human antibodies were used to characterize T_{REG} cells following isolation: Foxp3-Pacific Blue, Foxp3-PE [PCH101], Gata3-PE [TWAJ] and T-bet-PE [4B10; Invitrogen]; CD4-FITC, LAG-3-PE, CD39-APC and CD73-APC [BD Biosciences]; CD4-APC, CD25-APC, CD25-PE, CTLA-4-APC and PD-L1-APC [BioLegend]. Fixable Viability Stain 780 [BD Biosciences] was used to exclude dead cells according to manufacturer's instructions. Appropriate isotype control antibodies were used, and gating was based on < 5% isotype staining. Intracellular staining was performed using the Foxp3 Transcription Factor Staining Buffer Set [Invitrogen] according to manufacturer's instructions. Data was acquired on a BD Fortessa [Beckman Coulter] and data was analyzed using FlowJo software [TreeStar].

2.3.5 In vitro proliferation assay

Responder T cell proliferation assay was performed as previously described with minor modifications (Venken et al. 2007). Briefly, purified CD8+ T cells were labeled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE) [BD Biosciences]. Labeled CD8s were cultured at

a constant number of 6 x 10^4 cells/well either alone (1:0) or at a 4:1 ratio with either 0 Gy or 10

Gy treated iT$_{REG}$ cells 48hrs post radiation in a U-bottom 96-well plate with 5 μg/mL plate-

bound anti-CD3 and 1 μg/mL anti-CD28 in TexMACS media for 4-5 days. Proliferation was

determined by CFSE dilution on the flow cytometer and analyzed using FlowJo software.

2.3.6 *Statistical analysis*

Statistical differences between groups were calculated using the Student *t* test or a one-way

ANOVA with Bonferroni test for multiple comparisons using GraphPad Prism software.

Statistical significance was defined as *P* ≤ 0.05. *P* values: *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤

0.001.

2.4 Results

2.4.1 *Both natural T$_{REG}$ and induced T$_{REG}$ cells are more resistant to cell death by radiation than CD4+ conventional T cells*

It has been reported that T$_{REG}$ cells preferentially survive radiation treatment compared to

CD4+ conventional T (T$_{conv}$) cells in mice (Qu, Zhang, et al. 2010; Anderson et al. 2004;

Komatsu and Hori 2007). However, experiments utilizing human cells observed increased

sensitivity of T$_{REGS}$ to low dose radiation (< 2 Gy) (Cao et al. 2011). Most studies examining this

question have investigated the sensitivity of natural T$_{REGS}$ (nT$_{REGS}$) alone or the total T$_{REG}$

population, which potentially includes both natural and tumor induced T$_{REGS}$. As such, the

specific radiosensitivity of induced T$_{REG}$ (iT$_{REG}$) cells has not fully been explored. While both

nT$_{REG}$ and iT$_{REG}$ cells are functionally suppressive, Foxp3 expression is reportedly unstable in

iT$_{REGS}$ (Floess et al. 2007). Therefore, it is plausible that nT$_{REGS}$ may have different sensitivities

than T$_{REGS}$ induced in the periphery by tumors. Thus, we first compared the sensitivities of

natural and induced human T$_{REGS}$ to determine if there were differences in susceptibility to cell

death following exposure to radiation. We isolated CD4+CD25+ nT$_{REG}$ cells from human peripheral blood mononuclear cells (PBMCs) as described in the Material and Methods. To induce a T$_{REG}$ phenotype, naïve CD4+ T cells were cultured in the presence of TGF-β1 and ATRA for 6 days which results in expression of Fox3 and other T$_{REG}$ associated genes (Schmidt et al. 2016). nT$_{REG}$, iT$_{REG}$, or CD4+ T$_{conv}$ cells were subsequently exposed to low doses of radiation (5 Gy or 10 Gy) and evaluated 48 hours post-treatment for cell death. While CD4+ T$_{conv}$ cells exhibited significant increases in death after radiation, both nT$_{REG}$ and iT$_{REG}$ cells had lower relative amounts of cell death (Fig. 2.1). These results support the idea that human T$_{REG}$ cells are more radioresistant as compared to CD4+ T$_{conv}$ cells.
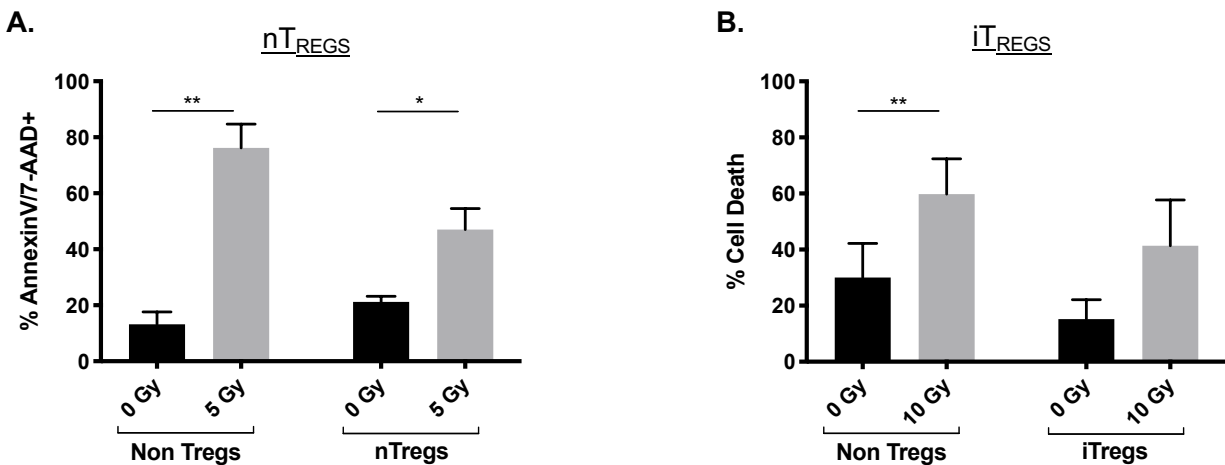
Figure 2.1 T$_{REGS}$ more radio-resistant than CD4+ T cells.
(**A**) Purified CD4+ conventional T cells or nT$_{REGS}$ were exposed to 5 Gy of radiation or mock irradiated (0Gy). After 48hrs, cells were stained with Annexin V and 7-AAD and analyzed by flow cytometry. (**B**) TGF-β1-induced T$_{REGS}$ or CD4+ T$_{conv}$ cells were treated with 10 Gy of radiation or mock irradiated. 48hrs post treatment cells were stained with a fixable viability dye (FVS) and CD4 or FVS, CD4, CD25, and Foxp3 to denote CD4+ T$_{conv}$ and iT$_{REG}$ cells, respectively. Data are representative of three independent experiments. Error bars represent SEM. *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ by paired, one-tailed Student t test.

2.4.2 Radiation decreases Foxp3 expression more robustly in iT$_{REGS}$ as compared to nT$_{REGS}$

T$_{REG}$ cells express the transcription factor Foxp3, a master regulator essential for their development and suppressive function (Shimon Sakaguchi et al. 2008). Several groups have reported different effects of radiation on Foxp3 expression. Murine studies have reported both an increase (Muroyama et al. 2017; Kachikwu et al. 2011) and decrease (R. Liu et al. 2010) in T$_{REG}$ frequency following radiation while a human study noted a reduction in CD4+CD25+ T$_{REG}$ cells (Cao et al. 2009). Similar to reports describing radiations impact on T$_{REG}$ radiosensitivity, most of the studies examining FoxP3 expression were performed in mice. Additionally, *in vivo* experiments in disease settings evaluated the total T$_{REG}$ population, which likely contain both nT$_{REG}$ and iT$_{REG}$ cells, while human experiments assessed only the nT$_{REG}$ cell population. We therefore evaluated human natural and induced T$_{REGS}$ for Foxp3 expression following exposure to low dose radiation, *in vitro*. Foxp3 expression in CD4+CD25+ nT$_{REGS}$ decreased after treatment with 10 Gy (Fig. 2.2A). FoxP3 was expressed in 88% of untreated cells on average and decreased to 68% in cells treated with radiation across 3 independent experiments (Fig. 2.2B). More cells expressed CD25 in iT$_{REGS}$ as compared to nT$_{REGS}$, however FoxP3 expression was still decreased by radiation within these cells (Fig. 2.2C). Foxp3 was detected in 38% of untreated iT$_{REGS}$ and was reduced to 8% following radiation across independent experiments (Fig. 2.2D). Interestingly, iT$_{REGS}$ showed a more robust decrease in Foxp3 expression when compared to nT$_{REGS}$. iT$_{REG}$ cells are characterized as expressing high levels of CD25. Evaluation of the CD4+CD25^{hi} population of iT$_{REGS}$ revealed that Foxp3 was more highly expressed in the untreated cells (69% on average) and that radiation significantly decreased Foxp3 expression within CD25^{hi} iT$_{REGS}$ down to 10% (Fig. 2.2E). Interestingly, the magnitude of decreased Foxp3 expression was greater within the CD25^{hi} population as compared to that observed in the total

CD25+ iT$_{REG}$ population. Compared to untreated cells, both nT$_{REGS}$ and iT$_{REGS}$ showed a

significant decrease in Foxp3 expression 48 hours after exposure to 10 Gy. Furthermore, the

percent of total CD4+ T cells remained unchanged (data not shown) within iT$_{REGS}$ suggesting

that radiation specifically downregulates the expression of Foxp3 and that iT$_{REG}$ cells were more

sensitive to this effect.
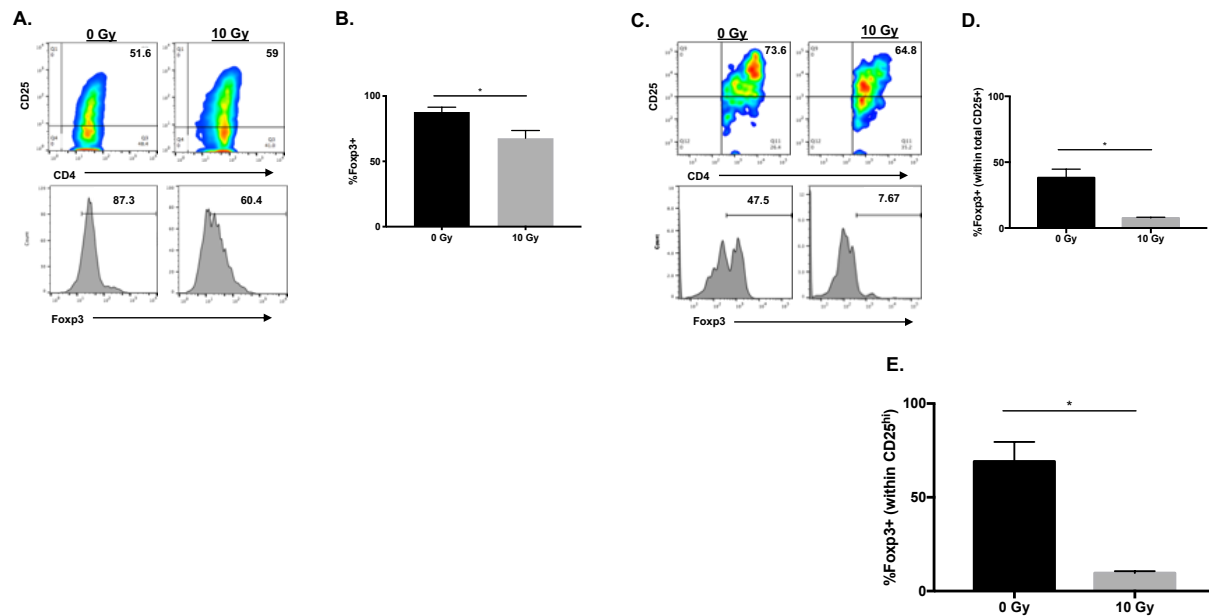
Figure 2.2 Radiation decreases Foxp3 expression in natural and induced human CD4+CD25+ T$_{REGS}$.
Natural and induced T$_{REG}$ cells were mock irradiated or exposed to 10 Gy of radiation. (**A**) After 48hrs, nT$_{REGS}$ were stained for expression of CD4 and CD25 by flow cytometry. (**B**) Expression of Foxp3 was evaluated within the CD4+CD25+ population. (**C**) 48hrs after irradiation, TGF-β1-induced T$_{REGS}$ were stained for expression of CD4 and CD25 by flow cytometry. (**D**) Expression of Foxp3 was evaluated within the total CD4+CD25+ population. (**E**) iT$_{REGS}$ were evaluated for the expression of Foxp3 within the CD4+CD25^{hi} population. Data are representative of three independent experiments. Error bars represent SEM. *$P \leq 0.05$ by paired, one-tailed Student t test.

2.4.3   *Irradiated iT$_{REGS}$ are not converted to another T cell subset following loss of Foxp3*

Plasticity is a unique characteristic of CD4+ T cells, allowing them to differentiate from

one T helper (T_H) subset to another when exposed to the right cytokine milieu (L. Zhou, Chong, and Littman 2009). Additionally, epigenetic changes in transcription factor activity induce changes in the type of CD4+ T cell needed for the appropriate immune response (Wei et al. 2009). Foxp3 is induced in regulatory T cells to limit cell cytotoxicity and autoimmunity (Haribhai et al. 2011). The transcription factors T-box transcription factor (T-bet) and GATA binding protein 3 (GATA3) drive T_H1 and T_H2 differentiation, respectively (Chakir et al. 2003). Because changes in the microenvironment can directly influence the phenotype of local CD4+ T cells (Butcher et al. 2016), we wanted to determine if irradiated iT_{REGS} were being converted into another T_H subset upon downmodulation of Foxp3 expression. While radiation robustly reduced Foxp3 expression in iT_{REG} cells, expression of T_H1-associated T-bet or T_H2-associated GATA3 did not exhibit a compensatory increase in expression 48 hours post-treatment (Fig. 2.3). Interestingly, while T-bet expression was low and remained low after radiation, GATA3 expression was detected in a subpopulation of the cultured cells and its expression was also reduced by radiation. These data suggest that while radiation can reduce expression of transcription factors in CD4+ T cells, irradiated iT_{REG} cells are not converted to a T_H1 or T_H2 subset but instead can be described as an "ex-Foxp3+" CD4+ T cell.
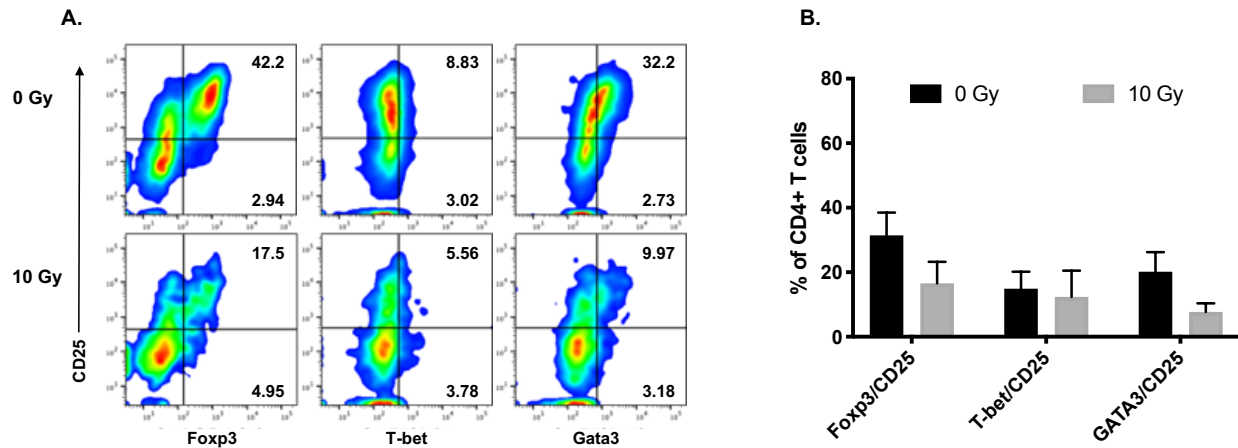
Figure 2.3 Irradiated iT$_{REGS}$ are not converted to a T$_H$1 or T$_H$2 subset after radiation. Induced T$_{REG}$ cells were mock irradiated or exposed to 10 Gy of radiation. (**A**) iT$_{REGS}$ were analyzed 48hrs post-treatment for CD25 and Foxp3, T-bet, or GATA3 expression within CD4+ T cells. Representative plots of CD4+ T cells and (**B**) mean frequency of each subset. Data are representative of three independent experiments. Error bars represent SEM. *P ≤ 0.05 by paired, one-tailed Student *t* test.

2.4.4   *Radiation induces differential changes in signature T$_{REG}$ molecules*

Aside from Foxp3, T$_{REG}$ cells express several signature molecules associated with their regulation and functional activity. CD25, the high-affinity IL-2 receptor, is highly expressed by iT$_{REGS}$. CD25 is also regulated by Foxp3 through binding at the *Cd25* promoter thus enhancing its expression (Camperio et al. 2012). Because we observed a decrease in Foxp3 following radiation treatment we wanted to determine if CD25 expression was also reduced. When iT$_{REGS}$ were evaluated for CD4+CD25+ double positive cells we observed a significant decrease in CD25 expression in irradiated iT$_{REGS}$ as compared to untreated cells (Fig. 2.4A). This was observed within the total CD25+ population as well as the CD25^{hi} cells. In contrast, while CD25 expression was reduced in irradiated CD4+ T$_{conv}$ cells compared to untreated cells, the change was not as significant as that observed in iT$_{REGS}$ (Fig. 2.4B). Because CD4+CD25^{hi} iT$_{REG}$ cells had the highest frequency of Foxp3+ cells we further evaluated this cell population for the expression of other surface proteins associated with T$_{REG}$ suppressive function. Cytotoxic T

lymphocyte antigen 4 (CTLA-4) and lymphocyte activation gene 3 (LAG-3) have been shown to block dendritic cell maturation and inhibit effector T cell proliferation (Huang et al. 2004; Kolar et al. 2009; Onishi et al. 2008; Liang et al. 2008). Concordant expression of the ectoenzymes CD39 and CD73 suppress effector T cell function by converting ATP into adenosine (Deaglio et al. 2007). Furthermore, the presence of PD-L1+ T_{REGS} has been correlated with exhausted effector T cells and a suppressive tumor microenvironment (S. P. Wu et al. 2018). Similar to CD25, CTLA-4 and LAG-3 have been reported to be regulated by Foxp3 (Xie et al. 2015; Sadlon et al. 2010) thus we wanted to determine if their expression would also be reduced following radiation. Radiation significantly down-regulated the expression of CTLA-4 in $CD4+CD25^{hi}$ iT_{REGS} from 57% to 44% (Fig. 2.4C). In contrast, LAG-3 (34% to 48%) (Fig. 2.4D) and CD73 (20% to 28%) (Fig. 2.4F) were moderately upregulated following treatment. Interestingly, radiation had no effect on the expression of CD39 (Fig. 2.4E) and PD-L1 (Fig. 2.4G). These results suggest that radiation-induced modulation of iT_{REG}-associated suppressive proteins may not be strictly dependent on Foxp3 regulation or that LAG-3 expression may be regulated by other mechanisms in iT_{REGS} following radiation treatment.
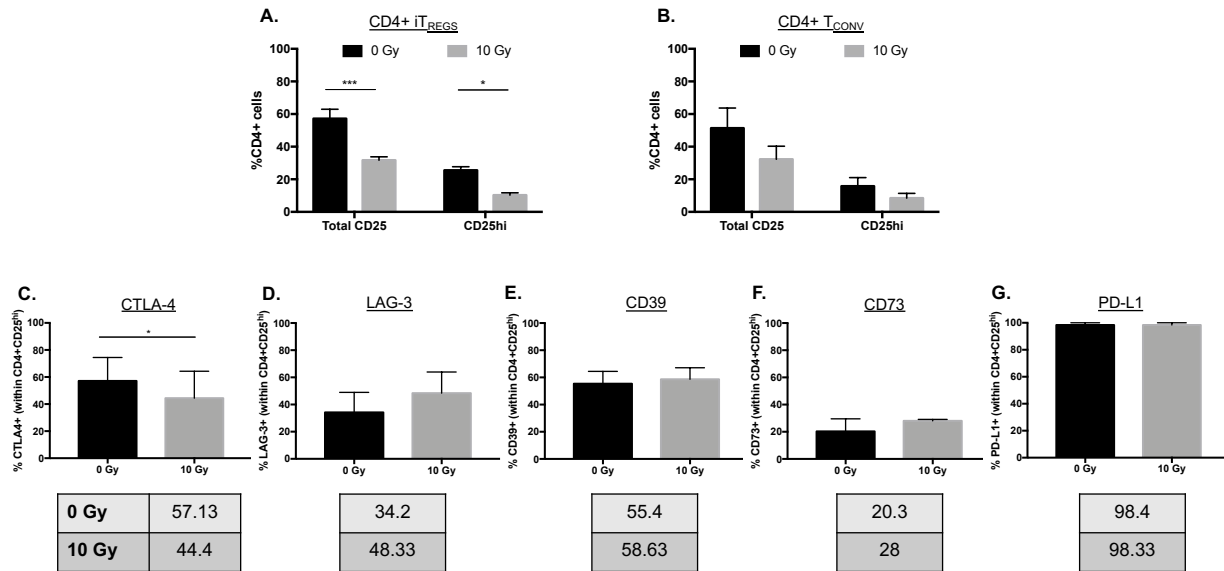
Figure 2.4 Phenotypic modulation of CD4+CD25^{hi} iT$_{REGS}$ by radiation.
(**A**) iT$_{REGS}$ and (**B**) CD4+ T$_{conv}$ cells were mock irradiated or exposed to 10 Gy of radiation.
48hrs post treatment CD4+ cells were analyzed for expression of CD25 by flow cytometry. Live
cells were gated on CD4+CD25^{hi} and the expression of (**C**) CTLA-4, (**D**) LAG-3, (**E**) CD39, (**F**)
CD73, and (**G**) PD-L1 was evaluated 48hrs after radiation; mean of target proteins shown below
(**C-G**). Data are representative of three independent experiments. Error bars represent SEM. *$P \le$
0.05; ** $P \le 0.01$; *** $P \le 0.001$ by paired, two-tailed Student t test.

Not all cells in the CD4+CD25^{hi} population were Foxp3 positive (Fig. 2.2E) so we next

evaluated changes to T$_{REG}$ suppressive molecules within Foxp3+ cells following radiation

treatment. For this analysis iT$_{REGS}$ were defined as CD4+Foxp3+ (Fig. 2.5A) and the expression

of suppressive molecules after radiation was measured. PD-L1 was expressed in over 80% of

CD4+Foxp3+ iT$_{REG}$ cells while CD39 was expressed in about 30% of cells. 48hrs post-radiation

we saw little change in CD39 (Fig. 2.5E) and PD-L1 (Fig. 2.5G) expression. Similar to the

change detected in CD4+CD25^{hi} cells (Fig. 2.4C), CD25 (Fig. 2.5B) and CTLA-4 (Fig. 2.5C)

expression also decreased upon radiation in CD4+Foxp3+ cells though the modulation did not

reach statistical significance. We detected a significant increase in LAG-3 expression from 24%

to 29% (Fig. 2.5D), as well as an increase in CD73 that neared statistical significance (Fig. 2.5F).

Overall, analysis of both CD4+FoxP3+ and CD4+CD25^{hi} iT$_{REGS}$ revealed that radiation reduced

expression of CTLA-4 and CD25, while conversely increasing expression of LAG-3 and CD73.

However, no change in the expression of CD39 or PD-L1 was induced by *in vitro* irradiation in

either cell population.
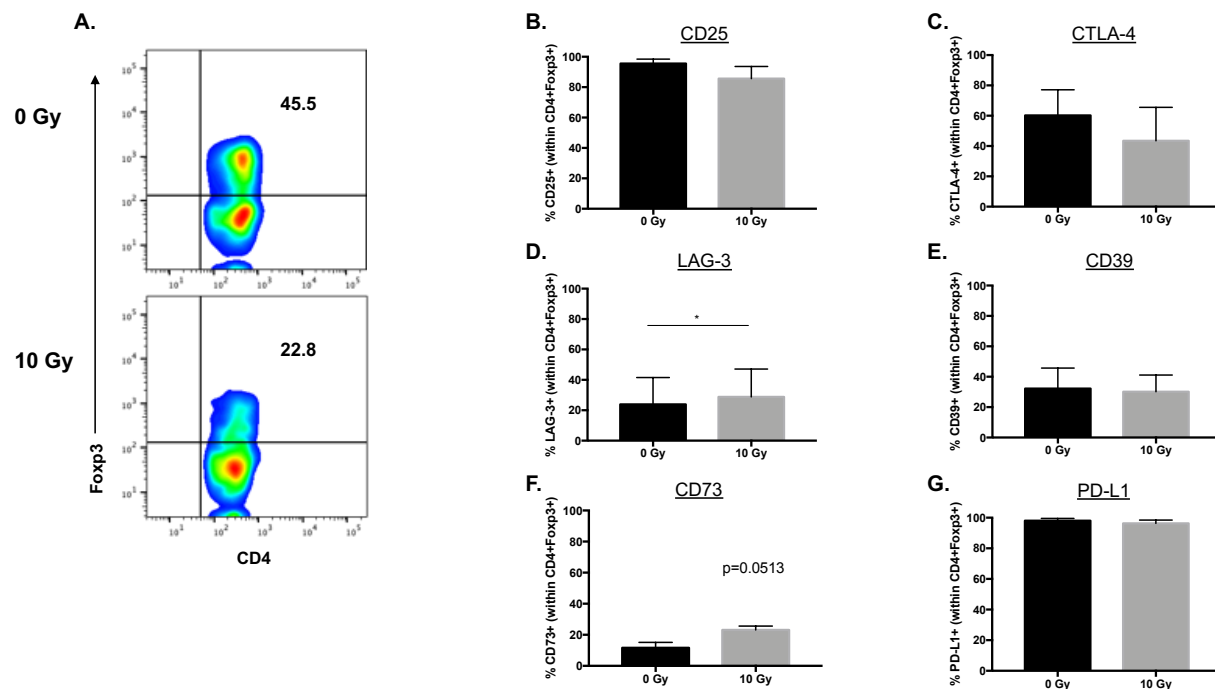
Figure 2.5 Phenotypic modulation of CD4+Foxp3+ iT$_{REGS}$ by radiation.
(**A**) iT$_{REGS}$ were mock irradiated or exposed to 10 Gy of radiation. 48hrs post treatment
CD4+Foxp3+ cells were analyzed for expression of (**B**) CD25, (**C**) CTLA-4, (**D**) LAG-3, (**E**)
CD39, (**F**) CD73, and (**G**) PD-L1. Data are representative of two-three independent experiments.
Error bars represent SEM. *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ by paired, two-tailed Student *t*
test.

2.4.5 *Radiation inhibits suppressive activity of iT$_{REGS}$*

We observed that radiation treatment reduces the expression of Foxp3 in iT$_{REG}$ cells and

that the expression of molecules associated with their functional capacity to suppress other

immune cells is modulated both positively and negatively post treatment (decreased expression

of CD25 and CTLA-4 versus increased expression of LAG-3 and CD73). Thus, we wanted to

directly investigate whether irradiated iT$_{REG}$ cells retained their suppressive function. We

compared the ability of irradiated and non-irradiated iT$_{REGS}$ to inhibit CD8+ T cell proliferation.

48hrs after treatment with radiation, viable iT$_{REGS}$ were counted and co-incubated with

autologous CFSE-labeled CD8+ T cells at a ratio of 1:4. Cells were co-cultured together for 4-5

days and CD8+ T cell proliferation was measured by CFSE dilution. CD8+ T cells had a mean

proliferation rate of 92% in the presence of 10 Gy treated iT$_{REGS}$, as compared to only 72%

following co-culture with non-irradiated iT$_{REG}$ cells (Fig. 2.6).
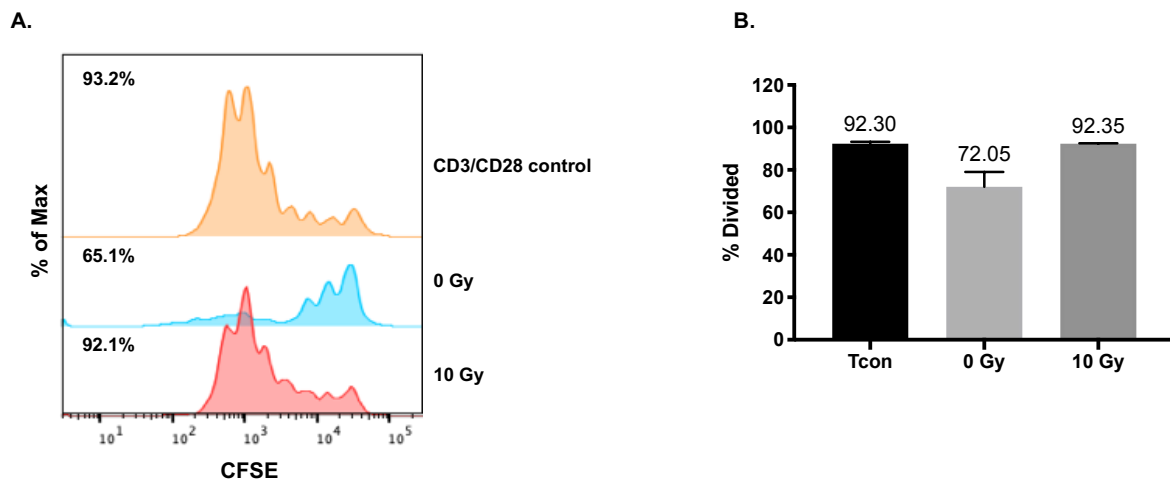
Figure 2.6 Irradiated iT$_{REGS}$ exhibit reduced suppressive capacity.
CFSE-labeled CD8+ T cells were stimulated with CD3 and CD28. Irradiated or non-irradiated
iT$_{REGS}$ were co-incubated with stimulated CD8+ T cells at a ratio of 1:4 for 4-5 days. (**A**)
Histogram overlay displaying CD8+ T cell CFSE dilution in the presence of 0 Gy (middle
portion of plot) or 10 Gy treated iT$_{REGS}$ (bottom portion of plot) at a suppressor:responder ratio
of 1:4. CD8 alone division is displayed in top portion of plot. (**B**) Percentage of divided CD8+ T
cells cultured with mock or 10 Gy treated iT$_{REG}$ cells or alone. Data are representative of two
independent experiments. Error bars represent SEM. *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ by a
one-way ANOVA with Bonferroni test for multiple comparisons.

2.5   Discussion

Radiotherapy is a common treatment modality for cancer and lower doses can effectively

enhance antitumor immune responses by modulating tumor phenotypes (Kumari et al. 2016).

However, the effect of radiation directly on T$_{REG}$ viability, phenotype, and function remains

controversial and less well evaluated in human cells. In this study, we compared the effect of radiation treatment on human natural versus induced T$_{REG}$ cell viability and expression of Foxp3. Furthermore, we examined iT$_{REGS}$ for radiation-induced changes in cell phenotype and suppressive capacity. We show that irradiated nT$_{REG}$ and iT$_{REG}$ cells are more viable than conventional CD4+ cells and that CD4+CD25+ T$_{REG}$ cells exhibit decreased expression of Foxp3 after exposure to ionizing radiation. Additionally, we demonstrated that iT$_{REG}$ cell phenotype is differentially modulated following radiation treatment and that their suppressive function is inhibited.

Earlier reports in mice showed that T$_{REGS}$ are more resistant to radiation treatment (Qu, Jin, et al. 2010; Komatsu and Hori 2007). Our results in human cells are in line with previously reported data that T$_{REG}$ cells are more resistant to radiation-induced cell death as compared to CD4+ T$_{CONV}$ cells. Additionally, we found that this resistance was seen in both nT$_{REG}$ and iT$_{REG}$ cells. However, these results contrast those previously observed in other studies of human T$_{REGS}$ (Cao et al. 2011). A significant factor that could explain this discrepancy in human T$_{REG}$ viability is radiation dose. Previous reports exposed T$_{REGS}$ to low doses of radiation (0.94 Gy and 1.875 Gy) resulting in significantly more cell death in T$_{REGS}$ as compared to CD4+ T$_{CONV}$ cells (Cao et al. 2011). In contrast, our study utilized higher radiation doses (5 Gy and 10 Gy) which showed a marked increase in CD4+ T$_{CONV}$ cell death compared to both nT$_{REG}$ and iT$_{REG}$ cells (Fig. 2.1). Radiation reportedly decreases human CD4+ T cell viability in a dose-dependent manner (Nakamura, Kusunoki, and Akiyama 1990), cells exposed to 5 Gy of radiation exhibited a robust decrease in live cells not seen in cells treated with ≤ 2 Gy. Therefore, it is plausible that human T$_{REGS}$ are more resistant to higher radiation doses whereas low dose radiation does not induce

significant death in CD4+ T_{CONV} cells. Doses above 2 Gy are more commonly used in cancer therapy than those below 2 Gy.

Much of what is known about T_{REG} phenotype and functional activity has been derived from murine models. However, little research has been conducted to elucidate if similar trends are observable in human cells. Thus, conflicting reports regarding the effect of radiation treatment on T_{REG} cells could be attributed to the use of murine versus human cells. Moreover, different experimental designs have been used. When evaluating T_{REG} frequency in murine models, the use of whole-body versus local radiation treatment could have a profound effect on both overall T_{REG} number and observed functional capacity. Mice treated with low-dose total body irradiation exhibited a decrease in the frequency and total number of nodal CD4+Foxp3+ T_{REG} cells (R. Liu et al. 2010), while mice that received local irradiation were found to increase the proportion of tumoral and splenic T_{REGS} (Kachikwu et al. 2011; Muroyama et al. 2017). In addition, T_{REGS} from locally irradiated mice retained their suppressive function.

It is important to distinguish natural versus induced T_{REG} cells. Though phenotypically similar, nT_{REG} and iT_{REG} cells display distinctly different regulatory functions (Haribhai et al. 2011). Despite this fact, we are unaware of studies to elucidate if radiation-induced modulations observed are attributed to natural and/or induced T_{REGS}. Here, our focus was on human TGF-β1-induced T_{REG} cells which would be similar to the tumor-induced T_{REGS} exposed to radiation as a part of cancer treatment. The reason for the differential observations remains unclear, however it is possible that the composition of natural versus induced T_{REGS} within the experimental systems may have an impact on the frequency of T_{REG} cells observed.

Several groups have reported both an increase and decrease in T_{REG} frequency when exposed to radiation (Muroyama et al. 2017; Kachikwu et al. 2011; R. Liu et al. 2010; Cao et al.

2009). Our findings showed a significant decrease in human T_{REG} cells 48 hours post treatment *in vitro*. Importantly, this decrease was observed in nT_{REG} and iT_{REG} cells as indicated by downregulation of Foxp3 expression (Fig. 2.2A-D). Among iT_{REGS}, this decrease was observable regardless of whether Foxp3 was analyzed among total CD25+ or CD25^{hi} T cells (Fig. 2.2D-E). Though iT_{REGS} downregulated Foxp3, this did not correlate to an increase in the T_H1 or T_H2-associated transcription factors T-bet and GATA3, respectively (Fig. 2.3). These results are significant because they suggest that radiation is not converting iT_{REGS} into another CD4 subtype and that CD4 expression is not affected.

Phenotypically, Foxp3 is not the sole protein characterizing T_{REGS}. High expression of CD25, as well as expression of CTLA-4, CD39, CD73, and LAG-3 have all been reported to be expressed by T_{REGS}. Additionally, some groups have reported the presence of PD-L1 on T_{REGS} in tumor models (S. P. Wu et al. 2018). To our knowledge, the effect radiation has on the expression of many of these molecules in T_{REGS} has not been reported, particularly in human derived iT_{REG} cells. T_{REG} cells are commonly defined as being Foxp3+ and CD25^{hi}. We therefore compared the phenotype of iT_{REGS} defined as either CD4+CD25^{hi} or CD4+Foxp3+. These experiments revealed that either phenotypic characterization of iT_{REGS} displayed similar trends in protein expression after radiation treatment (Fig. 2.4 and Fig. 2.5). Both gating strategies showed a decrease in CD25 and CTLA-4 while LAG-3 and CD73 expression increased. Lastly, CD39 and PD-L1 expression was unchanged with radiation treatment as assessed by either strategy. This observation is particularly noteworthy because it suggests that as Foxp3 regulated genes, CD25 and CTLA-4 expression may be directly tied to Foxp3 expression.

In addition to CD25 and CTLA-4, LAG-3 is also reported as being regulated by Foxp3 (Xie et al. 2015). Interestingly, we observed an increase in LAG-3 expression following low

dose radiation as opposed to the decrease seen in CD25 and CTLA-4 expression (Fig. 2.4 and

Fig. 2.5). Similarly, chemoradiation was shown to increase the proportion of CD4+LAG-3+

expressing T cells in head and neck cancer patients (Sridharan et al. 2016). It is not clear how

radiation treatment upregulates the expression of LAG-3 in iT$_{REGS}$. It is possible that radiation is

directly altering expression of this gene via epigenetic mechanisms as has been reported for

expression of some immune relevant genes in irradiated tumor cells (Kumari et al. 2013),

however further exploration into this possibility is needed. In addition, previous studies of

irradiated human nT$_{REG}$ cells noted enhanced expression of glucocorticoid-induced tumor

necrosis factor receptor (GITR), another Foxp3 regulated gene (Cao et al. 2009). These

observations combined with our results greatly suggest that Foxp3-associated genes may be

regulated by other mechanisms other than Foxp3 following radiation.

A possible orchestrator could be the transcription factor early growth response gene 2

(Egr2). Egr2 has been shown to convert naïve CD4+ T cells into LAG-3-expressing T$_{REGS}$

(Okamura et al. 2009). Notably, these LAG-3-expressing T$_{REGS}$ were characterized as being

Foxp3-. Our study demonstrates that radiation induces a CD4+Foxp3- T cell subset from

CD4+CD25^{hi}Foxp3+ iT$_{REGS}$ ("ex-Foxp3+ cells"). iT$_{REG}$ cell conversion in our study focused on

possible plasticity and conversion towards a T$_H$1 or T$_H$2 subset. However, it is plausible that

radiation treatment could convert Foxp3+ iT$_{REGS}$ to another regulatory T cell subset. Though

LAG-3 expression has been reported to confer Foxp3+ regulatory T cells with greater

suppressive capacity (Huang et al. 2004; Liang et al. 2008), we found that irradiated iT$_{REG}$ cells

were functionally less suppressive as compared to untreated cells (Fig. 2.6) despite an increase in

LAG-3 expression. This is in line with reports that showed Egr2-transduced CD4+ T cells, which

positively expressed LAG-3 and IL-10, insufficiently suppressed proliferation of responder T

cells *in vitro* (Okamura et al. 2009). Subsequent *in vivo* studies were able to demonstrate the suppressive capacity of Egr2-transduced CD4+ T cells which could suggest functional differences in LAG-3 activity *in vitro* versus *in vivo*.

LAG-3 on CD4+ and CD8+ T_{CONV} cells inhibits their expansion and effector function (Durham et al. 2014; Grosso et al. 2007). However, it isn't entirely clear how LAG-3 signaling impacts T_{REGS}. In a murine model of Type 1 diabetes LAG-3 was shown to limit T_{REG} function (Q. Zhang et al. 2017). Anti-LAG-3 blocking antibodies are currently being used in preclinical studies and phase 1 clinical trials in combination with anti-PD-1. Recent studies have revealed that dual treatment with anti-LAG-3 and anti-PD-1 blocking antibodies significantly enhances the proliferation of CD4+ and CD8+ T_{CONV} cells (Lichtenegger et al. 2018). Therefore, the combined use of radiotherapy and anti-LAG-3 blocking antibodies could greatly enhance the antitumor immune response in preclinical studies. However, it seems plausible that antagonistic antibodies that prevent LAG-3 signaling may enhance T_{REG} suppressive function at the same time that they are promoting effector T cell activity. Further studies are needed to elucidate the effect LAG-3 has on T_{REG} suppressive function.

It has been reported that the suppressive function of iT_{REG} cells is conferred by the expression of Foxp3 (Hori, Nomura, and Sakaguchi 2003), however it is unclear which suppressive mechanism(s) have been arrested in irradiated iT_{REGS}. In this study we showed that radiation is capable of modulating the expression of Foxp3 and several iT_{REG} suppressive surface molecules, though it is also possible that radiation also induces changes in T_{REG} associated cytokines, TGF-β1 and IL-10, which could be evaluated in further investigations.

2.6 Conclusion

In summary, our study found that both human nT$_{REG}$ and iT$_{REG}$ cells are more resistant to radiation-induced cell death and that radiation treatment reduces their expression of Foxp3. In addition, we demonstrate that radiation modulates iT$_{REG}$ cell phenotype and inhibits their suppressive activity. This data provides a rationale for the use of radiation in combination with current immunotherapies to increase antitumor immune responses by specifically targeting Foxp3+ iT$_{REG}$ cell function.

3 CHARACTERIZATION OF THE IMPACT OF RADIOTHERAPY ON REGULATORY T CELLS AND THE TUMOR MICROENVIRONMENT IN DIVERSE MURINE TUMOR MODELS

3.1 Abstract

The impact of radiation on T_{REG} cells remains controversial. Sub-lethal radiation has been shown by our lab and others to alter the phenotype of human tumor cells by enhancing their expression of the co-stimulatory molecules OX40L and 4-1BBL. Signaling through both OX40 and 4-1BB have been reported to reduce T_{REG} frequency and function. We therefore examined whether radiation-induced modulation of OX40L and 4-1BBL in murine tumor cells could influence the frequency of T_{REG} cells in tumor bearing mice. In addition, we evaluated differences in OX40L and 4-1BBL expression in two widely used murine tumor models for these experiments. 4T1 mammary and MC38 colorectal cells were implanted subcutaneously into mice and treated locally with 8-10 Gy of radiation. We found that radiation differentially modulated the expression of OX40L and 4-1BBL among the two tumor models, however this expression did not appear to correlate with the observed reduction in T_{REG} frequency seen in both tumor models. Further analysis aimed to assess differences in immune cell composition induced by radiation. We show that CD8+ effector T cells were increased in irradiated 4T1 but not in MC38 tumors. Further, we show that radiation induces differential changes in the immune cell gene profile among 4T1 and MC38 tumor models. Overall, our data suggests that radiation-induced changes in tumor expressed OX40L and 4-1BBL do not mediate changes in T_{REG} frequency within tumors. Moreover, this study highlights that differences in the tumor microenvironment and immune cell profile may contribute to variations in therapeutic responses of various murine models utilizing radiation.

3.2   Introduction

T cell immunity is an important factor in the elimination of tumor cells and inhibiting tumor growth. T cell activity can be inhibited by signals through T cell checkpoint proteins such as CTLA-4 and PD-1. The use of immunotherapeutic agents such as anti-CTLA-4 and anti-PD-1/PD-L1 blocking antibodies can effectively enhance anti-tumor responses (Curran et al. 2010; Fransen et al. 2013; Selby et al. 2013). Unfortunately, checkpoint blocking antibodies only benefit a small percentage of patients and are not always effective (Fares et al. 2019). A major contributor of tumor escape is the presence of immunosuppressive cells within the tumor microenvironment including regulatory T (T_{REG}) cells. T_{REGS} are a suppressive subset of CD4+ T cells that negatively regulate anti-tumor immunity. These cells function by inhibiting dendritic cell maturation and cytotoxic T lymphocyte (CTL) effector activity (Liang et al. 2008; Onishi et al. 2008; McNally et al. 2011; Bauer et al. 2014). Thus, the selective removal of T_{REG} cells or reduction in their suppressive capacity, as well as increasing CTLs could greatly enhance anti-tumor activity.

OX40 and 4-1BB are T cell co-stimulators belonging to the tumor necrosis factor superfamily that bind to OX40L and 4-1BBL, respectively (Croft 2009). Expression of OX40L and 4-1BBL is induced on professional antigen presenting cells, CD4+ T cells, and CD8+ T cells, as well as endothelial cells and smooth muscle cells under inflammatory conditions. OX40 and 4-1BB are inducibly expressed on activated CD4+ and CD8+ T cells and are involved in the promotion of effector T cell clonal expansion, survival, and cytokine induction (Croft 2009; Vinay and Kwon 2012). Conversely, signaling through both OX40 and 4-1BB have been shown to inhibit the conversion of naïve CD4+ T cells into T_{REGS}, abrogate Foxp3 expression, and inhibit T_{REG} suppressive function (Vu et al. 2007; Xiao et al. 2008; Kitamura et al. 2009; Smith,

Hoeizinger, and Dominguez 2011; Madireddi et al. 2012; X. Zhang et al. 2018). These are important signals for promoting tumor immunity and treatment of tumor-bearing mice with agonist antibodies to OX40 and 4-1BB have been reported to induce effective anti-tumor immune responses and decrease the activity of murine T_{REG} cells (Gough et al. 2008; Curran et al. 2011; Smith, Hoeizinger, and Dominguez 2011; Barsoumian, Yolcu, and Shirwan 2016). Radiation has been shown to induce the expression of OX40L and 4-1BBL in several human tumor cells (Bernstein et al. 2014; Kumari and Garnett-Benson 2016). Additionally, we previously reported that radiation-induced modulation of OX40L and 4-1BBL on human colorectal tumor cells increased killing by CTLs (Kumari and Garnett-Benson 2016), however it is unknown how such changes effect T_{REG} cells. Therefore, this study was designed to test the hypothesis that radiation-induced modulation of tumor expressed OX40L and 4-1BBL reduces T_{REG} number.

In addition, data regarding the efficacy of radiotherapy in combination with cancer immunotherapies, and the impact of radiation on T cell frequencies, has been based on observations in pre-clinical murine tumor models. Much of this data comes from two of the most commonly used tumor models, 4T1 and MC38. Here we also investigate differences in OX40L and 4-1BBL expression, as well as differences in the immune cell profile between these model systems. Our data revealed that radiation differentially modulates the expression of OX40L and 4-1BBL in 4T1 and MC38 tumor cells. Data also revealed that T_{REGS} are significantly reduced with radiation in both tumor models despite marked differences in OX40L and 4-1BBL expression between these models, suggesting that signaling through these molecules is not likely contributing to the reduction in T_{REGS} observed. Additionally, we showed that radiation differentially modulates the immune cell profile of 4T1 and MC38 tumors.

3.3   Materials & Methods

3.3.1   Tumor cell lines

Murine 4T1 (breast) and MC38 (colon) carcinoma cell lines were obtained from LTIB, NCI, NIH. Cells were cultured according to ATCC recommendations and periodically tested to ensure the absence of Mycoplasma. Cells were cultured at $37^{o}C$ with 5% CO_2.

3.3.2   Irradiation of cells in vitro

A RS-2000 biological X-ray irradiator [Rad Source Technology] was used to irradiate cells and mice. Irradiation was performed at a dose of 2 Gy/min by setting irradiator voltage to 160 kV and current to 25 mA. Tumor cells were washed and resuspended in fresh culture medium. Cells were kept on ice and irradiated (5 Gy or 10 Gy) or mock-irradiated (0 Gy). Immediately following irradiation, culture medium was replaced with fresh medium.

3.3.3   Irradiation of tumor-bearing mice

6-8 week old BALB/c or C57BL/6 mice were purchased from Charles River Laboratories. C57BL/6[41BBnull] mice were generated in-house. Mice were injected subcutaneously (s.c) in the right hind leg with 8 x 10^5 4T1 or 3 x 10^5 MC38 cells in 100 uL of 1x PBS. Tumor growth was measured every 2-3 days. When tumors reached 200-400 mm^3 mice were anesthetized using a cocktail of 100 mg/kg ketamine and 10 mg/kg xylazine before being locally irradiated (8 Gy) or mock-irradiated (0 Gy). Mice were restrained in a plexi-glass jig [Braintree Scientific] and placed under a lead shield such that only the tumor-bearing leg was exposed to radiation. Mice were sacrificed 24-48 hours post-irradiation and tumors harvested. Tumor samples were also flash frozen in O.C.T. Compound [Tissue-Tek] and sectioned for fluorescence microscopy staining or formalin fixed [Anatech].

3.3.4   Flow cytometry

Anti-mouse antibodies used: OX40L-APC and 4-1BBL-PE [BioLegend]. Appropriate isotype control antibodies were also used. Data was acquired on a BD Fortessa [Beckman Coulter]. FACS data was analyzed using FlowJo software [TreeStar].

3.3.5   Quantitative real-time PCR

mRNA was isolated from formalin-fixed paraffin embedded tumor tissue sections using the RNeasy FFPE Kit [Qiagen] according to manufacturer's instructions. mRNA concentration was measured using a NanoVue nanodrop [GE Healthcare]. cDNA was synthesized using 300ng of mRNA and amplified using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR [Thermo Scientific] according to manufacturer's instructions. qPCR was conducted using TaqMan gene expression assay [OX40L: Mm00437214; EIF1: Mm00783932_s1; Applied Biosystems]. Gene-specific primer sequences to mouse 4-1BBL were adopted from (Devarapu et al. 2017). The primers for mouse 4-1BBL consisted of forward primer (5'-GCGTTGTGGGTAGAGGAGCAAA-3') and reverse primer (5'-CCAAGTACCTTCTCCAGCATAGG-5') [GenBank: NM_009404]. Primers were obtained from Integrated DNA Technologies. All samples were run in duplicate. Data were collected using a 7500 Real Time PCR System and analyzed using the comparative ΔΔCt method (Livak and Schmittgen 2001). Target genes were normalized to the eukaryotic translation initiation factor 1 (EIF1) housekeeping gene. Expression levels of target genes were compared between non-irradiated and irradiated samples.

3.3.6   Fluorescence microscopy

Frozen tumor tissue sections were fixed with 4% paraformaldehyde at -20°C for 10 minutes and washed twice with mQH$_2$O. To conserve reagents, a hydrophobic barrier was drawn around each

tissue section. Tissue was blocked with 2% BSA in PBS in a humidified chamber on rocker for 1 hour at room temperature. After washing 3 times with TBS, tissues were surfaced stained by incubating with anti-rat CD4 [clone GK1.5; Thermo Scientific] or anti-rabbit CD8α [Santa Cruz Biotechnology] unconjugated antibodies overnight on rocker at 4°C. Tissues were washed 3 times before secondary antibody staining with Alexa Fluor 488 [Invitrogen] for 1 hour at room temperature followed by 3 additional washes. Intracellular staining was subsequently performed following fixation with 4% paraformaldehyde and blocking with 2% BSA in TBS containing 0.05% Tween-20 (TBST). Tissue was incubated with anti-mouse/rat Foxp3-eFluor 570 [clone FJK-16s; Invitrogen] overnight on rocker at 4°C. Slides were mounted with DAPI Fluoromount-G [SouthernBiotech] to distinguish nucleated cells. Images were acquired using a LSM700 confocal microscope [Zeiss]. 10 image fields per tumor section were randomly selected on Foxp3 (T_{REG}/CD4+ T cell analysis) or DAPI (CD8+ T cell analysis). The number of positive cells per image field were manually counted and the average recorded.

3.3.7   *NanoString*

mRNA was isolated from formalin-fixed paraffin embedded tumor tissue sections using the RNeasy FFPE Kit [Qiagen] according to manufacturer's instructions. mRNA concentration was measured using a NanoVue nanodrop [GE Healthcare]. Genes were quantified using an nCounter system (NanoString Technologies).

3.3.8   *Statistical analysis*

Statistical differences between groups were calculated using the paired or unpaired, one-tailed or two-tailed Student *t* test or a one-way ANOVA with Bonferroni test for multiple comparisons using GraphPad Prism software. Statistical significance was defined as $P \leq 0.05$. *P* values: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

3.4 Results

3.4.1 *Radiation differentially modulates the expression of OX40L and 4-1BBL on murine tumor cells*

Studies have shown that radiation treatment can modulate the expression of co-stimulatory molecules, including OX40L and 4-1BBL on human tumor cells from solid tumors including prostate, colorectal, and breast (Garnett et al. 2004; Bernstein et al. 2014; Kumari and Garnett-Benson 2016; unpublished data) and that expression of these proteins can significantly impact T cell activity. To expand upon these findings, we utilized two commonly used murine tumor models to investigate if radiation also effects the phenotype of tumors *in vivo*. Colorectal cancer is the 3rd leading cause of cancer-related death in U.S. men and women. Additionally, breast cancer remains the 2nd leading cause of cancer-related death in women indicating the continued need to develop effective treatments. MC38 colon and 4T1 breast are two murine tumor models often used to give insight into treatment of these diseases. We began by evaluating these murine tumor cells for changes in OX40L and 4-1BBL expression following *in vitro* irradiation to see if they would respond similarly to human cells treated *in vitro*. We exposed 4T1 breast and MC38 colon tumor cell lines to 5 Gy and 10 Gy of radiation and assessed co-stimulatory molecule expression levels 48hrs post treatment. OX40L was moderately expressed in untreated 4T1 cells (63%) while protein expression was lower in MC38 cells (26%) (Fig. 3.1A). OX40L expression was slightly reduced in 4T1 cells treated with 10 Gy (56%). Minimal change in OX40L was observed in treated MC38 cells. We further analyzed cells for changes in protein density by MFI. Following exposure to radiation, we observed a dose-dependent trend towards an increase in the density of OX40L expression in both 4T1 and MC38 cell lines though it did not reach statistical significance (Fig. 3.1B). 4-1BBL was highly expressed in both

untreated 4T1 and MC38 tumor cell lines, 100% and 99%, respectively (Fig. 3.1C). Radiation treatment had minimal effect on the frequency of 4T1 cells expressing 4-1BBL, however a slight decrease was observed in MC38 tumor cells. Interestingly, 4-1BBL was expressed at a higher density in 4T1 cells compared with MC38 cells (Fig. 3.1D). Tumor cells treated with 10 Gy of radiation showed an increase in the density of 4-1BBL, with expression in MC38 cells reaching statistical significance. However, we noted little to no change in 4-1BBL expression following exposure to 5 Gy which may indicate that higher radiation doses are needed to upregulate its expression. Overall, a higher frequency of 4T1 cells express OX40L compared to MC38 while both cell lines express high levels of 4-1BBL. These results indicate that similar to published reports in human cells, radiation can modulate the surface densities of OX40L and 4-1BBL co-stimulatory molecules on murine tumor cells, but expression is differentially modulated by cell line and radiation dose.
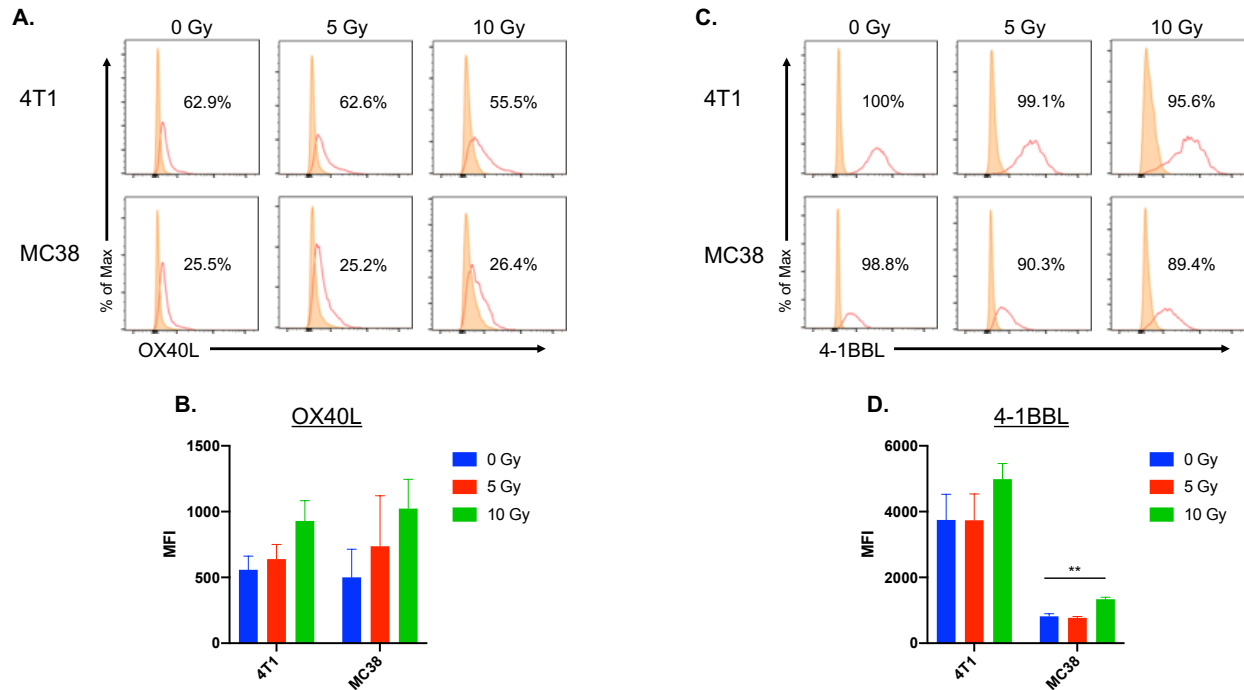
Figure 3.1 Role of radiation on tumor cells differs by model, *in vitro*.
4T1 and MC38 tumor cells were exposed to 5 Gy and 10 Gy of radiation or mock irradiated (0 Gy). (**A**) After 48hrs, cells were stained for expression of OX40L by flow cytometry. FMO control staining is shown in orange filled histogram. OX40L positive cells are shown in red lined histogram. Representative plots and (**B**) MFI. (**C**) Representative plots of tumor cells were stained for expression of 4-1BBL and (**D**) MFI. Data are representative of two-three independent experiments. Error bars represent SEM. *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ by unpaired, two-tailed Student *t* test.

We next determined whether radiation modulates co-stimulatory molecule expression, *in vivo*. 4T1 or MC38 cells were implanted into syngeneic mice, subcutaneously (s.c.), and allowed to grow to 300-500 mm³ before local treatment with 8 Gy. 24-48hrs after treatment, tumor tissue was harvested and formalin fixed. mRNA was isolated from sectioned tumor tissue and mRNA expression was measured by qRT-PCR. Local irradiation was shown to upregulate the expression of OX40L in 4T1 and MC38 tumors though neither reached statistical significance (Fig. 3.2A). In contrast to OX40L, there was no change in 4-1BBL mRNA induced following radiation treatment in both tumor models (Fig. 3.2B). This data shows that radiation induces OX40L expression in 4T1 and MC38 tumors but 4-1BBL expression is unchanged.
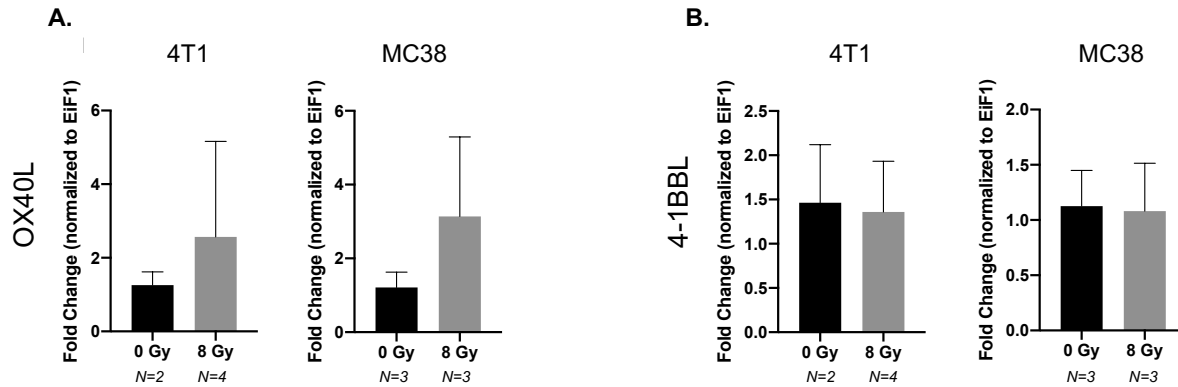
Figure 3.2 Effect of radiation on mRNA levels of OX40L and 4-1BBL tumor-bearing mice.
4T1 or MC38 tumor cells were s.c. implanted into syngeneic mice. Tumors 300-500 mm^3 in size received 8 Gy of local radiation under general anesthesia. 48hrs following treatment tumors were harvested and mRNA was isolated. Bar graph of quantitative RT-qPCR analysis of (**A**) OX40L and (**B**) 4-1BBL expression relative to EiF1 normalized to a single 0 Gy sample. Error bars represent SD. *$P \leq 0.05$.

3.4.2 Reduced number of CD4+Foxp3+ T$_{REGS}$ in 4T1 and MC38 tumor models following local irradiation does not appear to correlate with the radiation-induced expression of OX40L and 4-1BBL on tumor cells

Tumor cells are known to recruit T$_{REGS}$ to the tumor microenvironment, as well as induce CD4+ conventional T cells to a T$_{REG}$ phenotype (Facciabene, Motz, and Coukos 2012; Povoleri et al. 2013). This recruitment and conversion aids in the maintenance of an immunosuppressive tumor microenvironment by inhibiting dendritic cell maturation and CTL effector function (McNally et al. 2011; Bauer et al. 2014; Chen et al. 2017). We previously reported that signals transmitted by OX40L and 4-1BBL enhanced CTL effector function towards irradiated colorectal tumor cells (Kumari and Garnett-Benson 2016). In contrast to the positive effect on CTLs, signals through OX40 and 4-1BB have been shown to abrogate Foxp3 expression in T$_{REG}$ cells and inhibit their suppressive function (Vu et al. 2007; Kitamura et al. 2009; Smith, Hoeizinger, and Dominguez 2011; X. Zhang et al. 2018). Thus, we wanted to determine if radiation-induced expression of OX40L and 4-1BBL on tumor cells decreased T$_{REG}$ frequency *in*

vivo. We hypothesized that the higher percentage of OX40L expressing tumor cells, as well as the greater density of 4-1BBL on tumor cells in the 4T1 murine model as compared to the MC38 model would induce a significant decrease in T_{REG} frequency in the 4T1 model alone. To investigate this question, 4T1 mammary or MC38 colorectal tumor cells were implanted, s.c., into syngeneic mice and allowed to grow to 300-500 mm^3 before local treatment with 8 Gy. 24-48hrs after treatment, tumor tissue was harvested and flash frozen in O.C.T compound. The frequency of tumoral CD4+Foxp3+ T_{REG} cells was evaluated by fluorescent IHC (Fig. 3.3). Mice bearing 4T1 tumors exhibited a significant decrease in the average number of CD4+Foxp3+ T_{REGS} 48h post radiation (avg. 2.155) as compared to untreated mice (avg. 4.129) (Fig. 3.3B). Radiation did not induce a significant change in CD4+ T cell number (Fig. 3.3C). Similarly, MC38 tumor bearing mice showed a significant reduction in T_{REGS} following radiation treatment (avg. 3.2) as compared to control mice (avg. 8.175) (Fig. 3.3E). In addition, the number of CD4+ T cells in MC38 tumor bearing mice was also significantly reduced after treatment with 8 Gy (Fig. 3.3F).

Interestingly, the degree of T_{REG} reduction appeared greater in MC38 tumors compared to 4T1. We hypothesized that the modulation of OX40L and 4-1BBL on tumor cells could reduce the frequency of T_{REG} cells within the tumor microenvironment. Though we showed that radiation modulates the expression of OX40L in both tumor cell lines *in vitro* (Fig. 3.1), the frequency of MC38 cells expressing OX40L was relatively low and radiation slightly decreased the frequency of 4T1 cells expressing OX40L. Additionally, the frequency of 4T1 and MC38 tumor cells expressing 4-1BBL was high, however the density in MC38 tumor cells was low in comparison to 4T1 MFI (Fig. 3.1D). Despite these differences, exposure of 4T1 and MC38 tumors to radiation resulted in a reduction in T_{REG} cell number. These results indicate that

radiotherapy reduces T_{REG} cell frequency but differences in tumor expressed OX40L and 4-1BBL do not appear to facilitate in this reduction.
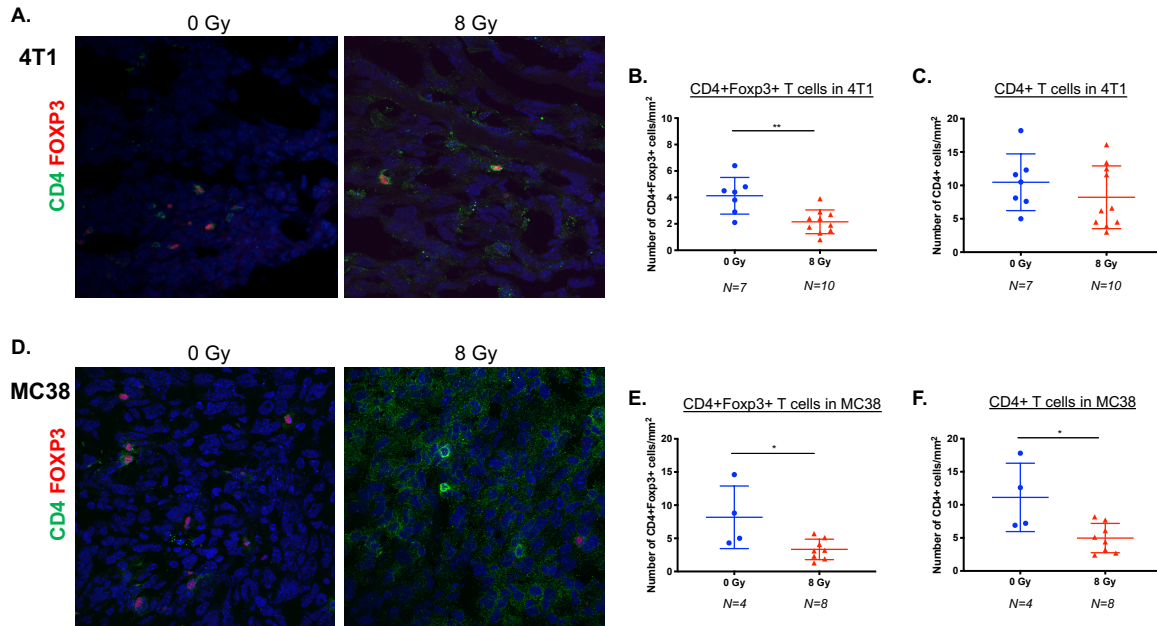
Figure 3.3 CD4+Foxp3+ T_{REGS} differentially reduced by radiation in 4T1 and MC38 murine tumors in vivo.
(**A**) 4T1 tumor cells were subcutaneously implanted into mice. Tumors 300-500 mm³ in size received 8 Gy of local radiation under general anesthesia. 48hrs following treatment tumors were harvested and tissue sections co-stained for CD4 and Foxp3 prior to analysis by confocal microscopy. 10 image fields per mouse were randomly selected and the total number of (**B**) CD4+Foxp3+ T_{REGS} and (**C**) CD4+ T cells were manually counted and the average plotted. (**D-F**) MC38 tumor cells. *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ by unpaired, two-tailed Student t test. *Green: CD4*; *Red: Foxp3*; *Blue: DAPI*.

3.4.3   *CD4+Foxp3+ T$_{REG}$ frequency is still reduced following radiation in the absence of 4-1BB signaling*

To further determine if signaling through 4-1BB can directly affect T$_{REG}$ frequency we utilized a 4-1BB KO mouse model. MC38 cells were implanted into C57BL/6$^{4-1BBnull}$ mice and tumors were locally irradiated (10 Gy) or untreated. 3 days after treatment the frequency of tumoral CD4+Foxp3+ T$_{REG}$ cells was evaluated by fluorescent IHC. Radiation treatment showed a moderate decrease in mean T$_{REG}$ frequency (1.5) as compared to control (2.8) (Fig. 3.4A). Surprisingly, this decrease was also observed in the CD4+ T cell population (Fig. 3.4B). Preliminary data evaluating immune cell frequency 6 days post radiation treatment indicated a rebound in T$_{REG}$ number (data not shown) which may suggest that the decrease observed in the total CD4+ T cell population is a direct result of the radiation treatment. This reduction was similar to that observed in WT mice (Fig. 3.3E and 3.3F) suggesting that signaling through 4-1BB is dispensable and has little effect on tumoral T$_{REG}$ frequency following radiation.
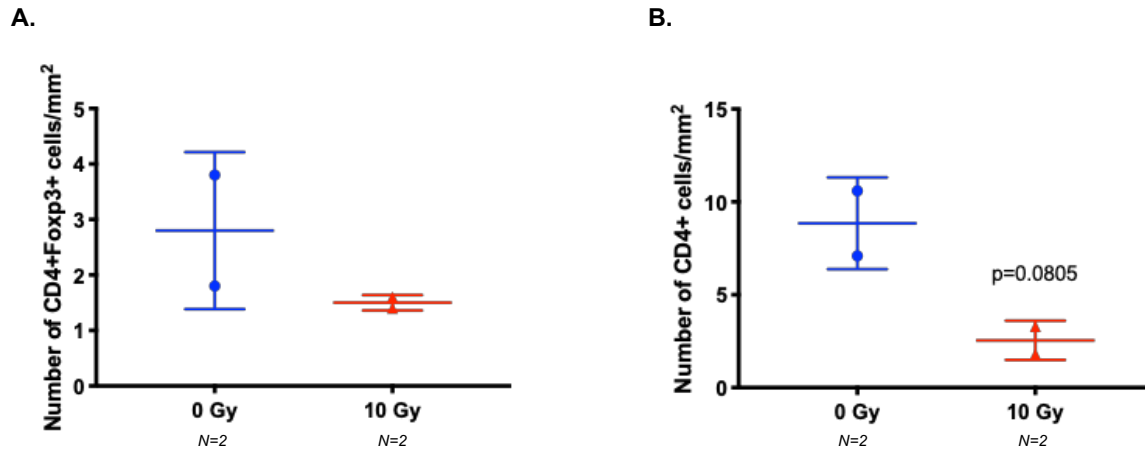
Figure 3.4 Loss of 4-1BB reduces T$_{REG}$ frequency following radiation.
MC38 cells were implanted into C57BL/6^{41BBnull} mice. Mice with tumors 200-300 mm^3 in size received 10 Gy of local radiation under general anesthesia. 3 days following treatment tumors were harvested and tissue sections stained with CD4 and Foxp3 prior to analysis by confocal microscopy. 10 image fields per mouse were randomly selected. The total number of (**A**) CD4+Foxp3+ T$_{REGS}$ and (**B**) CD4+ T cells were manually counted and the average plotted. *$P \leq$ 0.05 by unpaired, two-tailed Student t test.

3.4.4   CD8+ TILs increased in irradiated 4T1 but not MC38 tumors

All of the experiments described above examined the effect of radiation-induced

modulation of tumor expressed OX40L and 4-1BBL on T$_{REG}$ frequency. We were unable to

correlate the significant reduction in T$_{REG}$ number observed in both 4T1 and MC38 tumors with

the differential modulation of OX40L and 4-1BBL between these two models. In further

experiments, we expanded our studies to examine radiation-induced differences in the immune

cell profile of 4T1 and MC38 tumor models. We showed that local tumor irradiation

significantly reduces the frequency of tumoral T$_{REG}$ cells in both 4T1 and MC38 models (Fig.

3.3). We next examined the effect of radiation on CD8+ T cells within the tumor

microenvironment as T$_{REG}$ cells are known to inhibit CD8+ CTL activity. In 4T1 tumor-bearing

mice we observed an increase in CD8+ T cell number 48hrs after treatment (avg. 11.65) as

compared to untreated mice (avg. 6.02) though this increase did not reach statistical significance

(Fig. 3.5A). In contrast, minimal change in CD8+ T cell number was observed in MC38 tumors 24-48hrs following treatment (avg. 1.1 vs 1.26) (Fig. 3.5B). Interestingly, we noted that untreated 4T1 tumors contained more CD8+ T cells compared to MC38 tumors.

We further assessed changes in the ratio of CD8:T_{REG} cells after treatment with 8 Gy. We observed a significant increase in the CD8:T_{REG} ratio in irradiated 4T1 tumors (Fig. 3.5C) whereas the ratio in irradiated MC38 tumors remained unchanged (Fig. 3.5D). Overall, we concluded that radiation treatment differentially alters CD8+ T cell number based on tumor model.
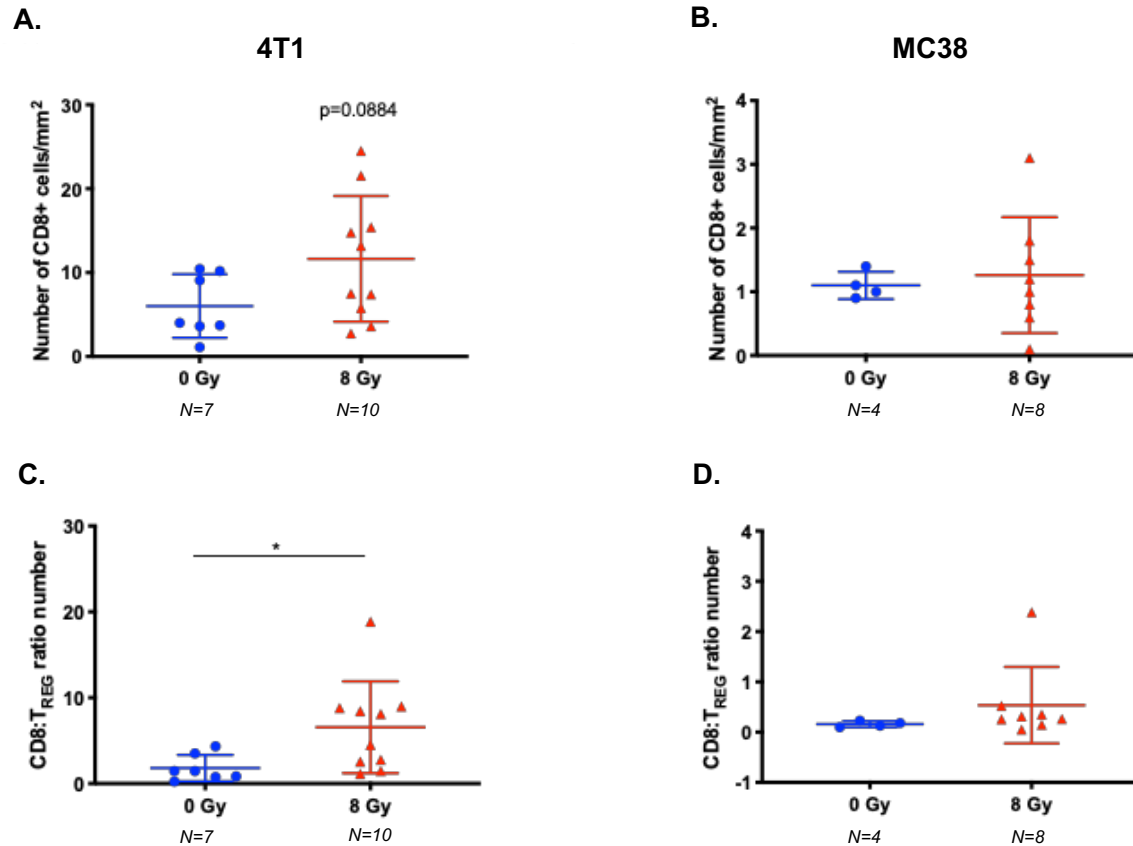
Figure 3.5 CD8+ T cell number increased following radiation treatment in murine tumor models.
24-48hrs after radiation exposure (8 Gy), tumor tissue sections from (**A**) 4T1 and (**B**) MC38 tumor-bearing mice were stained for CD8 and analyzed by confocal microscopy. 10 image fields per mouse were randomly selected and the total number of CD8+ T cells present in each of 10 randomly selected images were manually counted and the average plotted. CD8:T_{REG} ratio in (**C**)

4T1 and (**D**) MC38 tumor tissue sections. Ratio was calculated by dividing the average number of CD8 T cells by the average number of T_{REG} cells per mouse. *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ by unpaired, two-tailed Student t test.

3.4.5   *Effect of radiation on RNA immunoprofile*

Thus far, our data has shown that local irradiation reduces T_{REG} number in 4T1 and MC38 tumors, however an increase in CD8+ T cell number was only observed in 4T1 tumors. These results indicated the differential effect of radiotherapy on different tumor models. 4T1 tumors are widely considered to be poorly immunogenic in contrast to the immunogenic MC38 tumors . To further examine the immune landscape within the tumor microenvironment following radiotherapy we isolated mRNA from 8 Gy treated and untreated tumors from 4T1 and MC38 implanted mice. mRNA was analyzed for gene expression using NanoString technology. Differences in the expression of T cell-associated genes (probes: CD4 and CD8a) showed increased expression of the CD4 gene after radiation treatment in 4T1 and MC38 tumors (Fig. 3.6A). In contrast, the CD8a gene was increased in irradiated 4T1 cells but no change was observed in treated MC38 tumors, similar to the cellular composition observed in Fig. 3.5. We also examined differences in T_{REGS} following radiotherapy (probe: Foxp3). The Foxp3 gene was reduced in 4T1 tumors while no change was observed in MC38 tumors with radiation.

Additionally, we looked at genes associated with immune cell activation (probes: IFN-γ and Tnfsf4). IFN-γ is a pleiotropic cytokine that has been shown to regulate CD8 expansion and cytotoxicity, as well as induce antitumor effects (Whitmire, Tan, and Whitton 2005; Zaidi 2019). We found that IFN-γ gene expression was induced in irradiated 4T1 tumors but not MC38 tumors. This may likely correlate with the increased number of CD8+ T cells observed in irradiation 4T1 tumors but not in MC38 tumors (Fig. 3.5). Furthermore, radiation induced a similar fold change increase in Tnfsf4 (OX40L) in 4T1 and MC38 tumors. This data suggests

that radiation induces better effector T cell infiltration, immune cell activation and T_{REG} reduction in 4T1 tumors as compared to MC38 tumors. Further, the observed fold change increase in Tnfsf4 following radiation indicates that tumor expressed OX40L is likely not the mechanism of T_{REG} abrogation in either 4T1 or MC38 tumors.

We next analyzed mRNA for gene expression associated with a T cell inflamed tumor microenvironment as described by Gajewski *et al* (Gajewski et al. 2017). Both 4T1 and MC38 tumors differentially increased expression of chemokines related to T cell recruitment (probes: CCL5 and CXCL10) (Fig. 3.6B). Though both chemokine transcripts were increased with radiotherapy, a greater increase in CCL5 was observed in irradiated MC38 tumors compared to control while a higher fold change in CXCL10 was seen in irradiated 4T1 tumors. We further compared 8 Gy treated MC38 and 4T1 tumors for differences in chemokine gene transcripts. Interestingly, CCL5 and CXCL10 genes were highly expressed in irradiated MC38 tumors as compared to irradiated 4T1 tumors which may be due to MC38 tumors being more immunogenic from the onset.

Genes linked to immune inhibitory mechanisms and T cell dysfunction (probes: CD274, IDO1, and LAG-3) were also analyzed following radiation treatment (Fig. 3.4B). Irradiated tumors exhibited increased expression of CD274 (PD-L1), IDO1 and LAG-3 genes. Fold change differences in IDO1 and LAG-3 were similar in both tumor models following radiotherapy. A greater change in CD274 was seen in MC38 tumors treated with radiation. Finally, we compared 8 Gy treated MC38 and 4T1 tumors for changes in inhibitory genes and noted that irradiated MC38 tumors expressed more CD274 and LAG-3 gene transcripts as compared to irradiated 4T1 tumors. In contrast, 8 Gy treated 4T1 tumors express higher levels of IDO1 than radiation treated MC38 tumors. These data indicate that radiation treatment promotes a T cell inflamed tumor

microenvironment in both 4T1 and MC38 tumors, however the gene profile of irradiated MC38 tumors appears to highly mediate an inhibitory tumor microenvironment in comparison to 4T1 tumors.

A.

Genes	4T1 (8 Gy vs 0 Gy) Fold change	MC38 (8 Gy vs 0 Gy) Fold change
CD4	1.36	1.84
CD8a	1.73	1.02
Foxp3	-2.61	1
IFN-γ	2.39	1.16
Tnfsf4 (OX40L)	2.09	2.05

B.

Genes	4T1 (8 Gy vs 0 Gy) Fold change	MC38 (8 Gy vs 0 Gy) Fold change	MC38 vs 4T1 (8 Gy) Fold change
CCL5	2.58	5.07	11.2
CXCL10	5.23	3.54	8.26
CD274 (PD-L1)	1.84	2.79	1.46
IDO1	1.82	1.92	-2.17
LAG-3	2.21	2.36	1.61

Figure 3.6 Immune profiling analysis.
NanoString immune profiling analysis of tumor samples from 0 Gy and 8 Gy treated 4T1 or MC38 tumor-bearing mice. Fold change in gene expression is listed. (**A**) Profiling of tumor-associated immune cells and immune activating markers. (**B**) Profiling of immune genes expressed in T cell inflamed tumor microenvironment.

3.5   Discussion

We have previously reported on the direct effect radiation treatment has on modulating T_{REG} phenotype and reducing Foxp3 expression (manuscript submitted). However, the potential indirect effects of radiation treatment on T_{REGS} within the tumor microenvironment remains unclear. Our lab, and others, have shown that radiation can modulate the expression of co-

stimulatory molecules on human tumor cells (Bernstein et al. 2014; Kumari and Garnett-Benson 2016). Further, modulation of tumor expressed OX40L and 4-1BBL enhances CTL effector function (Kumari and Garnett-Benson 2016). In this study, we assessed the radiation-induced modulation of OX40L and 4-1BBL co-stimulatory molecules on T_{REG} frequency, as well as differences in the tumor immune cell profile in two commonly used murine tumor models. First, we showed that irradiated 4T1 and MC38 tumors differentially modulate the expression of OX40L and 4-1BBL co-stimulatory molecules. Additionally, we showed that radiation reduces the number of T_{REG} cells in 4T1 and MC38 tumors, however this reduction does not appear to be induced by tumor expressed OX40L or 4-1BBL. In addition, we observed an increase in CD8+ T cell number in irradiated 4T1 tumors but not MC38 tumors which we were unable to correlate with tumor modulation of OX40L or 4-1BBL. We further analyzed tumors for differences in the immune cell gene profile and showed that radiation differentially induces a more immunogenic microenvironment in 4T1 tumors, as well as induces a T cell inflamed tumor microenvironment in 4T1 and MC38 tumors.

Earlier reports in human tumor cells showed that radiotherapy can induce the expression of OX40L and 4-1BBL (Bernstein et al. 2014; Kumari and Garnett-Benson 2016). Our results in murine tumor cells are in line with previously reported data that radiation can modulate the phenotype of tumor cells by upregulating the expression of OX40L and 4-1BBL. While radiation did not induce any significant change in the percent of positive expressing cells in both the 4T1 and MC38 models we did observe increases in protein density indicating that the modulatory effects of radiation is cell line dependent.

OX40 and 4-1BB co-stimulation can reportedly abrogate Foxp3 expression and inhibit the suppressive function of T_{REG} cells (Kitamura et al. 2009; Smith, Hoeizinger, and Dominguez

2011; Bulliard et al. 2014; X. Zhang et al. 2018). Co-stimulation in published reports was achieved with the use of agonist antibodies. We aimed to determine if radiation-induced expression of OX40L and 4-1BBL on tumor cells can reduce T_{REG} number. We hypothesized that irradiated 4T1 tumors would induce a more significant decrease in T_{REG} number compared to the MC38 model as 4T1 cells contain a high percentage of OX40L and 4-1BBL expressing cells and express moderate to high density of both proteins that is increased with radiation. Our data showed a significant reduction in T_{REG} frequency following radiation treatment in both 4T1 and MC38 tumor models (Fig. 3.3). Interestingly, we found that radiation had no effect on total CD4+ T cell frequency in 4T1 tumors but significantly decreased CD4+ T cells in the MC38 model. Though our data showed an increase in OX40L and 4-1BBL protein density in 4T1 and MC38 tumor cells following radiation treatment we were unable to conclusively correlate protein modulation with the observed decrease in T_{REG} number. In fact, it is more likely that the modulation of OX40L and 4-1BBL had no effect on T_{REG} frequency. To further determine whether 4-1BB signaling can reduce T_{REG} number, we implanted MC38 tumor cells into a 4-1BB KO mouse model. Similar to results seen in WT mice, we observed a reduction in T_{REG} cell number and CD4+ T cells 3 days post-treatment (Fig. 3.4). This data further indicates that 4-1BB signaling had no impact on reduced T_{REG} frequency, *in vivo*.

Because we were unable to correlate radiation-induced changes in co-stimulatory molecules with a reduction in T_{REG} cells, we shifted our focus to assess radiation-induced differences in immune cell composition between our two mouse models. T_{REGS} are known to exert their suppressive function on several immune cells including CD8+ T cells. As we observed a significant reduction in T_{REG} number following radiotherapy in both 4T1 and MC38 tumor models we next evaluated tumor samples for changes in CD8+ T cell number. Our data

showed that radiation increased the frequency of CD8+ T cells, and subsequently the CD8:T_{REG}

ratio, in 4T1 tumors. Surprisingly, we saw no change in CD8+ T cell frequency in MC38 tumors

(Fig. 3.5). We also utilized NanoString technology to analyze the immune cell gene profile in

irradiated tumors (Fig. 3.6A). We analyzed irradiated 4T1 and MC38 for changes in the gene

transcript of CD4 and CD8a compared to untreated tumors. Radiation was found to upregulate

expression of the CD4 gene in both 4T1 and MC38 tumor models. Similar to observed changes

in T cell frequency, CD8a was increased in irradiated 4T1 tumors while gene expression was

unchanged in MC38 tumors. We also looked at radiation induced changes in the immune

activating genes IFN-γ and Tnfsf4 (OX40L). Radiation induced expression of IFN-γ was

observed 4T1 tumors while MC38 remained relatively unchanged. These data suggest that

radiation may induce a more immunogenic microenvironment in 4T1 tumors as compared to

MC38 tumors.

4T1 tumors are considered to be poorly immunogenic. One factor that could explain this

effect is the BALB/c mouse strain this model is used in. BALB/c mice have been reported to

contain more of the "pro-tumor" M2 macrophages as compared to C57BL/6 mice, used for

MC38 tumors, which skew more towards an M1 phenotype (Mills et al. 2000; Sellers et al.

2012). Furthermore, tumor-associated macrophages primarily consist of M2 macrophages.

Radiation treatment reportedly recruits macrophages to the tumor microenvironment (S. C.

Wang et al. 2013; Jones et al. 2018). Interestingly, low-dose radiation has been shown to

facilitate increased T cell recruitment to the tumor microenvironment by differentiating

macrophages to an M1 phenotype (Klug et al. 2013). Therefore, it may be possible that radiation

treatment alters the balance of M1 to M2 macrophages within the tumor microenvironment. As

the basal amount of M2 macrophages is much higher in BALB/c mice as compared to C57BL/6

mice the increased amount of M1 macrophages to irradiated 4T1 tumors may promote a more significant increase in CD8+ T cell infiltration than that seen in MC38 tumors.

We further extended our immune profile analysis to include genes associated with a T cell inflamed tumor microenvironment as described by Gajewski *et al* (Gajewski et al. 2017). 4T1 and MC38 tumors both induced expression of T cell recruiting chemokines (CCL5 and CXCL10) and inhibitory molecules (CD274, IDO1, and LAG-3) (Fig. 3.6B). We then compared gene expression levels between irradiated MC38 versus irradiated 4T1 tumors. Surprisingly, we found that the expression of all genes except IDO1 was higher in MC38 tumors, particularly CCL5 and CXCL10. Irradiated MC38 tumors express markedly greater expression of CCL5 and CXCL10 mRNA in comparison to 4T1 tumors, however that did not seem to correlate to an increase in the observed number of CD8+ T cells as assessed by IHC. While we noted an increase in chemokine associated mRNA we did not analyze tumor tissue for protein expression. It is possible that secretion of either chemokine was not significantly altered following radiotherapy which could affect T cell recruitment to the tumor. Therefore, changes in chemokine secretion could be examined in future studies.

Lastly, we were greatly intrigued by the observed increase in LAG-3 mRNA with radiation treatment. We previously observed an increase in LAG-3 expressing human iT$_{REG}$ cells following radiotherapy (manuscript submitted). Our data showed a reduction in tumoral T$_{REG}$ number post-radiation treatment, however LAG-3 mRNA is shown to be induced. This indicates that LAG-3 is also being expressed by other cells within the tumor microenvironment. It is unclear which cell subset is contributing to this increase in expression, however our data signifies the potential benefit for the use of an anti-LAG-3 blocking antibody. Combination treatment with anti-PD-1 and anti-LAG-3 blocking antibodies was shown to increase T cell

proliferation (Lichtenegger et al. 2018). Therefore, radiation and anti-LAG-3 combination treatment could further enhance the antitumor immune response in both 4T1 and MC38 tumor models.

In summary, our study provides valuable information on how radiation differentially modulates tumor phenotype in two commonly used murine models. These results demonstrate the inhibitory effect of radiation on T_{REG} number, *in vivo*, and indicates that tumor expressed OX40L and 4-1BBL does not appear to induce these changes. Furthermore, our study highlights differences in the immune profile of our two models and that radiation can induce a more immunogenic tumor microenvironment. These data could be helpful for assessing the effect of radiation on the immune cell gene profile in murine models and defining the usefulness of radiotherapy in preclinical studies.

4   CONCLUSIONS

Radiotherapy remains an important cancer treatment modality, as more than half of cancer patients receive treatment. The administration of radiation can be curative for some cancer types when used alone or in combination with other standard treatments such as surgery or chemotherapy ("Radiation Therapy Basics" n.d.). Unfortunately, treatment for patients with advanced-stage malignancies is frequently incurable. While the higher doses given for curative radiotherapy are intended to kill tumor cells through such mechanisms as DNA damage, several groups have reported on the immune enhancing effect of low dose radiation (Filatenkov et al. 2015; Lugade et al. 2005; Y. Lee et al. 2009; Spary et al. 2014; Gupta et al. 2012).

Low dose radiation has been shown to enhance the anti-tumor immune response through such avenues as increased infiltration of immune cells to the tumor, dendritic cell activation, and the modulation of several co-stimulatory molecules on tumor cells (Y. Lee et al. 2009; Gupta et al. 2012; Bernstein et al. 2014; Garnett et al. 2004; Kumari and Garnett-Benson 2016). While extensive research has expanded our knowledge on the increased activation and survival of effector T cells following radiotherapy, it is less clear how radiation effects human CD4+ T_{REG} cells. Our data in Chapter 2 focused on induced T_{REGS}, similar to those derived within the tumor microenvironment. We showed that natural and induced T_{REG} cells are more radioresistant as compared to CD4+ T_{CONV} cells and direct radiation significantly reduced their expression of Foxp3. Irradiated iT_{REGS} further modulated the expression of signature T_{REG} molecules. A decrease in CD25 and CTLA-4 expression was seen while LAG-3 and CD73 expression was upregulated. We observed no change in CD39 and PD-L1 expression. Furthermore, iT_{REGS} exposed to 10 Gy of radiation were less capable of inhibiting CD8+ T cell proliferation. Based

on our findings we identified the direct effect of radiation on human induced T_{REG} cell phenotype and suppressive function.

The effect of radiation treatment on T_{REG} cells has produced conflicting reports. One reason for such discrepancies could be the differences between natural versus induced T_{REGS}. Death staining and analysis of Foxp3 expression revealed that natural and induced T_{REG} cells are similarly affected by radiation treatment. We noted that the rate of Foxp3 expression following radiotherapy was greater in iT_{REGS} as compared to nT_{REGS}. This difference in Foxp3 reduction could be due to differences in methylation of the Foxp3 promoter. The Foxp3 promoter in nT_{REG} cells is fully demethylated in contrast to iT_{REGS} that exhibit partial demethylation. Additionally, acetylation of the Foxp3 promoter is important for the stability of Foxp3 expression (Kwon et al. 2012). We have previously shown that radiation is capable of epigenetically regulating gene expression (Kumari et al. 2013). Thus, future studies could investigate epigenetic changes in natural and induced T_{REG} cells following radiation treatment.

Furthermore, our study focused on iT_{REGS} as these cells are similar to those that would be induced within the tumor microenvironment. We found that radiation altered the phenotype of Foxp3+ T_{REGS}. Irradiated cells showed reduced expression of CD25 and CTLA-4, proteins that are regulated by Foxp3 (Sadlon et al. 2010). Of significant interest was the increased expression of another Foxp3 regulated gene, LAG-3. LAG-3 is an suppressive molecule that inhibits T cell proliferation (Okamura et al. 2009). The immunogenic effects of radiation are frequently discussed however it should be noted that radiation can upregulate the expression of inhibitory molecules such as PD-L1. It is quite possible that LAG-3 expression is similarly regulated with radiation. Though irradiated iT_{REGS} induced expression of LAG-3 these cells were still less suppressive as compared to untreated cells. Currently, anti-LAG-3 blocking antibodies are being

tested in preclinical studies. Future experiments could investigate whether combination treatment with radiation and anti-LAG-3 to iT$_{REGS}$ further inhibits their suppressive function.

In Chapter 3, we investigated the role of radiation on tumor cell modulation and its effect on T$_{REG}$ cells *in vivo*. In addition, we examined tumors for radiation-induced differences in the immune cell profile of two commonly used murine tumor models. Previous data from our lab demonstrated the modulatory effects of radiation on tumor cells (Garnett et al. 2004; Kumari and Garnett-Benson 2016). Of significance, it was reported that radiation can induce the expression of the co-stimulatory molecules OX40L and 4-1BBL on colorectal tumor cells. Signals from these tumor expressed molecules were found to enhance CTL effector activity (Kumari and Garnett-Benson 2016). Additionally, it has been reported that signaling through OX40 or 4-1BB can inhibit Foxp3 expression in T$_{REGS}$ and reduce their suppressive function (Kitamura et al. 2009; X. Zhang et al. 2018; Bulliard et al. 2014; Smith, Hoeizinger, and Dominguez 2011). Our data demonstrates the differential modulation of radiation in 4T1 and MC38 murine tumor models. We found that radiation differentially modulated the expression of OX40L and 4-1BBL on 4T1 and MC38 tumor cells. We also revealed that the number of tumoral T$_{REGS}$ was significantly reduced in 4T1 and MC38 tumors 24-48hrs after exposure to radiation. Additionally, radiation increased the number of CD8+ T cells in 4T1 tumors, however no change was observed in MC38 tumors. Finally, we report that the immune profiles of the 4T1 and MC38 tumor models are modulated differently following radiation treatment. Irradiated 4T1 tumors appear to be more immunogenic as compared to MC38 tumors. Lastly, radiation was shown to increase the expression of genes associated with a T cell inflamed tumor microenvironment in both 4T1 and MC38 tumors though expression was higher in MC38. Overall, these data indicate that local tumor irradiation can significantly affect the tumor immune profile, however these

changes were highly variable between two commonly utilized murine tumor models. Moreover, we can report that tumor expressed OX40L and 4-1BBL does not appear to mitigate T_{REG} reduction or enhance the presence of CD8+ T cells following radiation treatment.

The study presented in Chapter 3 is the first to evaluate the effect of tumor expressed OX40L and 4-1BBL on T_{REG} cells. Several studies have reported the inhibitory effect of OX40 or 4-1BB co-stimulation on Foxp3 expression and T_{REG} suppressive function in murine models, however many of these studies utilized agonist antibodies. We attempted to induce expression of OX40L and 4-1BBL on murine tumor cells using radiotherapy. In line with results observed in human tumor cells, 4T1 and MC38 murine tumor cell lines differentially modulated the expression of OX40L and 4-1BBL. While radiation increased the density of OX40L in both cell lines, 4T1 cells contained a higher frequency of positive cells as compared to MC38 cells. Additionally, radiation slightly decreased the frequency of OX40L positive 4T1 cells while the frequency of MC38 cells remained unchanged. In contrast, both cell lines expressed a high frequency of 4-BBL expressing cells but the density of 4-1BBL was markedly higher in 4T1 cells compared to MC38 cells. We also examined the expression of OX40L and 4-1BBL *in vivo*. mRNA from tumor samples revealed that local tumor irradiation induced OX40L in both 4T1 and MC38 tumors but 4-1BBL expression was unaltered in both. While we observed a significant decrease in T_{REG} cells from 4T1 and MC38 tumors our data did not substantiate an obvious link between induced OX40L and 4-1BBL expression and T_{REG} reduction. Though we were able to perform preliminary experiments on MC38 tumor cells in 4-1BB KO mice, additional experiments to evaluate 4-1BBL expression and its effect on T_{REGS} was difficult. The use of Nanostring technology allowed us to evaluate the gene profile of immune cells with the tumors from both models, however a probe for 4-1BBL was not available. Additionally, we were

unable to obtain 4-1BB KO mice on a BALB/c background within our experimental timeframe.

To directly study the tumor induced effect of OX40L and 4-1BBL, future experiments could

evaluate the frequency and suppressive function of T_{REG} cells co-cultured with OX40L or 4-

1BBL over-expressing tumor cells, *in vitro*, or examine T_{REGS} isolated from 4T1 tumors in OX40

KO or 4-1BB KO mice.

We further compared differences in the immune cell gene profile following radiation

treatment using NanoString technology to examine if there were any obvious disparities (1)

induced by radiation within each tumor model and (2) between the tumor models that could

account for differences in T_{REG} and CD8+ T cell frequency. We found that radiation induced the

gene transcript of CD4 and CD8a in 4T1 tumors while only CD4 was increased in MC38. This

data correlates with the increase in CD8+ T cell number observed in irradiated 4T1 tumors but

not MC38 (Fig. 3.5). Radiation was further shown to greatly decrease the gene transcript of

Foxp3 in 4T1 which correlated with the significant decrease in CD4+Foxp3+ T_{REG} cell number

(Fig. 3.3). Surprisingly, the Foxp3 gene transcript was unaltered with radiation in MC38 tumors

despite seeing a significant decrease in tumor T_{REG} number. It is not immediately clear why

changes in the Foxp3 gene transcript did not coincide with the observed decreased in tumor T_{REG}

number. Experimental repeats would be needed to investigate this further. We also looked at two

immune activating genes, IFN-γ and Tnfsf4 (OX40L). Only irradiated 4T1 tumors increased

expression of the IFN-γ gene while only a minimal increase was seen in MC38 tumors.

Interestingly, both tumor models increased expression of the OX40L gene transcript. This

correlated with the increase observed in OX40L mRNA isolated from *in vivo* murine tissue. This

data indicates that irradiated 4T1 tumors are more immunogenic as compared to MC38 tumors.

Additionally, this data, in concert with the increase in OX40L mRNA, suggests that radiation

induced expression of OX40L on tumor cells does not appear to correlate with our observed decrease in T_{REG} cells in both tumor models or the increased expression of CD8+ T cells in 4T1 tumors.

Currently, clinical ICB treatment is only approved for use as a monotherapy. For those patients that do respond well, ICB treatment has been shown to increase patient survival (Hodi et al. 2010; Topalian et al. 2019). Unfortunately, most eligible patients do not respond to treatment signifying a major hurdle for scientists and clinicians. Combination treatment with radiation is currently being tested in clinical trials to help address this problem. Several preclinical studies have reported significantly delayed tumor growth and enhanced antitumor responses with combination therapy compared to ICB treatment alone demonstrating a promising role for radiation in combination with ICB (Dewan et al. 2009; Deng et al. 2014; Sharabi et al. 2015; K. J. Kim et al. 2017). In addition, combined ICB and radiation treatment has been shown to induce an abscopal effect in murine tumor models (Dewan et al. 2009; Rodriguez-Ruiz et al. 2016). An abscopal response has also been observed in human patients, however this effect is rare (Hiniker et al. 2012; Golden et al. 2013; Grimaldi et al. 2014). Though the mechanism inducing this phenomenon is not known, however it is understood that a competent immune system is required to obtain a response. Therefore, it is possible that the enhanced antitumor response induced by radiation is able to enhance CTL activity while simultaneously inhibiting T_{REG} function.

Despite the significant success of CTLA-4 and PD-1/PD-L1 checkpoint inhibitors in cancer treatment its use is ineffective in most eligible patients, while some that initially responded well to treatment later acquire resistance (Pitt et al. 2016; Koyama et al. 2016). Thus the development of new combination treatment regimens, as well as new therapies targeting alternative inhibitory receptors, is currently being conducted. The use of immunocompetent

preclinical murine tumor models supports the development of cancer therapeutics by allowing for the study of interactions between tumor and immune cells. However, differences in the immune cell composition of various murine models can have a significant effect on therapeutic results. Additionally, the effect of common cancer treatment modalities, such as radiation and chemotherapy, can differentially alter the immune landscape of murine tumor models. 4T1 (poorly immunogenic) and MC38 (highly immunogenic) are two commonly used murine tumor models that exhibit different responses to radiation treatment. Our mRNA results indicate that radiotherapy can induce a more immunogenic tumor microenvironment in the 4T1 tumor model, as well as enhance the expression of genes associated with a T cell inflamed microenvironment in both models, particularly MC38.

Overall, our study demonstrates the inhibitory effects of radiation on T_{REG} cells. We also show that the radiation-induced modulation of the co-stimulatory molecules OX40L and 4-1BBL had no effect on observed changes in immune cell frequency. Interestingly, we found that radiation differentially modulates the immune cell gene profile 4T1 and MC38 tumors. These murine models are commonly used for preclinical immunotherapeutic studies in combination with radiotherapy. Therefore, these data will be helpful for assessing the usefulness of radiation treatment in preclinical cancer immunotherapy studies and how the immune cell gene profile in murine models may affect these studies.

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