The Role of Radiation Therapy in Generating Effective Anti-Tumor CD8+ T Cell Responses

by

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List of Abbreviations

4-1BB	Tumor necrosis factor receptor superfamily member 9, TNFRSF9, CD137
APC	Antigen Presenting Cell
ATM	Ataxia-Telangiectasia Mutated protein
ATP	Adenosine-5'-Triphosphate
ATR	ATM- and Rad3- related kinase
ATRIP	ATR-Interacting Protein
B2M	β2M gene
Batf3	Basic leucine zipper transcription factor ATF-like 3
BCG	Bacillus Calmette-Guerin
Bcl-2	B-cell lymphoma 2 protein
BED	Biologic Equivalent Dose
BLS	Bare Lymphocyte Syndrome
BRCA1	Breast Cancer type 1 susceptibility protein
BRCA2	Breast Cancer type 2 susceptibility protein
CCR	C-C chemokine Receptor
CD	Cluster of Differentiation
CD4	CD4+ T cell
CD8	CD8+ T cell
CD25	Cluster of Differentiation 25, interleukin-2 receptor alpha chain
CD28	Cluster of Differentiation 28, receptor for B7-1 and B7-2
CD40	Cluster of Differentiation 40
CD40L	Cluster of Differentiation 40 Ligand, CD154
CD45	Cluster of Differentiation 45, protein tyrosine phosphatase receptor type C
CD80	Cluster of Differentiation 80, B7-1
CD86	Cluster of Differentiation 86, B7-2
CDR	Complementarity-Determining Region
cGAS	cyclic GMP-AMP synthase
cGAMP	cyclic GMP-AMP
Chk	Cell cycle checkpoint kinase
CIITA	MHC Class II Transactivator
CITA	MHC Class I Transactivator, NLRC5
CP	Catalytic Particle
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRT	Calreticulin
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Associated protein 4
CXCL	C-X-C chemokine Ligand
CXN	Calnexin
DAB	3,3'-Diaminobenzidine
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DC1	DC type 1, conventional dendritic cells
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
DNA pol	DNA polymerase
DSB	Double-strand break

dsDNA	double-stranded DNA
EF-1α	Eukaryotic translation Elongation Factor 1 alpha
EGFR	Epidermal Growth Factor Receptor
EGFRvIII	Epidermal Growth Factor Receptor variant III
ER	Endoplasmic Reticulum
ERAAP	ER-Associated Aminopeptidase
ERAdP	ER Adaptor Protein
ERp57	ER protein 57
G_1	Gap 1
G_2	Gap 2
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GITR	Glucocorticoid-Induced Tumor necrosis factor Receptor
GMP	Guanosine Monophosphate
GTP	Guanosine-5'-Triphosphate
Gy	Gray: SI unit of radiation defined as absorption of one joule of radiation energy per kilogram of matter
HC	Heavy Chain
HepB	Hepatitis B virus
HLA	Human Leukocyte Antigen, analogous to H-2 in mice
HMGB1	High Mobility Group Box protein 1
HPV	Human Papilloma Virus
HR	Homologous Recombination
IFNβ	Interferon-beta
IFNγ	Interferon-gamma
IFN-I	Type I Interferon
IFNγR	Interferon-gamma Receptor
IFNAR	Interferon-alpha/beta Receptor
IL-2	Interleukin-2
IP-10	Interferon gamma-induced protein 10, CXCL10
IRF	Interferon Regulatory Factor
ISG	Interferon-Stimulated Gene
ISRE	Interferon-Stimulated Regulatory Element
JAK	Janus Kinase
Lag-3	Lymphocyte-activation gene 3, CD223
Lck	Lymphocyte-specific protein tyrosine Kinase
LCMV	Lymphocytic Choriomeningitis Virus
LGP2	Laboratory of Genetics and Physiology 2 protein, RIG-I-like receptor 3
Lm	*Listeria monocytogenes*
LmEGFRvIII	*Lm* vaccine expressing EEKKGNYV peptide
LmOva	*Lm* vaccine expressing SIINFEKL peptide
LmSIY	*Lm* vaccine expressing SIYRYYGL peptide
LMP2	Low-molecular Mass Protein-2, β1i
LMP7	Low-molecular Mass Protein-7, β5i
LRR	Leucine-Rich Repeat
M phase	Mitotic phase
MCA	Methylcholanthrene
MDA5	Melanoma Differentiation-Associated protein 5
Mdm2	Mouse double minute 2 homolog
MDSC	Myeloid-Derived Suppressor Cell
MECL-1	Multicatalytic Endopeptidase Complex subunit 1, β2i

MHC	Major Histocompatibility Complex
MHC-I	Major Histocompatibility Complex Class I
MHC-II	Major Histocompatibility Complex Class II
Mre11	Meiotic Recombination 11 homolog
MRN	Mre11-Rad50-Nbs1 complex
mTOR	Mechanistic Target of Rapamycin
NBD	Nucleotide Binding Domain
Nbs1	Nibrin
NHEJ	Non-Homologous End Joining
NK	Natural Killer
NLRC5	NOD-Like Receptor family member CARD domain containing 5
NOD	Nucleotide-binding Oligomerization Domain
NSCLC	Non-Small Cell Lung Carcinoma
NT	Not Treated
Nur77	Nuclear receptor subfamily 4 group A member 1
Ova	Ovalbumin
OX40	Tumor necrosis factor receptor superfamily member 4, TNFRSF4, CD134
PA28	Proteasome Activator protein 28
PARP	poly(ADP-ribose) polymerase
PD-1	Programmed cell Death receptor 1, CD279
PD-L1	Programmed cell Death receptor ligand 1, B7-H1, CD274
PD-L2	Programmed cell Death receptor ligand 2, B7-DC, CD273
Pdcd1	PD-1 gene
PIKK	Phosphoinositide-3-Kinase-related protein Kinase
PLC	Peptide Loading Complex
pMHC	peptide-MHC complex
RAG	Recombination Activating Gene
RFX	Regulatory Factor X
RIG-I	Retinoic Acid-Inducible Gene I
ROS	Reactive Oxygen Species
RPA	Replication Protein A
RT	Radiation Therapy, radiotherapy
S phase	DNA Synthesis phase
SBRT	Stereotactic Body Radiation Therapy
SCCVII	murine cell line Squamous Cell Carcinoma VII
SHP-2	Src-homology 2 domain containing protein
SL8	SIINFEKL peptide, component of ovalbumin protein
SSB	Single-strand break
ssDNA	single-stranded DNA
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of Interferon Genes
TAP	Transporter associated with Antigen Processing
TBK1	TANK-Binding Kinase 1
TCGA	The Cancer Genome Atlas
TCR	T Cell Receptor
TDLN	Tumor-Draining Lymph Node
TIGIT	T cell immunoreceptor with Immunoglobulin and ITIM domains
Tim-3	T-cell Immunoglobulin- and Mucin- domain containing 3
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TopBP1	DNA Topoisomerase-Binding Protein 1

TP53	p53 gene
Tpn	Tapasin
Treg	T regulatory cell
Ub	Ubiquitin
UV	Ultraviolent
V(D)J	Variable (Diversity) Joining
WT	Wild-Type
XCR1	X-C motif Chemokine Receptor 1
XLF	XRCC4-Like Factor
XRCC4	X-ray repair cross-complementing protein 4
αPD-L1	PD-L1 blocking antibody
αβ T cell	T cell expressing TCRα and TCRβ subunits
β2m	beta-2 Microglobulin
γδ T cell	T cell expressing TCRγ and TCRδ subunits

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Abstract

Effective treatments for cancer have traditionally been divided into three separate arms: surgery, chemotherapy, and radiotherapy. Recently, however, a fourth arm, known as cancer immunotherapy, has been incorporated into treatment regimens and clinical trials with remarkable efficacy. These immunotherapies, designed to generate anti-cancer immune responses, have emphasized the importance of the immune system in controlling cancer and have placed greater focus on the the immune consequences of more traditional therapies. In this thesis, we have attempted to elucidate the way that radiotherapy can stimulate the interactions between the immune system, specifically CD8+ T cells, and cancer cells to induce lasting clearance of tumors.

In the field of radioimmunology, it is commonly thought that radiotherapy may work in part by functioning as an *in situ* vaccine, boosting CD8+ T cell numbers in circulation and generating systemic anti-tumor immunity. This model is supported in part by observations of abscopal effects, where localized irradiation of cancer can stimulate immune-mediated regression of tumors outside of the treatment field. In order to test the hypothesis that radiation can induce regression of tumors by acting as a vaccine, we modeled radiation-induced vaccination with a live-attenuated *Listeria monocytogenes* vaccine capable of generating order-of-magnitude higher numbers of tumor-specific CD8+ T cells. We demonstrated that vaccine-induced T cells have cytotoxic capacity and effectively traffic to tumors; however, we found that vaccination alone or in combination with PD-1/PD-L1 checkpoint blockade was insufficient to replicate the efficacy of radiation. Significantly, we observed that while cancer cells were resistant to killing by CD8+ T cells, antigen-specific T cell responses could be enhanced by pretreatment of cancer cells with cytokine stimulation or irradiation. These results

suggest that vaccination by radiation is not sufficient to explain enhanced CD8+ T cell-mediated control, but that radiation can augment the susceptibility of cancer cells to killing by T cells.

While the traditional aim of radiotherapy is to directly kill cancer cells, sublethal doses of radiation can trigger phenotypic alterations in these cells; among the most compelling of these potential alterations for the enhanced response of T cells in irradiated tumors is the augmentation of antigen presentation on MHC-I molecules, due to the integral role of antigen presentation in permitting recognition by CD8+ T cells. In order to determine whether enhanced antigen presentation by radiation was sufficient to generate effective anti-tumor T cell responses, we attempted to determine the mechanism by which radiation upregulates MHC-I on cancer cells. Contrary to previous reports, we found that MHC-I induction in our model was not dependent on signaling through STING or IFNAR, but instead found that radiation was able to induce expression of the MHC-I transactivator, NLRC5, independently of these pathways. Significantly, we found that increased expression of NLRC5 was sufficient to enhance MHC-I expression on cancer cells. Finally, we demonstrated that while NLRC5 upregulation by cancer cells did not improve CD8+ T cell recognition of cancer cells, T cells were better able to control NLRC5^{hi} cancer cells than NLRC5^{lo} controls. Together, these results support a model where radiation permits CD8+ T cell mediated control by upregulation of MHC-I via NLRC5.

Chapter 1: Introduction

1.1 Cancer and Adaptive Immunity

Cancer is a disease that provides a set of unique challenges for its control and eradication. Tumors generally form from healthy tissues that have been transformed through the acquisition of somatic mutations, which arise through assaults to DNA by exposure to carcinogens, UV radiation, or through the normal stress of repeated cell divisions that occur as a body ages. The transformation of a single healthy cell to a progenitor of millions of malignant cells proliferating at the cost of the organism as a whole has been studied extensively (1). Very broadly, oncogenic transformation requires activating mutations in tumor promoting oncogenes (hastening the rate at which cells replicate) and deactivating/suppressive mutations in tumor suppressor genes (preventing recognition of, or response to, deregulated cell growth). Collectively, the mutations that confer selective advantages to the growth of cancer cells are called *driver mutations*.

Comprehension of cancer biology requires an extension of our understanding of normal tissue biology under intense Darwinian selection. Early in their development, cancer cells look very similar to healthy cells in their tissue of origin. The 2000 seminal article by Douglas Hanahan and Robert Weinberg, “The Hallmarks of Cancer,” listed six key acquired characteristics of cancer, differentiating it from cells at the origin: limitless replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, tissue invasion and metastasis, and evasion of apoptosis (2). The most self-evident of these features is the enhancement of replicative potential since cancer is, above all, an abnormal accumulation of cells forming a tumor. Hanahan and

Weinberg expanded on the six original Hallmarks with the addition of two “emerging” Hallmarks in 2011: deregulation of cellular energetics (metabolism) and, significantly for this thesis, evasion of immune destruction (3).

The first line of defense against malignant transformation requires cell-intrinsic sensing of significant alterations to DNA sequences and/or structure or its consequence: changes in the shape or abundance of proteins produced; the mechanisms and outcomes these processes will be discussed further in this chapter. Cellular sensing of alterations to DNA trigger pauses in cell division at set checkpoints in the cell cycle and activation of DNA repair pathways; if the damage is too severe to be repaired the cell signals to itself to begin the process of *apoptosis*, or cell suicide. Key for the development of cancer are disruptions in the ability of cells to sense or respond to DNA damage; accordingly, the vast majority of human cancers lose function in one or more of these pathways, allowing for continued proliferation and accumulation of pro-tumorigenic mutations.

A second major line of defense in higher organisms is extrinsic to transformed cells: the immune system. It is now known that the immune system heavily shapes the progression of cancer, in ways that are both advantageous and disadvantageous to the body. In the early 1900s, Paul Ehrlich proposed that the precursors to cancer were likely generated at some frequency throughout the lifespan of an individual, but that most of these cells were eradicated early on in development by immune cells (4). Ehrlich’s proposal was an extension of his work on organ transplantation where it was known that despite gross anatomical similarities, the immune system was able to recognize differences between the graft and host tissue: because transplantation is an artificial condition that does not exist in nature it is otherwise perplexing that such a violent inflammatory response should be inbuilt against seemingly subtle differences derived

from the host source of transplanted tissues. Many of the immunological barriers to organ transplantation appear to be symptoms of protective immunity against malignant transformation including recognition of altered major histocompatibility complex (MHC) expression, which will be discussed further in this work. More than fifty years later, the description of this process of was expanded on independently by Lewis Thomas and Frank Macfarlane Burnet and termed *cancer immunosurveillance* (5-7).

Cancer immunosurveillance is in large part conducted by T cells, a member of the adaptive immune system, but other members of that system, B cells and NK cells, and members of the innate immune system can also play roles in the body's response to carcinogenesis (8-11). Cells of the adaptive immune system differentiate themselves from innate immune cells in their ability to clonally respond to a specific antigen and to confer lifetime immunity to that specific signal. For example, each T cell generates by genetic recombination a unique T cell receptor sequence that is able to bind to recognize specific peptide structures in the context of MHC molecules. When a naïve T cell meets a cognate peptide-MHC complex (pMHC) in the appropriate context it becomes activated, proliferates, and eventually forms an expanded clonal population of memory cells that persist throughout the life of the organism and are capable of rapidly responding to the signal should it appear again. By contrast, cells of the innate immune system do not undergo genetic recombination, respond to a range of broad stimuli such as bacterial cell wall components and cytosolic DNA, and typically favor more rapid responses (12).

Early experiments attempting to link T cells and resistance to cancer overall failed to prove a definitive connection. Logically, if components of the immune system were able to control tumorigenesis, then hosts with impaired immunity would exhibit increased incidences of induced carcinogenesis by viral infection, exposure to

carcinogens, or in animals with genetic dispositions to developing spontaneous tumors. Studies in nude mice, conducted by Osías Stutman and others, which fail to develop functional thymuses key to the development of T cells, overwhelmingly demonstrated no significant differences in the development of carcinogen methylcholanthrene (MCA) induced tumors between nude and immunocompetent animals. In retrospect, these findings are more likely due to incomplete understanding of degree of immunocompetence of the model (nude mice have detectable numbers of functional αβ T cells, some intact subsets of γδ T cells, and NK cells, which can facilitate anti-tumor immunity but do not require the thymus for development) and susceptibility to MCA-induced tumorigenesis (CBA/H nude mice express an enzyme that rapidly activates the drug's carcinogenic capacity, potentially overwhelming the ability of the immune system to control transformed cells) than to logical failure of the immunosurveillance hypothesis (13, 14). However, among the most significant findings from this period were that mice with induced immune deficiencies were highly susceptible to lymphomas and virally-induced carcinomas, although these were generally explained by attenuated responses to viral pathogens and chronic activation and exhaustion of lymphocytes, resulting in cancer (13).

In the 1990s, studies by Robert Schreiber and others demonstrated clear links between the ability of host lymphocytes to exert functional control by T cells or NK cells secreting interferon-gamma (IFNγ) and perforin and the susceptibility of animal models to develop tumors (15-17). The key role of T cells in cancer immunosurveillance was revealed when the $Rag2^{-/-}$ model was developed, wherein mice completely lack mature T and B cells due to their inability to perform V(D)J recombination; in this setting, mice are far more sensitive to the development of MCA-induced tumors with this effect being exacerbated when they are also unable to produce interferons ($Rag2^{-/-}STAT1^{-/-}$) (8).

Together with studies deciphering the roles of other adaptive lymphocytes such as NKT and γδ T cells and innate subsets such as NK cells, Schreiber proposed a model incorporating immune surveillance of carcinogenesis: *The Three Es of Cancer Immunoediting* (13, 18). The Three Es describe a process in which immune cells *eliminate* potentially carcinogenic cells by the process of immune surveillance as proposed by Burnet and Macfarlane. In circumstances where cancerous cells evade early immune screening and form stable masses, the immune system later can form an *equilibrium* within the tumor maintaining the size or severity of disease, but effectively selects for cancer cells capable of further evasion: cells which are capable of this evasion can then *escape* the equilibrium stage and overwhelm the immune system, leading to disease progression and potentially death of the host.

The *Three Es of Cancer Immunoediting* describe a Darwinian interplay between the developing tumor and the host immune system wherein the host eliminates cells capable of easy immune control and thus selects for cells better able to evade detection. As evidence of this, it was observed that implanted MCA-induced tumors developed in both $RAG2^{-/-}$ and immune competent control mice shared similar growth kinetics in $RAG2^{-/-}$ mice; however, when implanted in immune competent hosts, a portion of the tumors derived in $RAG2^{-/-}$ mice established but were then rejected, indicating that tumors arising in the absence of immune-mediated selective pressures are more immunogenic (8). This study highlighted the ability of tumors to adapt immune evasion strategies as they develop under the selective pressure of the immune system; some of these mechanisms will be elaborated upon throughout this chapter.

1.1.1 CD8+ T cells as Effectors of Anti-Tumor Immunity

Cytotoxic T lymphocytes (also known as CTLs, CD8+ T cells, CD8s, or cytotoxic T cells) are among the most therapeutically relevant effector cells in the adaptive immune system in controlling transformed cells (19-21). T cells are defined by their expression of the protein complex known as the T cell receptor (TCR), which contains a randomly-generated binding sequence capable of recognizing a specific shape and affinity of antigen, generally bound to a specific major histocompatibility complex (MHC) molecule; the requirement for T cell antigens to be bound to MHC molecules in order to be recognized is termed *MHC restriction*[1] (22-24). CD8+ T cells are defined by their expression of the CD8 co-receptor, which binds MHC class I (MHC-I), whereas another major population of T lymphocytes, CD4+ T cells (also known as CD4s or helper T cells), use the CD4 co-receptor to bind MHC class II (MHC-II) (22). The binding of these co-receptors facilitates localization of signaling molecules to the intracellular domain of the TCR and enhances signaling (22, 25). Cytotoxic CD8+ T cells are able to directly induce cell death in target cells presenting the cognate pMHC through the ligation of death receptors (Fas-FasL) (26), release of inflammatory cytokines (interferon-gamma, IFNγ, and tumor necrosis factor alpha, TNFα) (27), and transfer of cytotoxic granules (serine proteases known as granzymes) via perforin-lined pores generated in the target cell membrane (22). Together these abilities allow CD8+ T cells to selectively target tumor cells expressing mutated protein antigens (*tumor-associated antigens* or *neoantigens*) with relatively little damage to normal tissue.

Humans begin to develop T cells very early in life as lymphoid progenitor cells leave the bone marrow and traffic to the thymus where interactions with the stroma

[1] Broadly, T cells with TCRs containing α and β subunits (αβ T cells) require their cognate antigen to be presented on an appropriate MHC molecule in order for it to be recognized. However, certain minority T cell subjects, for example those with TCRs containing γ and δ subunits (γδ T cells) can recognize antigens resulting in activation outside of the context of MHC restriction (Janeway 2012).

trigger differentiation into T cell subsets, the majority of which will go on to become αβ T cells, expressing TCRα and TCRβ subunits. As these precursor cells develop in the thymus, they upregulate RAG1 and RAG2 genes to allow genetic rearrangement of the TCRβ locus followed by rearrangement of the TCRα locus in a process termed V(D)J recombination; simultaneously, expression of both CD8 and CD4 are upregulated. The process of rearrangement generates a vast array of unique TCR binding sequences that enable recognition of a wide range of antigens. Once the rearrangement process has produced a stable mature TCR, cells undergo *positive selection* in which their ability to bind MHC-I (with CD8) or MHC-II (with CD4) is determined and expression of the extraneous co-receptor is downregulated. These single-positive cells then move into the thymic medulla where *negative selection* occurs, the process by which cells that bind high-affinity self-peptides expressed by medullary thymic epithelial cells are triggered to activate apoptotic signaling (28). This process of eliminating self-reactive T cell clones results in *central tolerance*, reducing the risk of developing autoimmune diseases. Notably, an exception to the process of negative selection allows the generation of a population of CD4+ T cells which differentiate into T regulatory cells (Tregs) that are able to produce immune suppressive signals in the peripheral tissue, contributing to *peripheral tolerance* and further reducing the risk of developing autoimmune disease (29). By the end of thymic development only about 2% of the initial input of progenitor cells have passed selection and are exported from the thymus to form the peripheral T cell repertoire (22).

Outside of the thymus, CD8+ T cells exist in various stages of activation throughout the body and can differentiate into many subtypes depending on timing and location. The initial output of CD8+ T cells from the thymus are termed *naïve CD8+ T cells* as they have not yet met their cognate antigen and patrol the body by circulating

throughout the blood, lymphatics, and lymphoid organs. In order to enact their immune functions, CD8+ T cells must undergo the multistep process of activation. Once activated, CD8+ T cells proliferate over the course of several days in a process of clonal expansion, expanding the number of cells capable of responding to that antigen by many thousands, and differentiating into a population of *effector CD8+ T cells* which only require pMHC ligation in order to exercise cytotoxic functions on presenting target cells (22). These effector cells are relatively short-lived, with cell numbers peaking 5-7 days after acute stimulation and sharply declining in the absence of sustained or repeated antigen exposure. Appropriately activated CD8+ T cells can also differentiate into *memory T cell* populations which are maintained throughout the lifespan of the individual and are able to respond more quickly and strongly to repeated antigen exposure due to 100-1000-fold increased frequency above naïve T cell levels, increased sensitivity for pMHC:TCR signaling, increased capacity for expansion, and increased capacity for production of effector molecules (22, 30-33).

The activation of CD8+ T cells is a tightly regulated process requiring multiple positive signals (referred to here as Signal 1, Signal 2, and Signal 3) although the strength of each of these signals necessary for activation and differentiation of T cells depends on the type of T cell and the presence of negative signals. Briefly, Signal 1 refers to the ligation of the TCR with its cognate pMHC complex. All nucleated cells in the body express MHC-I molecules, presenting peptides derived from cytosolic proteins: this continuous process allows circulating CD8+ T cells to patrol for mutated (potentially cancer-derived), viral, bacterial or parasitic proteins; however, the amount of MHC-I expressed can vary, depending in part on inflammatory context and viral or oncogenic transformation. As mentioned previously, T cells are broadly restricted by MHC, meaning that they respond not only to the structure of the antigen but the structure of the MHC

molecule presenting the antigen; variability in T cell responses between individuals due to MHC haplotypes can thus be wider in humans, which are often heterozygous for these alleles, compared to laboratory mice which are commonly homozygous. Antigen presentation on MHC-I will be discussed further later in this chapter.

In order for CD8+ T cells to become appropriately activated by antigen recognition they must also receive *costimulation* by mature dendritic cells (DCs) (Signal 2). Although nearly all cells present self-derived peptides on MHC-I molecules, only DCs are capable of presenting exogenously-derived antigen on the cell surface to stimulate CD8+ T cell immunity, known as *cross-presentation* (34, 35). Among professional phagocytes, which also include macrophages and neutrophils, dendritic cells are specialized in their ability to preserve antigen structure by reduced proteolytic degradation (36, 37): this, combined with their ability to shuttle protein from the extracellular space into phagolysosomes and the endoplasmic reticulum (ER) to allow presentation on MHC-I, allows DCs to efficiently *cross present* MHC-I antigens that are not self-derived along with the exogenous MHC-II antigens which are common to professional antigen presenting cells (APCs) (38). Following activation in peripheral tissue by microbial contact or inflammatory cytokine, DCs halt antigen uptake, undergo maturation and migrate to the draining lymph node in a CCR7-dependent manner (39, 40). Once in the lymph node, mature DCs presenting antigens on MHC-II molecules will undergo licensing through CD40-CD40L interactions (L denotes the canonical ligand for the receptor) with helper CD4+ T cells (41-43) and upregulate costimulatory molecules CD80 and CD86 for CD8+ T cell activation: the interaction between CD28 on CD8+ T cells and CD80/CD86 on DCs represents the dominant costimulatory Signal 2 for naïve T cells (44).

While mature DCs may activate CD8+ T cells in the absence of CD4+ T cell help, the threshold for activation is high and requires strong adjuvant signals (45) and evidence suggests that CD4+ T cell help is required for the generation of effective long-term memory responses (22, 46-49). Notably, an absence of any costimulation by mature DCs appears to result in CD8+ T cell tolerance where T cells, despite receiving Signal 1, are not able to enact cytotoxic functions (50), demonstrating the essential role for Signal 2 in activating effective T cell responses.

Following activation by Signal 1 and Signal 2, T cells upregulate the α chain of the interleukin-2 (IL-2) receptor (CD25) to allow high-affinity binding of the Signal 3 molecule IL-2 to drive proliferation and differentiation (51); simultaneously, activated T cells produce and secrete IL-2 and thus form a feedback loop (52). In addition to IL-2, other proinflammatory cytokines (including type I interferons and interleukin-12) may act as Signal 3 molecules, enhancing the proliferation and memory formation of activated CD8+ T cells (53-55). Having received all three activation signals, T cells can divide 2-3 times a day for several days peaking in number around seven days following acute stimulation (12). The duration of T cell expansion is affected by the duration of antigen exposure as well as the persistence of adjuvant signals providing inflammatory context.

1.1.2 Therapeutic Interventions to Enhance Anti-Tumor T Cell Responses

Despite the ability of CD8+ T cells to mount potent and effective responses against cancer, there are significant barriers to T cell-mediated tumor killing. Studies have demonstrated that the presence of tumor-infiltrating CD8+ T cells is associated with improved prognosis (56, 57); however, these patients still have cancer despite the presence of these cells and require treatment in order to survive. These circumstances

raise important questions: how can we ensure that patients have sufficient CD8+ T cells capable of responding to tumor antigens and, in patients with sufficient T cells, how can we ensure that the T cells are able to kill the cancer? Some of the barriers to T cell-mediated treatment and ongoing efforts to subvert them are described below, but due to the recent explosion in research many strategies currently under exploration will not be addressed. As a whole, therapies such as these, aimed at stimulating immune responses against cancer, fall within the field of *cancer immunotherapy*.

1.1.2.1 Cancer Vaccines

The most successful vaccine used in the treatment of cancer is *Bacillus Calmette-Guerin* (BCG), which was developed more than 100 years ago to protect against tuberculosis infection and is still used for that effect. A connection between cancer survivors and cleared or active tuberculosis infections was observed in the 1920s but interest in using BCG to combat cancer waned when safety concerns arose regarding preparations of the vaccine (58). It wasn't until a 1959 study by Lloyd Old and others, demonstrating intravenous injection of BCG enhanced resistance of mice to the establishment of model tumors, that interest was renewed (59). Further studies demonstrated that BCG protects against tumors by activating macrophages and promoting local delayed hypersensitivity to induce clearance of cancer cells, thus requiring close contact between cancer cells and in location of inoculation (58). BCG vaccination is therefore in the same treatment category as Coley's vaccine, a preparation of killed *Strepococcus pyogenes* and *Serratia marcescens*, which was successfully used in the localized treatment of cancer in the late 19th century due to its adjuvant quality (60). These "vaccines" are powerfully inflammatory but function primarily to activate innate immunity rather than target adaptive immunity to specific targets and thus broadly fail to generate lasting immunological memory (61). As a result, over the

last 40 years BCG has been primarily successful in bladder cancer where cystoscopic injection of the vaccine engages inflammatory responses in the thin tissue of the organ and promotes local tumor eradication (58).

A second category of cancer vaccines, which prophylactically target oncogenic viruses, have also been successful. Prophylactic vaccination targeting Hepatitis B (HepB) was added to the routine vaccine schedule for infants in the mid-1990s and is about 70% effective in preventing the development of hepatocellular carcinomas (62). More recently, vaccination against human papilloma virus (HPV) has been recommended to be given to children ages 11 and 12 and is largely effective in preventing HPV-linked head and neck, cervical, penile, vulvar, and anal cancers (63). These vaccines confer lifelong protective immunity by activating adaptive immune subsets against virus-specific peptides: in the case of HPV vaccination the exterior of viral particles (L1 major capsid protein) is targeted to allow early clearance of an infection. Notably, successful vaccination strategies have only been developed to protect against establishing infection; clearing viral infections once they have been established has proved to be a far more difficult task (64).

Therapeutic vaccination strategies against established cancer, similarly, have largely proved ineffective in patients. It is clear that many tumor types express antigens that may, in theory, be sufficient to stimulate potent anti-cancer adaptive immune responses; regardless, targeting proteins overexpressed by cancer cells or mutated versions of proteins exclusively expressed by cancer cells (*neoantigens*) have failed to elicit tumor regression once the disease has established in the majority of patients treated (65). Many of these vaccines generate large populations of cells with cytotoxic capacity; however, by necessity tumors develop effective immune evasion strategies early in their development including: downregulation of tumor antigen presentation by

decreasing TAP1, β2M and MHC-I expression (8, 66), establishment of hypoxia and acidity affecting T cell metabolism (67), and upregulation of inhibitory immune checkpoints on tumor cells and infiltrating innate immune cells (68); as a result, theoretically tumor-reactive CD8+ T cells are often rapidly or chronically *tolerized* to tumor proteins. Many of these evasion strategies mean that vaccination to generate large populations of tumor-reactive cells will be ineffective because the T cells will not be able to "see" the cancer cell targets or be able to react appropriately due to immunological context.

1.1.2.2 Checkpoint Inhibitors

Perhaps the most significant breakthrough in cancer immunotherapy came in the form of checkpoint inhibitor therapy, developed based on increased understanding of how the immune system dampens self-destructive autoimmune responses and the ways in which tumors can hijack these processes. As described above, T cell responses can be potently effective at eliminating transformed or virus-infected cells, but particularly in cases of incomplete tolerance where self-reactive T cells survive thymic selection, T cells can be detrimental in the destruction of normal tissue. In order to protect against these unfavorable responses, along with the complement of proteins T cells express to enhance cytotoxic abilities, T cells express a number of self-inhibitory receptors which, when ligated, increase the downstream signaling threshold required to exert cytotoxic function. Additionally, tissue infiltrating immune cells such as regulatory T cells (Tregs) express protein receptors on their surfaces which can inhibit the ability of surrounding T cells to function providing an additional mechanism of peripheral T cell tolerance.

The first major therapeutic breakthrough in checkpoint inhibitors came with the blockade of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (69, 70). Throughout the 1990s, James Allison and colleagues published work characterizing the function of

this newly-discovered protein expressed on the surface of CD4+ T cells, originally described as a murine homolog to human CD28 (71-74); however, further exploration demonstrated that CTLA-4 and CD28 have opposing effects in the response to T cells to antigen stimulation (75). Mice lacking CTLA-4 develop a severe lymphoproliferative and autoimmune condition which leads to death 2-3 weeks after birth (76, 77). Part of the immune suppressive capability of CTLA-4 is a result of its rapid and relatively higher avidity binding to the costimulatory molecules CD80/CD86 with comparably higher avidity than CD28 (78, 79); this results in effective sequestering of costimulatory signal and dampened T cell effector responses. Allison's group observed that blockade of CTLA-4 was able to enhance anti-tumor immunity in mouse models, later finding that this effect was due to relatively high expression of CTLA-4 on Tregs allowing for selective depletion of Tregs *in vivo* (80, 81). In humans, however, there is little evidence to suggest that the depletion of Tregs observed in mice is the primary mechanism of efficacy for therapeutic CTLA-4 blockade (82); instead, signaling through the Fc domain of CTLA-4 blocking antibodies appears to be relevant (83).

A second major breakthrough in checkpoint inhibition came with the development of programmed death receptor 1 (PD-1) blockade. Tasuku Honjo and colleagues discovered PD-1 while exploring mechanisms of T cell death (84); however, the function of the protein as a T cell inhibitory receptor was not elucidated until the generation of PD-1 deficient mice ($Pdcd1^{-/-}$) which develop lupus-like autoimmune disorders, indicating a functional role in the development of peripheral tolerance (85, 86). PD-1 expression is low on naïve T cells but is upregulated in response to antigen stimulation; as a result, T cells that have been chronically exposed to their cognate antigen without eliminating the source of exposure, as in the case of chronic viral infection or within tumors, have characteristically high expression of PD-1 (87, 88). Ligation of PD-1 with its canonical

ligands PD-L1 or PD-L2, expressed on tumor infiltrating immune cells or on tumor cells (68, 89-92), results in suppression of intracellular signaling downstream of costimulatory CD28 (93, 94) and increases the threshold of activation for T cells to exert cytotoxic capacity. In human patients, tumor expression of PD-L1 can correlate with decreased functioning of T cells and negative prognosis (85, 95, 96); antibody-mediated blockade of the PD-1/PD-L1 axis has led to remarkable improvements in the treatment of many types of cancer including: melanoma, Hodgkin's lymphoma, non-small cell lung carcinoma (NSCLC), renal cell carcinoma, urothelial cancer, and head and neck cancers (97, 98).

While checkpoint blockade with CTLA-4 and PD-1/PD-L1 inhibitors have had unprecedented clinical successes, there are lingering questions regarding why some cancers still evade control by immunotherapy and what interventions can turn non-responders into responders. Additional checkpoint inhibiting agents including those targeting Lag-3, Tim-3 and TIGIT (99) are being explored, while another class of therapeutic antibodies targeting activating costimulatory receptors such as OX40, GITR, and 4-1BB (100) aim to increase positive signaling rather than alleviate negative signaling to overcome immune suppression; some of these newer checkpoint inhibitors have indeed been shown to combine with CTLA-4 or PD-1/PD-L1 blockade in preclinical models (101-103). Importantly, traditional cancer therapies such as radiotherapy also appear to be able to alleviate checkpoint blockade resistance in mouse models and in patients (102, 104-113); the immune modulatory effects of radiation therapy and how they may synergize with checkpoint inhibition are discussed below.

1.1.2.3 Radiotherapy

In cancer therapy, radiotherapy (RT) is primarily used to initiate localized death of cancer cells by targeted administration of beams of radiation (typically x-rays or γ-rays)

to tumors with the goal of reducing tumor burden at that location. Approximately 50% of cancer patients undergo RT at some point during their course of treatment in part because RT can be administered with either palliative or curative intent. RT is uniquely attractive in patients with inoperable tumors or incompletely resected tumors, although treatment doses are limited by the sensitivity of normal tissue to radiation and associated toxicities (114, 115).

In order to limit toxicity in healthy tissue and enhance efficacy in tumor tissue, radiation doses are often fractioned into many smaller doses, as will be discussed later on in this chapter in the context of the Four Rs of Radiobiology (116). Today, a standard conventional course of radiation therapy consists of daily fractions of 1.8-2 Gy over the course of 6-7 weeks, resulting in a cumulative dose of 50-70 Gy administered to the tumor (114, 117). This contrasts with experimental *accelerated* or *hyper- fractionated* courses, which may consist of yet smaller doses of radiation, administered more than five times weekly or 2-3 times daily (discussed further in a following section). More recently, however, *hypofractionated* regimes, with fewer doses administered on the order of 8-30 Gy per dose, have become accepted and in some cases the standard of care for cancers, particularly NSCLC (118-121).

One compelling argument for the utilization of hypo-, rather than hyper- or conventionally, fractionated radiation is that lymphocytes are particularly sensitive to cell death induced by radiation and thus any immune responses initiated to the tumor by radiation may be ablated before becoming effective (21, 116). Indeed, in mice it has been observed that following a high-dose of radiation with a series of smaller doses is actually less effective at inducing tumor clearance than the single high-dose alone, correlating with a decrease in radiosensitive CD8+ T cells and increase in more radioresistant myeloid-derived suppressor cells (MDSCs) (20).

It has long been established that lymphocytes accumulate around regressing tumors following irradiation (122), and radiotherapy is often poorly effective in non-immune competent mouse models (123). There is a growing body of evidence supporting the conclusion that many radiation responses are dependent on antigen-specific adaptive immunity (21, 124-128). In the following section we discuss the role of radiation in unleashing anti-tumor immunity as well as some of the established mechanisms of immune-related protection of tumors enhanced by radiation.

1.2 Radiation as Cancer Therapy

Radiotherapy (RT) has been used to control the growth of tumors for well over 100 years following observations by Pierre Curie, Antoine Becquerel and others that prolonged exposure to radioactive materials produced lesions that required weeks to heal (129). It was not until much later, however, that the mechanism of radiation-induced tumor regression was discovered. *Radiation* refers to the emission of energy in the form of waves or particles, and the type of radiation used to treat tumors in patients is termed *ionizing radiation*, denoting its ability to excite electrons in atoms to higher energy states. When atoms in cells are exposed to ionizing radiation, some high-valence electrons are ejected from the outer shell and react non-discriminately to induce damage to protein and DNA, followed by damage repair responses and potentially cell death.

Approximately half of the damage caused to cells by ionizing radiation is generated by water radicals (reactive oxygen species (ROS): $OH\cdot$, $H\cdot$ and e_{aq}^-) that rapidly react to disrupt protein structures and damage DNA (indirect effects), while the other half of damage is induced by direct ionization of non-water molecules such as protein and DNA (direct effects)[2] (129-131). Disruption of proteins following irradiation can initiate damage response pathways (132, 133) and may be responsible for a portion of post-RT cell death; however, protein degradation occurs naturally and frequently during the lifespan of a cell and damaged proteins may be turned over without lasting effects, while alterations to DNA have the potential to be passed on through cell divisions and cause lasting damage to the organism. Thus, there is strong evidence that damage to DNA is the primary mediator of RT-induced therapeutic responses (129, 134)

[2] Although water comprises about 80% of cells, the role of water radicals in initiating damage after irradiation is dampened by the presence of natural ROS scavengers (Sevilla 2016)

and mammalian cells have multiple, overlapping pathways designed to maintain the integrity of DNA sequences.

1.2.1 Radiation and the DNA Damage Repair Response

For mammalian cells, a relatively small dose of ionizing radiation (1-2 Gy) can trigger the formation of thousands of DNA lesions, primarily in the form of base damage or single-strand breaks (SSBs) which can be rapidly repaired using the corresponding DNA strand as a template (129). Double-strand breaks (DSBs) occur less frequently but form when SSBs occur within 10-20 base pairs of each other on opposite strands and can lead to chromatin cleaving into two fragments (135), and this type of lesion is considered to be the most significant DNA lesion in inducing cancer cell death following radiotherapy. Both normal and cancer cells undergo sensing and repair pathways for DSBs which can result in downstream signaling responsible for delaying or stopping cell division and inducing senescence, mitotic catastrophe and/or cell death.

In mammalian cells, DSBs are sensed shortly after irradiation and most lesions are repaired by non-homologous end joining (NHEJ) within two hours. DSBs cause relaxation in the chromatin fiber and post-translational modification of histones (e.g. acetylation) (134) and allow the initial direct sensors of double-stranded breaks, Ku70/80 heterodimers, to bind the blunt end of the DNA phosphate backbone (136). Bound Ku complexes recruit the DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs), which auto-phosphorylates and recruits DNA polymerase (DNA pol μ and pol λ), nuclease (Artemis), and ligase (DNA ligase IV, XRCC4, and XLF) subunits to fill in overhang regions, clip away residual or damaged single-stranded DNA bases and ligate the previously broken ends together (137, 138). Notably, although NHEJ is the preferred

method of mammalian cells to repair damage throughout the cell cycle (139, 140), the process is more prone to inserting or deleting bases in the repaired strand than homologous recombination, discussed below, and may result in significantly altered transcript structure or function. This potential disadvantage is counterbalanced, however, by the relative speed with which DNA-PK-dependent NHEJ resolves DSBs meaning that this method of repair does not have a clear role in slowing cancer cell cycle progression (141, 142).

While NHEJ occurs most commonly when DSBs occur during the majority of the cell cycle, its high-fidelity counterpart homologous recombination (HR) occurs mainly during late S/G_2 phase, after the chromatids have been replicated and joined by a centromere (139). HR is less error-prone than NHEJ due to the presence of the duplicate sister chromatid as a template for repair (143). The precise mechanism for the recruitment and activity of HR machinery is still under investigation, but it appears that after Ku proteins bind to DSBs, if NHEJ fails, the cell attempts to repair the damage through HR (144-146). Components of the Mre11-Rad50-Nbs1 (MRN) complex bind to homoduplex DNA, scan past nucleosomes to locate free DNA ends, remove Ku heterodimers, and allow loading of the DNA exonuclease Exo1 to initiate single-stranded DNA resection (147, 148). Activation of the MRN complex requires the recruitment of Ataxia-Telangiectasia Mutated kinase (ATM) dimers to the c-terminus of Nbs1, where the kinase is converted to the activated monomer form through an unknown mechanism (148, 149). ATM/MRN complex signaling triggers a complex cascade of recruitment and activation of proteins facilitating homologous recombination, including Rad51 and BRCA2, which are responsible for DNA end resection, invasion of the template strand by free DNA ends, synthesis of new DNA using the template, and reconstitution of DNA on the previously damaged chromosome (145, 148, 150). ATM is abundant in cells but is

rapidly inhibited by DNA-PKcs when NHEJ, not HR, is underway (151). In patients with ataxia-telangiectasia, the namesake condition describing mutational inactivation of the ATM kinase, cells are extremely sensitive to radiation-induced damage, to the point where radiotherapy can be lethal (152).

An alternate pathway of DSB repair by homologous recombination following irradiation occurs solely during S phase (interphase) while chromosomes are duplicated into sister chromatids. As the template helix is unwound at the replication fork, DNA lesions can cause stalling of DNA-synthesizing polymerases triggering extension of long stretches of single-stranded DNA (ssDNA) or collapse of the replication fork resulting in single-ended double-stranded breaks on one side of the fork (153). In these cases, ssDNA is rapidly coated by replication protein A (RPA) complexes and the ATM- and Rad3- related kinase (ATR) localizes to the lesion via its necessary binding partner ATR-interacting protein (ATRIP) (154). Once bound to the lesion, ATR is activated primarily by DNA topoisomerase II binding protein 1 (TopBP1) and initiates Rad17-dependent homologous recombination, similar to that mediated by ATM (143, 155).

The apical kinases of these DNA DSB repair pathways, DNA-PKcs, ATM and ATR, are members of the phosphoinositide-3-kinase-related protein kinase (PIKK) family and share sequence homology and activity (143), although the pathways of activation are distinct as described above. These kinases have numerous roles in a vast array of activities coordinating DNA repair, cell cycle progression, senescence, apoptotic signaling and more, and significant interplay exists between the kinases (143, 156). Inhibition of cell cycle progression is a significant consequence of activation of DSB repair pathways, particularly ATM- and ATR- dependent mechanisms which are implicated in the phosphorylation of over 900 sites on over 700 proteins (157). Both ATR, via phosphorylation of Chk1, and ATM, via phosphorylation of Chk2, halt cell cycle

progression during S/G_2 phase to allow for repair prior to mitotic progression (158, 159). Activation of cell cycle checkpoints allows time for the cell to repair DNA damage but in cases where the damage is too significant to be repaired cell death may occur.

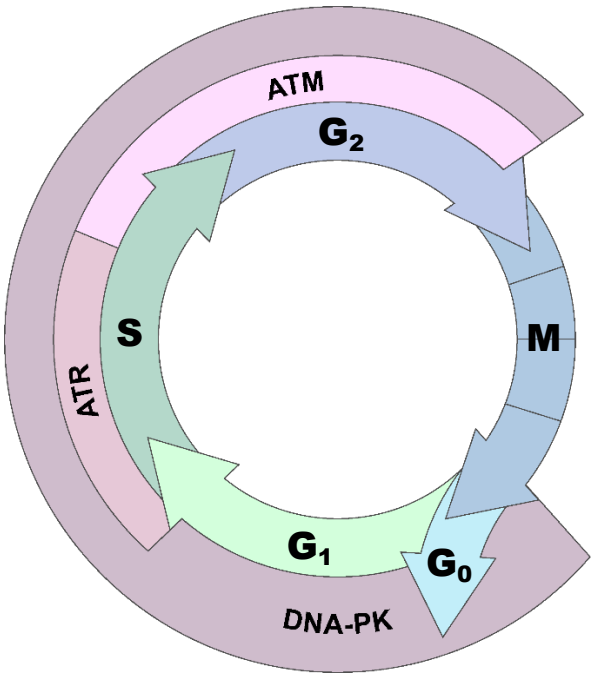

Fig. 1-1 The cell cycle and DNA double-strand break (DSB) repair pathways. Cell division occurs over the course of four phases: G_1 (gap 1), S (synthesis), G_2 (gap 2), and M (mitosis), while cells not actively undergoing cell division reside in G_0 phase. The replicative cell cycle begins in G_1, where cells increase in size and prepare for DNA synthesis in S phase, where DNA condenses and is replicated to form pairs of sister chromatids. In G_2, cells continue to grow and produce machinery required for the completion of mitosis. During M phase (divided further into prophase, metaphase, anaphase, and telophase), sister chromatids bind to spindles, line up at the metaphase plate, and are pulled to opposite poles before the cell divides into two daughters in a process of cytokinesis. DSBs generated by cell irradiation can be repaired by different processes depending on the cell cycle phase. During the majority of the cycle, DSB repair is completed rapidly by DNA-PK-dependent non-homologous end joining (NHEJ). While DNA is being replicated in early S phase, ATR-dependent homologous recombination occurs at the DNA replication fork. During late S and G_2 phase while chromatids are copied, ATM-dependent homologous recombination can occur.

1.2.2 Lethal Radiation and the Induction of Cell Death

A major target of ATM/ATR activation is the phosphorylation of the protein p53 at serine-20 and serine-15, respectively, allowing for the stabilization and conversion into the tetrameric transcription factor form. Ionizing radiation is a well-established initiator of p53 activity, both through activation of the protein directly and the inhibition of its inhibitors, including Mdm2 (160). P53 has been extensively studied due to its role as a key tumor suppressor; indeed its gene, *TP53*, is one of the most commonly mutated genes in human cancers, demonstrating the selective advantage cancer cells have in the absence of regulation by p53 (161). The protein has many overlapping roles in basic cellular pathways including in DNA damage repair, cell cycle progression, apoptosis, senescence, angiogenesis and metastasis (162, 163). How p53 switches between functions is not yet clear but appears to involve epigenetic modification to the protein following stabilization to trigger arrest and repair or initiate activation of cell death pathways (164, 165).

Studies in wild-type and p53-deficient murine thymocytes demonstrated that p53-dependent mechanisms are key in the induction of apoptosis following cell irradiation (166, 167). Following cellular irradiation, apoptosis can occur within several hours as the transcription factor p53 activates pro-apoptotic genes, including those of the Bcl-2 family, which play a role in permeablizing the outer membrane of mitochondria allowing for formation of the apoptosome. This complex, activated by cytosolic cytochrome c, mediates activation of caspase-9, allowing for cleavage and activation of the effector caspases 3, 6 and 7 (168). Caspase-dependent apoptosis results in degradation of chromosomal DNA, degradation of nuclear and cytoskeletal proteins, nuclear fragmentation, formation of apoptotic bodies and ultimately cell death (169). p53 also initiates cell-extrinsic pathways through the upregulation and export of death receptors,

which are also mediated by caspases 3, 6 and 7 (168) and will be discussed briefly in the section below on sublethal radiation and induction of anti-tumor immunity.

Lymphoid and myeloid lineage cells are among the most radiosensitive populations, where apoptosis appears to be the main form of cell death initiated by radiation. It seems, however, that apoptosis is less significant in cells of epithelial origin, from which many solid tumors are derived (168). Additionally, many cancer cells harbor inactivating mutations in p53-dependent pathways and are dysfunctional in normal apoptotic signaling. In these cases, cell death by p53-independent mitotic catastrophe is more likely to occur (170).

Mitotic catastrophe is considered to be the major pathway of cell death following radiation in tumor cells of non-hematopoietic origin. Cells which are undergoing mitotic catastrophe are morphologically distinct from those undergoing apoptosis most notably by their greatly increased size: this process results from aberrant mitosis where cells do not appropriately segregate chromosomes and is characterized by the formation of multiple nuclei, abnormally structured nuclei, and/or micronuclei: small extra-nuclear structures containing fragments of DNA (some of the immunological consequences of micronucleus formation are discussed in a future section). Mis-segregation of chromosomes leading to micronuclei formation frequently occurs as a result of mis-repair or incomplete repair of chromosomes following DSB induction by radiation resulting in lagging chromosomes or chromosome fragments during anaphase separation (171), but may also be triggered by overduplication of centrosomes generating division of chromosomes over more than two poles (172).

Mitotic catastrophe appears to be initiated in part by uneven condensation of chromatin around nucleosomes, characteristic of premature entry into mitosis prior to completion of S/G_2 as a result of faulty cell cycle checkpoint signaling (172). Following

irradiation, this may occur as a result of incomplete cell signaling where, for example, a deficiency in p53 results in premature entry into M phase past a dysregulated G_2/M checkpoint (168). Mitosis will then progress but halt at the spindle assembly checkpoint, also known as the mitotic checkpoint, prior to anaphase; in part because many normal cell processes are paused during mitosis, this delay can lead to apoptotic signaling in competent cells and be fatal (173, 174). However, transformed cells are often able to adapt to and progress past the mitotic checkpoint to complete M phase, but fail to undergo cytokinesis and enter subsequent the G_1 phase with tetraploid or greater chromosomes (168). Therefore, aberrant cells which will undergo death by mitotic catastrophe may complete one or more division cycles prior to cell death, which may occur as a delayed response 2-6 days following the initiation of radiotherapy.

1.2.3 The Rs of Radiotherapy

As mentioned previously, radiotherapy has been used for the treatment of cancer for more than a century with great debate about the most effective way to administer radiation treatments. Initial treatments of patients mostly utilized what is now called *hypofractionated* radiation with single or very few treatment doses administered but with very little control or quantification regarding the dose. By 1910, radium-contact therapy, where a radioactive source was placed near the tumor for 24 hours at a time repeated at an interval of six weeks, was popular and was still in use by 1951 when it was famously used in the treatment of cervical cancer in Henrietta Lacks, from whom the cell line HeLa was derived (175).

Very gradually, sentiments shifted away from single-, high-dose radiotherapy. At the turn of the 20th century, Claudius Regaud began experiments in rodent testes where it was first observed that undifferentiated cells and those undergoing mitosis were more

sensitive to cell death by irradiation (175). He expanded on these results in the 1920s with the observation that while a single dose exposure of ram's testes was sufficient to induce severe skin injury, it did not cause sterilization; however, breaking that dose into fractions did sterilize the animal (176). By the late 1920s and early 1930s, studies primarily in head and neck cancer patients using fractionated regimes presented by Henri Coutard generated interest in dose fractionation over periods longer than forty days with significantly improved results in patients (175, 177, 178). Dose fractionation has become the standard of care for the majority of malignancies in the time since Coutard's seminars following these observations and studies describing the sparing effect of dose fractionation on normal tissue.

In 1975 H. Rodney Withers published the foundational article "The Four R's of Radiotherapy" upon which many students of radiobiology base their mechanistic understanding of how fractionated radiation influences treatment outcomes (116). The four Rs are, briefly described, as follows:

1. *Repair*: Damage to cells following irradiation is distributed randomly and thus the probability of inducing cell death is equal for all cells in the treatment field receiving the same dose. In the examples provided, and in accordance with the findings of others (179), cells irradiated *ex vivo* have similar sensitivity to radiation-induced death based on the tissue of origin rather than malignant status; that is, cancer cells are not more sensitive to radiation than normal cells. All cells, if unable to sufficiently repair the initial injury, are more sensitive to the induction of cell death following subsequent doses of radiation due to the accumulation of "sublethal (multihit) events." Repair of sublethal injury to DNA, which has been discussed above, is dysregulated in cancer cells as a necessary component of the Hallmarks of Cancer in the evasion of cell death (2, 3) and thus multiple hits to key genes may be required

before the damage is catastrophic enough to induce cell death. Cells in normal tissues are unable to complete mitosis without repairing damage and thus are less susceptible to the accumulation of sublethal hits.

2. *Reoxygenation*: It has been observed since the 1950s that hypoxia enhances the ability of cancer cells to evade cell death following irradiation (180). This effect is predominantly due to the chemical reactions resulting in DSBs: radicalized DNA (DNA·) generated by ionization or reaction with free radical molecules derived from water (131) generates lesions at the deoxyribose sugar moieties that are only fixed in the presence of oxygen (O_2); however, under reducing conditions DNA radicals are repaired by siphoning hydrogen from sulfhydryl (SH) groups present in nearby proteins (181, 182). It is estimated that in order to affect the same degree of DNA damage in a hypoxic environment as an aerobic environment, 2-3 times the dose of radiation is required (116, 181). In dose fractionation schemes, however, incremental reoxygenation of the tumor occurs as cells die (reducing O_2 consumption) and the mass is revascularized (116). This mechanism appears to be key to the efficacy of fractionated radiotherapy, gradually reducing the dose required for therapeutic efficacy.
3. *Redistribution*: One of the founding principles of radiobiology, the “Law of Bergonié and Tribondeau,” was proposed in 1906, stating: “X-rays act on cells inasmuch efficiently as cells have a greater reproductive activity, their karyokinetic fate is longer, their morphology and function are at least definitively fixed. Hence from this law it is easy to understand that roentgenisation [radiation] destroys tumors without destroying healthy tissues…” (179). This broad statement that rapidly proliferating cells, with longer mitotic phases and less well-differentiated statuses, may be in part mechanistically explained by the third R: *redistribution*. As Withers describes, some stages of the mitotic cycle confer greater radiosensitivity to dividing cells than others:

cells in G_1, G_2 and M phases are more radiosensitive than those in late S phase. When a non-synchronized pool of cells is irradiated (as in a tumor), those cells in more sensitive phases are more readily eliminated causing redistribution (synchronization) of the remaining cells into radioresistant portions of the cell cycle. When subsequent doses of radiation are administered, cells that were previously resistant will have progressed into more-sensitive phases as the population regenerates. Accordingly, cells which are rapidly dividing, as cancer cells, are more radiosensitive than slowly dividing cells or those that are terminally differentiated (non-dividing) (175) due to the enhanced frequency with which they reach mitosis, triggering the potential for mitotic catastrophe and checkpoint-mediated apoptosis in the induction of cell death by irradiation.

4. *Regeneration*/*Repopulation*: Within the field of radiation, both normal cells and cancer cells receive equivalent doses of radiation damage in a somewhat random distribution. As stated previously, cells that are rapidly dividing are more prone to dying in response to that damage and because of that, ideally, cancer cells die in greater proportions than normal cells following irradiation. Unfortunately, rapidly proliferating cells can also *repopulate* much faster than normal cells, outpacing the intervals at which fractionated radiation is administered. In some cases, tumor masses continue to grow despite radiation treatment and radiation may be given more frequently (termed *accelerated fractionation* or *hyperfractionation*[3]) in order to

[3] In a 1983 follow-up paper, H Rodney Withers and others (Thames 1983) defined *accelerated fractionation* as “overall time shorter than conventional, achieved by giving 2 or 3 doses daily; total dose and fraction size similar to conventional” and *hyperfractionation* as “fractional doses smaller than conventional, given 2 or 3 times daily to achieve an increase in the total dose given in the same overall time as conventional.” Both accelerated and hyper- fractionated radiation are administered more than five times weekly but the doses given in hyperfractionated regimes are also smaller than conventional regimes; thus, accelerated regimes take the least amount of time to achieve a fixed dose while hyperfractionated and conventional regimes can take similar amounts of time.

attempt to control cancer cell repopulation (183). Ultimately, the principle of *repopulation* guides the design of radiation therapy toward timing doses to reduce as much as possible the number of surviving clonogenic cancer cells which may ultimately lead to recurrence of disease, with the understanding that early-responding normal tissue may be increasingly damaged as treatment is accelerated[4]. As a result, fractions are typically administered no fewer than six hours apart in order to allow for the repair of sublethal hits in normal tissue which are estimated to resolve within that time period (184).

The "Rs of radiotherapy" have now been expanded to include *radiosensitivity*, describing the intrinsic difference in cell-type specific responses to radiation defined by the steepness of a curve mapping the radiation dose versus the surviving fraction of cells (185). Calculating the dose response of irradiated tissue is notoriously complex, making comparison of treatments across tumor types particularly difficult. It is well established in radiobiology, for example, that dose fractionation effects are not linear; that is, fifteen doses of 2 Gy is not equivalent to one dose of 30 Gy. An approximation of dose equivalence is represented by the *biologic equivalent dose* (BED), which is based on a combination of linear and quadratic modeling:

$$BED = (total\ dose) \times (relative\ effectiveness)$$

$$BED = (nd) \times (1 + \frac{d}{\alpha/\beta})$$

[4] The terms *early responding tissue* and *late responding tissue* refer to the time it takes for normal tissue to demonstrate radiation-induced toxicities (e.g. loss of function, death). Early responding tissues (skin, mucosa, intestinal lining) express peak injury 2-3 weeks after completion of standard radiotherapy regimes; late responding tissues (spinal cord, kidney, lung) may take months or years to express peak injury. (Hall 2012)

where n is the number of fractions, d is the dose per fraction, *α* describes the linear component of the curve (reflecting death induced by a single lethal hit) and β describes the quadratic component (reflecting death resulting from multiple sublethal hits) (129, 186). The α/β component is used to define the intrinsic radiosensitivity of affected tissue types, where early-responding tissues such as skin and mucosa have α/β ratio estimated around 10 Gy and slow-proliferating late-responding tissues such as the spinal cord are estimated to have a ratio around 2-3 Gy; this value corresponds to the dose at which single- and multiple- hit killing is approximately equal. Notably, this modeling is more accurate at conventionally low doses of radiation where normal cells are able to repair damage in between dose administrations; at higher doses (as used in hypofractionated regimes such as SBRT), the killing of non-cancer components of the tumor such as endothelium can enhance the destruction of tumor architecture (187).

Together, these five principles help to mechanistically describe how fractionated radiotherapy takes advantage of differential responses of normal and cancerous tissues to maximize radiation-induced cell death. However, as described briefly above, immunity plays a significant role in the control of cancers following radiotherapy *in vivo* and immunological factors help to explain why in some cases administering more radiation does not necessarily mean more effective tumor killing.

1.2.4 Sublethal Radiation and the Induction of Anti-Tumor Immunity

Models of radiotherapy traditionally focus on maximizing lethal radiation: that is, how radiation can be administered in order to directly kill as many cancer cells as possible while keeping normal tissue toxicity at a manageable level. However, as the field of radiobiology builds a greater understanding of how the immune system interacts

with tumor cells a greater appreciation for the role of sublethal radiation is developing. While the direct induction of cancer cell death and reduction of tumor burden undoubtedly plays a very significant role in eradication of disease following administration of radiation therapy, it cannot be ignored that almost inevitably some cancer cells will remain viable after treatment.

It is tempting to introduce a sixth R of radiobiology into the discussion of appropriate dose fractionation to enhance the tumor killing: for example *reactivation* of anti-tumor immune responses as proposed by Boustani et al (188). Total-body irradiation, as is used in preparation for organ transplantation, has clearly suppressive effects to the degree that about 3.5-4.5 Gy can inhibit the body response to new antigens by ablating lymphocyte populations (129). However, in localized therapy tumor irradiation can set the stage for an influx of potent immune cells able to take advantage of the newly altered environment.

1.2.4.1 Activation of cGAS-STING

As mentioned previously, radiation responses frequently depend on the ability of the host to form a CD8+ T cell response to tumor antigens (21, 124-128, 189-191); a key driver of this response is intratumoral secretion of type I interferon (IFN-I) following activation of the cGAS-STING pathway.

In eukaryotic cells, DNA is typically restricted to the cell nucleus and nucleic acid sequences are transmitted through the cytosol with messenger RNA transcripts; DNA in the cytoplasm is therefore sensed as pathogen invasion or host damage. In the cytosol, nucleic acids may be sensed through retinoic acid-inducible gene I (RIG-I)-like receptor family members including RIG-I, MDA5 and LGP2 (192), ERAdP (193), and in the case of double-stranded DNA cGAMP synthase (cGAS) (194, 195) upstream of the secondary

messenger receptor STING (196). The cytosolic DNA sensor cGAS, upon ligation with double-stranded DNA, produces from GTP and ATP the secondary messenger molecule [G(2'-5')pA(3'-5')p] (cGAMP), a non-canonical cyclic dinucleotide (197). Cyclic dinucleotides, either 2'3'-cGAMP from cGAS or cyclic di-GMP or 3'3'-cGAMP secreted by intracellular bacteria, trigger conformation changes in the ER resident protein STING (198), which in turn activates the transcription factor interferon regulatory factor 3 (IRF3) through the protein kinase TANK-binding kinase 1 (TBK1) (196, 199). Most notably, activation of STING and IRF3 lead to production and secretion of IFN-I, namely IFNβ (200).

Activation of ISGs through STING leads to the production and secretion of proinflammatory type I IFNs α and β and binding of IFNs to the interferon-alpha/beta receptor (IFNAR) on the cell surface. For cells secreting IFN-I or bystander cells, ligation of IFNAR can trigger upregulation of antigen processing and presentation on major histocompatibility complexes (MHC-I and MHC-II) (201-204), can enhance T cell proliferation and activation by providing additional Signal 3-type signaling (54), and for dendritic cells specifically can enhance maturation signals and expression of CD40, CD80, and CD86 (205, 206). IFNAR/IFN-I signaling is critically important for regulation of the immune response to pathogen invaders, playing additional roles in the production of anti-pathogenic effector molecules and chemokines (207-209), and has been demonstrated to play an equally essential role in the efficacy of some anti-cancer therapies including radiotherapy (125). This may be in part due to the enhancement of T cell recruitment to interferon-inflamed tumors via production of CXCR3 ligands including IP-10 (CXCL10) (128). T cell activation frequently occurs in secondary lymphoid organs such as lymph nodes rather than at the site of infection or within tumors: this process, termed *cross presentation*, is described further in a later section. In order for activated T

cells to exact cytotoxic function they must then traffic from the lymphoid organ into the peripheral blood and be actively recruited by chemokines into inflamed tissue. Notably, T cell recruitment is not antigen-specific and following irradiation, TCR diversity has been observed to increase, indicating the recruitment of a wide variety of potentially reactive T cells (210, 211).

Following cell irradiation, as previously described, DSBs form in chromosomal DNA leading to the formation of micronuclei. During subsequent rounds of cell replication, micronucleus membranes are frequently disrupted (212), exposing the DNA content to cytoplasmic sensors. Cancer cells with micronuclei can respond to damaged DNA themselves (194, 195), or they can transfer it to tumor infiltrating phagocytes (213, 214). Significantly, activation of STING following radiotherapy promotes the induction of effective anti-tumor immunity in numerous tumor models (125, 194, 214, 215), demonstrating the key role of cytosolic sensing of damaged DNA downstream of tumor irradiation. The induction of IFN-I in the radiation-induced anti-tumor immune response appears to be essential but its role is unclear and may be multifaceted, from enhancing dendritic cell functions to increasing antigen presentation on cancer cells (discussed further in Chapter 3) (201, 216).

1.2.4.2 Vaccination *in situ*

Radiation has long been used as a local therapy in the treatment of cancer but observations that radiation can (rarely) contribute tumor regression outside of the field, known as the *abscopal effect*, highlight that irradiation of one site can have systemic effects transmitted by the immune system (217, 218). Leaders in the field of radiation immunology including Sandra Demaria and Silvia Formenti have built a case that radiation-induced vaccine effects *in situ* are a key mediator of tumor clearance *in vivo* (219, 220).

Effective vaccination requires a source of antigen, in the case of T cells necessarily a unique peptide, and an adjuvant to stimulate a pro-inflammatory adaptive immune response rather than a short lived “clean up” response. Thus, radiation-induced vaccination effects are thought to result in part from the generation of danger-associated molecular pattern (DAMP) signals by damaged or dying cells following irradiation, including cell surface exposure of endoplasmic reticulum (ER) resident chaperones calreticulin (CRT) and Erp57, translocation of the nuclear protein HMGB1, and extracellular release of ATP (221-223) which function as immunological adjuvants. Additionally, release of tumor-associated DNA and induction of STING signaling in phagocytes appears to play a significant role in the radiation-induced CD8+ T cell response (125, 214). Along with the uptake of tumor associated antigens, these damage signals may drive dendritic cell maturation, trafficking to tumor draining lymph nodes (TDLNs), and activation of tumor-reactive CD8+ T cells (126, 190, 224-226). Following tumor irradiation *in vivo*, the number and proportion of activated tumor-reactive CD8+ T cell increases in the TDLN, indicating the activation of a vaccination response (126, 227).

While radiotherapy is a localized therapy that primarily modulates immunity within the field of radiation, i.e. the tumor, there is some evidence to suggest that sufficient generation of effector T cells downstream of tumor radiation may be able to boost anti-tumor responses systemically to control metastatic disease (228). At the beginning of this thesis project, radiation-mediated vaccination was the dominant mechanistic rationale for synergy between radiotherapy and the immune system. In Chapter 2, we address the question of whether vaccination effects might be responsible for radiation-mediated tumor control by CD8+ T cells in mouse models of cancer.

1.2.4.3 Augmentation of Cancer Cell Susceptibility to Control by T Cells

In addition to generating CD8+ T cells able to respond to tumor-specific neoantigens through *in situ* vaccination effects and enhancing recruitment of these cells to the tumor through the production of pro-inflammatory chemokines and cytokines, radiation can alter the susceptibility of tumors to killing by T cells. Among the most compelling models for increased sensitivity of cancer cells to T cell-mediated killing is the upregulation of MHC-I molecules due to the integral role of MHC-I in directing CD8+ T cells. It is clear that radiation increases the expression of MHC-I on cancer cells (201, 229, 230) and specifically presentation of tumor-associated neoantigens (227). Proposed mechanisms of radiation-induced MHC-I upregulation are discussed in the following section and the significance of radiation-induced upregulation of MHC-I is explored in Chapter 3.

1.3 Major Histocompatibility Complex I (MHC-I)

As described previously, CD8+ T cells recognize cognate antigen only when it is bound to MHC-I molecules. This phenomenon, known as *MHC restriction*, was first realized by Rolf Zinkernagel and Peter Doherty when they found immune spleen cells (T cells) could only lyse lymphocytic choriomeningitis virus (LCMV) infected cells if the MHC-I haplotype from the source matched (24, 231). CD8+ T cells require antigen sources (proteins or protein fragments) to be processed internally for presentation: typically only a small fragment of the protein, an 8-10 amino acid long peptide chain, is presented on MHC-I. This model of recognition is unique and contrasts, for example, with antigen recognition by B cells, another member of the adaptive immune system, which can bind intact exposed antigen regardless of context (22).

MHC-I molecules are glycoproteins expressed on all nucleated cells in most vertebrates with the essential immune function to present up-to-date samplings of intracellular proteins to CD8+ T cells. The constant cycling of peptides on MHC-I allows the immune system to survey tissue for novel peptides resulting from infection, predominantly from intracellular viruses, and genetic mutations. This process provides the immune system with a mechanism of detection for potentially carcinogenic mutations by allowing recognition of novel peptide sequences resulting from altered gene sequences as neoantigens (genetic mutations are not directly surveilled by immune cells). Thus, antigen presentation on MHC-I plays an essential role in the recognition of cancer cells by the adaptive immune system.

1.3.1 Antigen Processing and Presentation on MHC-I

Antigen presentation on MHC-I is a tightly regulated process that has been progressively described over the more than 80 years since the discovery of the *H-2 antigen* in mice and more than 60 years since the discovery of its equivalent, the *HLA complex*, in humans. The original studies of major histocompatibility molecules described their function first in the context of transplantation, where different MHC alleles caused rejection of a transplanted tumor in mice or febrile response to blood transfusions in humans (232). Since these early discoveries, much has been elucidated about the structure and significance of MHC-I in health and disease.

1.3.1.1 MHC-I Structure

MHC-I molecules are composed of two subunits: the polymorphic heavy chain, known as H2 in mice and HLA in humans, and the monomorphic light chain β2-microglobulin (β2M) whose structure is also species-specific. The MHC-I heavy chain is composed of a long polypeptide strand with three domains, α1 and α2 which compose the antigenic peptide binding superdomain, and c-terminal α3 which contains the transmembrane domain and β2M binding domain. The superdomain consists of eight antiparallel β sheets supporting two α helix structures, one contributed by each α1 and α2: together, these helix structures form the walls of the peptide binding groove (12). MHC-I complexes present peptides of 8-10 amino acids in length, although 5-10% of bound peptides may exceed this length and extend past the edge of the binding cleft (23). Polymorphism within the superdomain is responsible for variable antigenic peptide binding affinities to different MHC-I haplotypes; this variability is thought to decrease the susceptibility of an entire population to an infectious agent by increasing the chance that some individuals will bind and present immunogenic peptides (12).

1.3.1.2 Antigen Presentation Pathways

Assembly of MHC-I complexes occurs in the endoplasmic reticulum (ER) following translation of the MHC-I heavy chain (HC) and β2M into the ER membrane. Once formed, HC molecules stabilize by associating with the membrane-bound chaperone calnexin (CXN), commonly bound to Erp57. Once bound to β2M, the HC swaps CXN for the soluble chaperone calreticulin (CRT) bound to Erp57: notably, CXN and CRT are also involved in quality control for the glycosylation of the MHC-I HC (12, 233). Together this complex associates with the peptide loading complex (PLC), described below (12).

1.3.1.2.1 Generation, Presentation, and Recognition of Antigens

In the absence of inflammation, candidate peptides are processed by the constitutively expressed 26S proteasome, a large barrel-shaped proteolytic complex made up a 20S subunit flanked by two 19S subunit “lids.” Proteins which are damaged, aged, or misfolded are tagged for degradation by the addition of ubiquitin (Ub) molecules by a cascade of E1, E2, and E3 Ub ligases. PolyUb chains of about four Ub moieties allow binding of the substrate to receptor components of the 19S regulatory particle (RP) subunit known as the proteasome lid; these Ub-tagged proteins are deubiquitinated and unfolded by ATPases by the RP as they pass into the 20S catalytic particle (CP). The CP consists of four stacked rings, two outer α rings and two inner β rings, each made up of seven protein subunits (12, 234, 235). Within the β rings are six catalytic active sites (β1, β2, and β5 on each ring) which cleave peptide bonds to ultimately form polypeptide chains 3-22 residues long: some portion of these peptides go on to become presented at antigens on MHC-I (236, 237).

In the context of inflammatory cytokine stimulation (including by IFN-I, IFNγ, and TNFα), alternate proteasome subunits are produced to favor processing of peptides appropriate for antigen presentation, generating a modified structure is known as the

immunoproteasome (235, 238). Within the 20S proteasome subunit rings β1, β2, and β5 are replaced by β1i (LMP2), β2i (MECL-1), and β5i (LMP7) which associate more rapidly than constitutive 20S components to preferentially assemble the immunoproteasome complex (235). In place of the 19S lid, the IFNγ proteasome activator PA28 can associate with the immunoproteasome to enhance the generation of MHC-I binding peptides; although this subunit is not essential for function it appears to enhance the yield of peptides able to bind MHC-I and may reduce generation of polypeptide chains too short for binding (12, 235, 239). Overall, immunoproteasome subunits demonstrate enhanced cleavage after hydrophobic and basic amino acid resides, favoring generation of peptides with C-terminal residues preferred for binding by many MHC-I alleles (12).

In order to be presented on MHC-I, peptide chains generated by the proteasome/immunoproteasome must first enter the ER through the transporter associated with antigen processing (TAP), a heterodimeric complex made up of TAP1 and TAP2. TAP transports peptide chains 8-12 amino acids long most efficiently but has been observed to transport chains as long as 40 residues long (12, 240); protein strands which are too long are trimmed once in the endoplasmic reticulum at the N-terminal end to an appropriate length for MHC-I presentation by the ER-associated aminopeptidase (ERAAP) ERAP1. TAP is an essential subunit of the PLC and associates with MHC-I/CRT/Erp57 via binding to tapasin (Tpn). Tpn stabilizes the open peptide-binding groove of MHC-I until a peptide of appropriate affinity binds and initiates the final folding of the ligand-binding groove; this stabilization of the structure releases MHC-I from the PLC and triggers export of the fully assembled complex through the Golgi apparatus to the cell surface for extracellular antigen presentation (12).

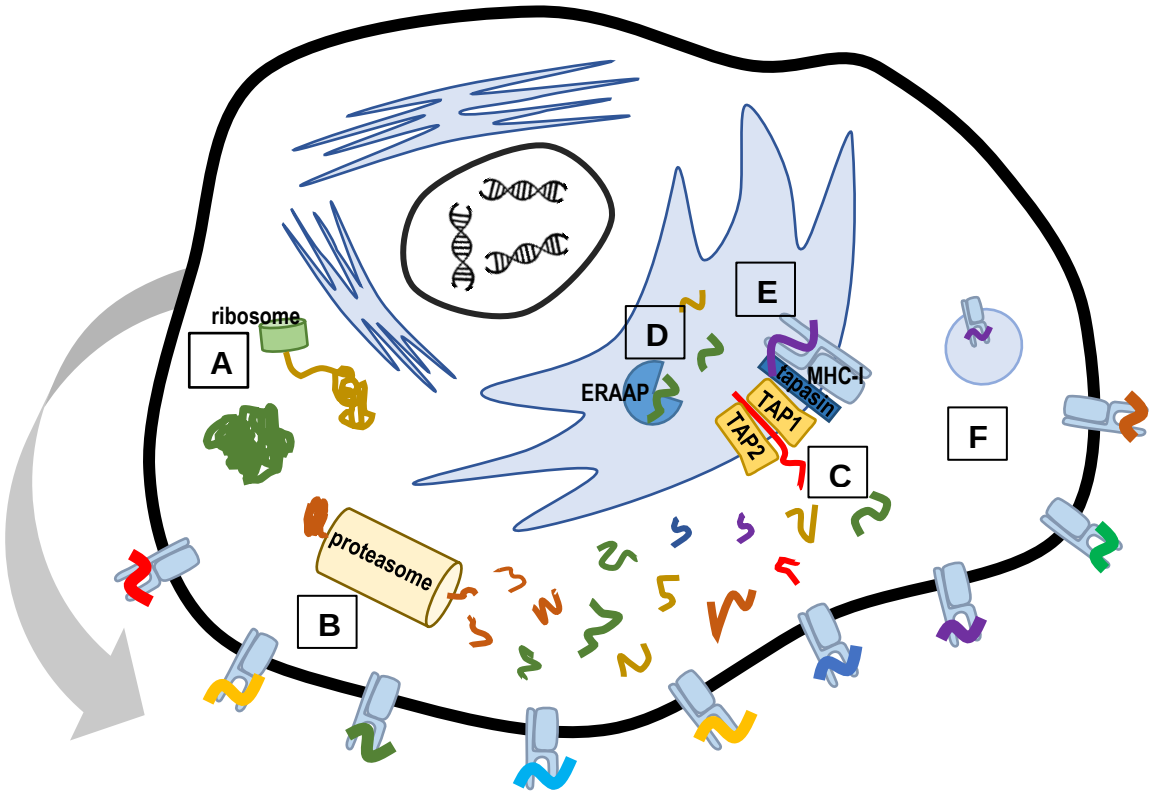

Fig. 1-2 Antigen processing and presentation on major histocompatibility complex class I (MHC-I). A) Ribosomes in the cytosol or endoplasmic reticulum (ER) synthesize new proteins, up to 30% of which are considered defective and are tagged with ubiquitin substrates for refolding or degradation. B) Defective ribosomal products, old proteins, and damaged proteins are degraded into polypeptide fragments by the proteasome or, in the context of inflammatory stress, the immunoproteasome. C) Peptide fragments can be further degraded or can be transported in an ATP-dependent manner into the ER for MHC-I presentation through heterodimeric channels made up of TAP1 and TAP2 protein subunits which favor the transport of polypeptides 8-12 amino acids long. D) Peptide fragments within the ER can be further clipped for optimal protein loading by ERAAP. E) Tapasin proteins stabilize the open peptide-binding groove of MHC-I molecules to allow for cycling of polypeptides until one of sufficient affinity stabilizes the complex and initiates dissociation from the peptide loading complex. F) Stable peptide-MHC-I complexes (pMHC) are exported from the ER and presented at the cell surface for recognition by immune cells.

The T cell receptor (TCR) interacts with MHC-I complexes bound to peptide (pMHC) via the variable regions of the subunits TCRα and TCRβ, known respectively as Vα and Vβ. At the terminal end of the Vα and Vβ structures are six complementarity-determining regions (CDRs), CDR1, CDR2 and CDR3 for each the α and β chains, forming peptide loops which bind the pMHC; CDR3 contains the greatest polymorphism derived from V(D)J recombination and thus the CDR3α and CDR3β loops are responsible for antigen recognition within the MHC peptide binding groove (12, 23, 241).

1.3.1.2.2 Cross Presentation

Among antigen presenting cells, dendritic cells (DCs) have the unusual ability to present exogeneously-derived protein antigens on MHC-I in a process termed *cross presentation*. Cross presentation allows DCs to be *licensed* by CD4+ T helper cells through ligation of DC-expressed peptide-MHC class II (pMHC-II) complexes, resulting in upregulation of costimulatory (Signal 2) molecules, and to prime naïve CD8+ T cells via ligation of cross-presented pMHC-I. Notably, some subsets of DCs are better able to cross present than others, the most efficient of which appears to be Batf3-dependent CD8+ XCR1+ DCs also known as inflammatory DCs or DC1s (242, 243).

DCs sample their surroundings through a process of phagocytosis, engulfing extracellular fragments in a membrane-bound vesicle called the phagosome. Typically, phagosomes undergo a process of acidification through fusion with lysosomes called maturation, leading to the destruction of microbial contents; however, in the presence of immune adjuvants such as TLR ligands, DCs slow the process of acidification to favor preservation of antigen structure (37). Upon receiving adjuvant signals, DCs undergo a process of *maturation*, downregulating phagocytosis and presentation of activation receptors while upregulating expression of costimulatory molecules (CD80/86) and

chemokine receptors responsible for lymph node trafficking (244). Significantly, T cells stimulation by DCs in the steady state (i.e. DCs not stimulated by inflammatory signals) results in T cell tolerance (12, 245).

Protein antigens preserved from the phagosome can enter the ER for protein loading through either of two pathways, the first of which is the vacuolar pathway. In the vacuolar pathway, peptides degraded within acidified phagosomes are loaded onto post-Golgi MHC-I molecules in a TAP-independent manner. This process requires phagosome-derived peptides to outcompete peptides loading through the classical TAP-dependent pathway and occurs most efficiently in TAP-expressing cells (242). In the second pathway for ER trafficking, the cytosolic pathway, protein fragments are exported from the phagosome into the cytosol either through an unknown transport channel protein or through disruption of the phagosomal membrane (242). Once in the cytosol, exogenously-derived proteins are processed through the classical proteasome- and TAP- dependent antigen processing pathway and are loaded onto MHC-I as described previously (246).

Significantly, antigen presentation on APCs is essential for priming optimal CD8+ T cell responses but antigen presented in the context of a DC does not guarantee elimination/killing of the original source of the antigenic protein. This means that even in cases where a vaccine generates a potent CD8+ T cell response through presentation of tumor antigens on cross presenting DCs, cancer cells can still evade immune detection through downregulation of MHC-I antigen presentation pathways. Known mechanisms of MHC-I evasion are discussed further in this section.

1.3.2 Genetic Regulation of MHC-I

MHC-I presentation requires the simultaneous expression of multiple genes in the same cells with many of these genes clustered together in the *Mhc* locus. In humans, the *Mhc* gene region is located on the short arm of chromosome 6 and is subdivided into the MHC-II region, MHC-III region, and MHC-I region; the MHC-I region contains genes coding for classical HLA-A, HLA-B, and HLA-C heavy chain molecules as well as non-canonical HLA heavy chains. In mice, the *Mhc* gene region is located on chromosome 17 and is similarly divided into regions by MHC class; however, in rodents a portion of the MHC-I region has been translocated so from the centromere the gene regions order: MHC-I, MHC-II, MHC-III, and MHC-I. In mice, the classical heavy chain molecules are H-2D, H-2K, and H-2L. For both mice and humans, non-MHC genes which are integral to antigen presentation on MHC-I are also contained within the *Mhc* region, including the genes for tapasin, TAP1, TAP2, and LMP7; however, in both humans and mice the gene for β2m is located on a separate chromosome (chromosome 15 and 2, respectively) (12).

Expression of classical MHC-I elements is mediated by three major regulatory elements: enhancer A, IFN-stimulated response element (ISRE), and the SXY module. Within MHC-I promoters, enhancer A elements are bound by NF-κB/rel family members and ISRE elements are bound by interferon regulatory factors (IRFs) including IRF1; these transcription factors notably mediate transcription of MHC-I proteins downstream of IFNγ and TNFα stimulation (247-249). The SXY module, comprised of S/W, X1, X2 and Y boxes, binds a number of nuclear factors including RFX (comprised of RFX5, RFXAP, and RFXANK/B), CREB/ATF, and NF-Y, which require a transcriptional regulator, either the Class I Transactivator (CITA, or NLRC5) or the Class II Transactivator (CIITA), to coordinate enhanceosome assembly (247, 250, 251).

NLRC5 and CIITA are NOD-like receptor (NLR) family members, a group of proteins characterized by common structural elements including central nucleotide binding domains (NBD, a component of a NACHT domain) and C-terminal leucine-rich repeat (LRR) domains (252). While the majority of research on NLRs has focused on those with pattern recognition or inflammasome activities in the cytosol, NLRC5 and CIITA primary target the nucleus and are known as master transcriptional regulators of MHC-I and MHC-II, respectively. Notably, NLRC5 and CIITA lack DNA-binding protein domains and can only exert coactivating function through binding to DNA-binding adaptor proteins (253).

CIITA was first described as a key MHC-I transactivating element in the early 1990s after being found defective in a form of MHC-II deficiency known as Bare Lymphocyte Syndrome (BLS) (254), a rare and heterogeneous condition grouping mutations in CIITA and members of the RFX protein complex. The role of CIITA in controlling MHC-I expression was less clear, however, and complicated by the overlapping adaptor proteins utilized by both the CIITA and NLRC5 enhanceosomes: in human patients with BLS, MHC-I expression may also be significantly reduced (255-257). CIITA was found to contribute to transcription of β2M and MHC-I heavy chains but not TAP1 or LMP2 (253, 257) and is not critical for MHC-I expression in the same way it is for MHC-II (258, 259).

After the discovery of NLRC5 as a regulator of MHC-I in cell lines (259), the relative contributions of NLRC5 and CIITA to MHC-I expression were best elucidated in genetic knockout mouse models. In these models, the contribution of NLRC5 or CIITA to MHC-I expression depends predominantly on cell type, where cells with high basal CIITA expression (APCs, including DCs and macrophages) are more resistant to MHC-I abrogation by NRLC5 deficiency than cells with low basal CIITA expression

(lymphocytes, including B cells and T cells) (250, 258, 260). Notably, expression of NLRC5 can be induced by IFNγ and, accordingly, NLRC5 deficiency reduces IFNγ-mediated induction of MHC-I; however, it does not abrogate it (261), demonstrating regulation of MHC-I expression is layered with partially overlapping mechanisms.

1.3.3 Tumor Suppression of MHC-I as a Mechanism of Immune Evasion

Tumor evolution is shaped by positive selection of genes advantageous to tumor growth and negative selection of genes inhibiting growth or enhancing immune-mediated control; due to the potency of adaptive immunity in detecting and controlling tumors, downregulation of antigen presentation on MHC-I is a common immune evasion mechanism employed by tumors (9). While decreased antigen presentation is a common feature of cancer, total loss of MHC-I expression (i.e. biallelic loss of B2M) is less common (262), likely due to selective pressure from natural killer cells whose cytotoxic function is inhibited by the presence of MHC-I. Antigen presentation on MHC molecules can be reduced without total ablation by epigenetic suppression or genetic loss of factors regulating MHC-I expression (e.g. NLRC5) (263-265), downregulation of molecules involved in peptide loading onto MHC-I (266-268), loss of specific HLA alleles (269), or suppression of cytokine-activated pathways for augmenting MHC-I expression (e.g. loss of IFNγR/IFNAR or downstream JAK/STAT molecules) (270-272).

Significantly, reduction of cognate pMHC expression on target cell surfaces can affect the magnitude and efficacy of CD8+ T cell responses (273); thus, rescue of MHC-I expression is a significant target for cancer immunotherapy. In cases where total genetic loss of B2M prevents augmentation of MHC-I expression, T cell enhancing therapies are

unlikely to effective and use of immunotherapy must be tailored to take this into account (270, 271).

1.3.4 MHC-I Upreguation on Cancer Cells by Radiation

Due to the common immune evasion tactic of cancer cells to downregulate antigen presentation on MHC-I, it is appealing to explore cancer therapies able to enhance visibility of these cells to tumor-reactive CD8+ T cells. As discussed previously, irradiation of cancer cells can increase their susceptibility to T cell-mediated control, including by upregulation of MHC-I expression. Multiple mechanisms have been proposed for MHC-I induction following radiation which may occur simultaneously.

In 2006, Eric Reits and colleagues proposed that MHC-I expression after radiation is enhanced due to increased availability of intracellular peptides available for loading (229). In their model, damage sustained by irradiation triggered activation of the key metabolic kinase mTOR and increased transcription of genes related to the damage response, including DNA repair genes. The increase in bulk transcription following a spike in damaged proteins generated by irradiation triggered an increase in proteins processed through the proteasome/immunoproteasome and thus higher concentrations of peptides available for TAP-dependent ER transport and loading onto MHC-I molecules.

An alternate mechanism of MHC-I induction by cancer cell irradiation is dependent on activation of the cGAS/STING and IFN-I pathways. As described previously, cancer cell irradiation triggers the release of dsDNA into the cytosol, triggering activation of the cGAS/STING pathway and triggering extracellular release of IFN-I including IFNβ (194). Ligation of IFNAR with IFN-I triggers downstream signaling

via STAT1, STAT2 and IRF9 and leads to transcription of ISGs including MHC-I related proteins (201, 274); in some cases, upregulation of MHC-I may be entirely dependent on IFN signaling (275).

The significance of radiation-induced upregulation of MHC-I on MHC-I^{lo} cancer cells is unclear, although augmentation of MHC molecules has been used as justification for studies of cancer immunotherapy (276, 277). In Chapter 3, I explore mechanisms of radiation-induced upregulation of MHC-I and the significance of MHC modulation on cancer cells to CD8+ T cell responses.

Chapter 2: Establishment of the significance of *in situ* vaccination by radiation

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2.1 Abstract

It has been established in numerous cancer models that radiation-driven tumor clearance requires the presence of CD8+ T cells, but how radiation drives these T cell responses is unclear. In these studies, we built on previous work in our lab examining the role of radiation as an *in situ* T cell vaccine, which found that radiation required the pre-existing tumor immunity generated by tumor inoculation to exert tumor control. Thus, we hypothesized that radiation might function to enhance CD8+ T cell responses by boosting existing T cell numbers. For these experiments a live-attenuated *Listeria monocytogenes* vaccine platform expressing tumor model antigens was used to generate large tumor-reactive CD8+ T cell populations in the absence of tumor irradiation. we demonstrated that while T cells generated by *Listeria* vaccination traffic to tumors, recognize antigen *in situ* and have cytotoxic potential, the generation of antigen-reactive CD8+ T cells is not sufficient to replicate the efficacy of radiation in controlling tumors. These experiments suggest that radiation functions poorly as an *in situ* vaccine and that other mechanisms are necessary to explain how radiation improves tumor susceptibility to control by CD8+ T cells.

2.2 Introduction

Vaccines capable of stimulating strong T cell responses require an antigen capable of being recognized by the host's T cells and an adjuvant capable of stimulating antigen-presenting cells (APCs) to take up and present the antigen to antigen's cognate T cells (22). In the field intersecting radiation biology and immunology, the ability of tumor irradiation to stimulate adaptive immunity by T cells is under investigation. It is clear that the irradiation of tumor tissue results in the release of tumor-associated antigens (TAAs) and strong inflammatory damage signals (DAMPs) which can act as immunological adjuvants; whether radiation-induced vaccination is necessary or sufficient to immune-mediated tumor clearance is therefore of interest.

Previous work from our lab demonstrated that the efficacy of radiation in combination with checkpoint inhibitors (antibody blockade of CTLA-4 and PD-1/PD-L1) requires the initial T cell priming event of tumor inoculation (124): silencing the initial activation of T cells at tumor challenge by CD8+ T cell depletion, blockade of dendritic cell (DC) maturation by αCD40L (278), or blockade of T cell egress through lymphatic vessels by FTY720 (279) resulted in the failure of therapy. Blockade of *de novo* T cell priming at the time of radiation, conversely, does not significantly affect therapeutic responses. From these results, we found that T cell priming resulting from tumor irradiation is not required for tumor clearance by radiation and checkpoint inhibitors.

These studies did not address, however, the significance of radiation-mediated CD8+ T cell *boosting*, and whether this effect might be able to account for the ability of radiation to initiate immune-dependent control of tumors. Efficient priming of CD8+ T cells, as described in Chapter 1, requires the presence of cognate antigen on presented on MHC-I (Signal 1), costimulation by mature dendritic cells (Signal 2), and pro-inflammatory cytokines triggering proliferation (Signal 3). Following an acute priming

event, the effector T cell population contracts and a small population of cells become long-lived memory T cells; this population can then be reactivated, or *boosted*, to re-expand and control the antigen source. It is unclear whether this reactivation of tumor-specific CD8+ T cells might account for the efficacy of T cells in controlling tumors following irradiation.

In order to determine whether radiation-mediated CD8+ T cell boosting can explain immune control of tumors, we modeled radiation-mediated *in situ* vaccination with a live-attenuated *Listeria monocytogenes* vaccine (280) expressing tumor model antigens. This vaccine is capable of generating significantly greater CD8+ T cell numbers than radiation-induced vaccination systemically, but these cells are unable to control tumors. Instead, we found that cancer cells are intrinsically resistant to killing by T cells but that radiation directly improves the susceptibility of cancer cells to immune control. In Chapter 3, I describe experiments exploring how radiation might augment the ability of CD8+ T cells to control cancer.

2.3 Materials and Methods

Animals and Cell Lines

Animal protocols were approved by the Earle A. Chiles Research Institute IACUC (Animal Welfare Assurance No. A3913-01). All experiments were performed in accordance with relevant guidelines and regulations. 5-8 week old C3H/HeJ mice (Stock #00659) and 5-8 week old C57BL/6 mice (Stock #000664) were purchased from the Jackson Laboratory (Bar Harbor, ME) for use in these experiments. Nur77^{GFP} reporter mice were kindly provided by Dr. Weinberg (Earle A. Chiles Research Institute, Portland, OR)(281). 2C transgenic mice were kindly provided by Dr. Gajewski (University of Chicago, Chicago, IL), bred in-house and crossed with Nur77^{GFP} reporter mice. OT-I transgenic mice were gifted by Dr. Redmond (Earle A. Chiles Research Institute). Pdx-Cre mice (Stock #014647, Jackson Laboratory) were crossed with B6.129S4-Gt(ROSA)26Sor$^{tm3(CAG-luc)Tyj}$/J (Stock #009044, Jackson Laboratory) to generate animals tolerant to SIYRYYGL peptide. B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ mice expressing CD45.1 were obtained from the Jackson Laboratory (Stock#002014) for *in vivo* cytoxicity assays. Survival experiments were performed with 3-8 mice per group as noted in figure legends.

Cell lines were cultured in RPMI-1640 (HyClone, Fisher Scientific, Hampton, NH) supplemented with 10% heat inactivated fetal bovine serum (Cat#10082147, Thermo Fisher Scientific, Waltham, MA), 2mM L-glutamine (Cat#SH3003401, HyClone, Fisher), 10mM HEPES (Cat#HOL06, Caisson Labs, Smithfield, UT), 100U/mL penicillin-streptomycin (Cat#PSL01, Caisson), 1X non-essential amino acids (Cat#SH3023801, Fisher), 1mM sodium pyruvate (Cat#PYL01, Caisson).

The parental squamous cell carcinoma line SCCVII was kindly provided by Walter T. Lee (Duke Cancer Center Institute, Durham, NC). To generate SCCVII cells expressing EGFRvIII, cancer cells were transfected using Lipofectamine 2000 Transfection Reagent (Life Technologies, Carlsbad, CA) with MSCV-loxp-dsRed-loxp-eGFP-Puro-WPRE (Addgene plasmid #32702, gifted from Hans Clevers) and MSCV-XZ066-EGFRvIII (Addgene plasmid #20737, gifted from Alonzo Ross) and sorted on a BD FACSAria II cell sorter (Becton Dickinson, Franklin Lakes, NJ) for high expression of GFP (SCCVII-EGFRvIII) and RFP (SCCVII-control) as previously described (280). The parental murine pancreatic adenocarcinoma cell line Panc02 was kindly provided by Dr. Woo (Mount Sinai School of Medicine, New York, NY). Panc02 expressing the model antigen SIY was kindly provided by Dr. Weishelbaum (University of Chicago, Chicago, IL), as used previously (124), and expresses GFP-SIY in approximately 40% of cells. Panc02SIY100 was derived and expanded from a high GFP expressing single clone within Panc02SIY on a BD FACSAria II cell sorter.

Antibodies and Reagents

Viability staining was performed in PBS using Zombie Aqua Fixable Viability Kit (BioLegend, San Diego, CA) for 15 minutes prior to staining with fluorescently-conjugated antibodies for flow cytometry. Monoclonal antibodies were used against: CD3 [17A2], CD4 [RM4-5], CD8α [53-6.7], CD11b [M1/70], CD45.1 [A20], CD45.2 [104], CD69 [H1.2F3], CD90.1 [HIS51], IFNγ [XMG1.2], H-2Kb [AF6-88.5.5.3], and TNFα [MP6-XT22]. Fluorescently-conjugated MHC-multimer complexes were used as follows: tetramer-EEKKGNYV (tEGFRvIII, NIH Tetramer Core, Atlanta, GA), tetramer-SIINFEKL (tOva, NIH Tetramer Core), and pentamer-SIYRYYGL (pSIY, ProImmune, Sarasota, FL).

Immunohistochemistry was performed on Zinc-fixed tumors embedded in paraffin preserved as described previously (282). Five micron sections were stained with primary αCD3 (SP7, Cat#ab16669, Abcam, Burlingame, CA) diluted in blocking buffer, secondary goat anti-rabbit IgG conjugated to HRP (Cat #AP1879, EMD Millipore, Burlingame, MA), and ImmPACT DAB Peroxidase (HRP) Substrate (Cat#SK-4105, Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin 7211 (Cat#S7439-1, Cardinal Health, Dublin, OH). CD3 infiltration was quantified using Aperio ImageScope (Aperio, Sausalito, CA).

For *in vitro* stimulation of cell lines, recombinant mouse IFN-gamma protein (Cat#14-8311-63, Thermo Fisher) was used at a final concentration of 20ng/mL.

The *in vivo* cytotoxicity experiment was conducted as described previously(283). Briefly, wild-type mice were vaccinated with *LmSIY* or *LmOva* seven days prior to injection of congenic splenocytes labelled with CFSE (Cat#C34554, Thermo Fisher) and pulsed with peptide (A&A Labs, San Diego, CA). Six hours later, recipient spleens are harvested and analyzed by flow cytometry.

Listeria monocytogenes **vaccination**

ActA deleted (***ΔactA***) *Listeria monocytogenes* (*Lm*) strains used for vaccination were engineered to express the EGFRvIII peptide EEKKGNYV (*LmEGFRvIII*) as described previously (280), the ovalbumin peptide SIINFEKL (*LmOva*), or SIYRYYGL peptide (*LmSIY*) cloned in-frame with the *actA* N-terminal fragment. Bacteria were grown in brain-heart infusion broth, washed twice in PBS and administered by retro-orbital injection at a dose of 1×10^5 (C3H mice*)* or 1×10^7 (C57BL/6 mice) CFU in 100µL total volume. Effective vaccination was confirmed seven days later by MHC-multimer binding of peripheral blood as described above.

Immunotherapy and Radiation Therapy of Tumors

Tumors were inoculated at a dose of 2 x 10^6 for SCCVII-control and SCCVII-EGFRvIII tumors, 5 x 10^6 for Panc02SIY tumors and 10 x 10^6 for Panc02SIY100. Tumor size was determined via caliper measurements of the longest length x the longest perpendicular width. Survival endpoint was defined as tumor size greater than or equal to 150mm^2 or when the mouse appeared moribund.

For *in vivo* experiments, 12 Gy of CT-guided radiation was administered to tumor isocenters using a Small Animal Research Radiation Platform (SARRP) (Xstrahl, Suwanee, GA) and Murislice software (Xstrahl), 14 days after tumor implantation. 250µg per dose αPD-L1 checkpoint blockade (Cat#BE101, BioXCell, West Lebanon, NH) was administered intraperitoneally at day 7, 14 and 21 post tumor implantation. For vaccine protection studies, *Listeria* vaccines were administered in prime-boost regimes 21 days and 7 days prior to challenge with SCCVII tumor cells; for Panc02 derivative cell lines, vaccines were administered 7 days prior to tumor challenge. For therapeutic early vaccination, SCCVII tumors were treated at 3 days post tumor challenge and Panc02 derivative tumors were treated at 6 days post tumor challenge. For studies where therapeutic *Listeria* vaccines were used in comparison with radiation the vaccines were administered at day 14 following tumor challenge to coincide with radiation controls.

Tumor analysis

For flow cytometric analysis of tumor-infiltrating cells, tumors harvested seven days after treatment were chopped into small fragments and dissociated in a solution of 250 U/mL collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ) and 30 U/mL DNase (Millipore Sigma, St Louis, MO) using a GentleMACS tissue dissociator (Miltenyi Biotec, Auburn, CA). After 30 minutes incubation at 37°C, the digest was quenched in

RPMI-1640 (Cat#SH30027LS, Fisher) supplemented with 10% FBS (Cat#16000069, Thermo Fisher) and 2mM EDTA (Cat#324504, Millipore Sigma) and strained through 100μM and 40μM cell strainers. Filtered cells were rinsed in cold PBS twice prior to counting and staining for analysis on a BD LSR II flow cytometer (Becton Dickinson).

In vitro T Cell Coculture Assays

Prior to coculture with cancer cells, donor CD8+ T cells were activated *in vivo* with *Listeria* vaccines. Seven days after vaccination, spleens were harvested and dissociated using a 70μM cell strainer and syringe plunger then red blood cells were lysed with ACK Lysing Buffer (Lonza, Basel, Switzerland). CD8+ T cells were sorted from splenocytes using a CD8α+ T cell negative isolation kit (Cat#130-104-075, Miltenyi) and counted prior to use *in vivo* or in *in vitro* cell coculture assays.

Cancer cell growth was monitored using an Incucyte (Sartorius, Goettingen, Germany) and Zoom software (Incucyte, Sartorius). Briefly, cancer cells were plated in the presence of cytokines or following *in vitro* irradiation. After 24 hours, adherent cells were rinsed twice in 10% complete RPMI supplemented with βME. Purified CD8+ T cells from *Lm*-vaccinated animals were added to cancer cell cultures at a 25:1, 50:1, and 100:1 effector:target ratios. Non-adherent cells were allowed to settle for 15 minutes at room temperature prior to hourly tracking in the Incucyte, housed at 36.5°C/5%CO_2, until untreated cells reached confluence. In order to isolate cancer cells from T cells for analysis, endogenous green fluorescence was used as a proxy for cell confluence as described in Figure 2-6A.

Statistics

Data was analyzed and graphed using FlowJo (Tree Star, Ashland, OR) and Prism (GraphPad Software, La Jolla, CA). Individual data sets were compared using Welch's T

tests. Growth curves and analysis across multiple groups were analyzed using ANOVA (two-way and one-way, respectively). Kaplan Meier survival curves were compared using log-rank tests.

2.4 Results

2.4.1 Radiation acts as an *in situ* cancer vaccine

In order to confirm that radiation can act as an *in situ* vaccine and boost systemic T cell responses as has been reported previously (284), C57Bl/6 mice were challenged with the murine pancreatic tumor cell line Panc02 expressing the model antigen SIYRYYGL, Panc02SIY. Two weeks following tumor implantation, tumors were irradiated using a CT-guided external beam radiation platform and seven days later peripheral blood was harvested to measure model antigen-reactive CD8+ T cells by fluorescent MHC-multimer binding and flow cytometry (Fig. 2-1Ai). At around 10-14 days, the initial peak in T cell numbers generated by the priming event of tumor cell inoculation has subsided and the resulting memory populations are able to be boosted by subsequent presentation of their cognate antigen. Following irradiation, we observed a significant increase in tumor-reactive CD8+ T cell numbers compared to unirradiated controls, consistent with a boosting effect driven by vaccination (Fig. 2-1Aii). Notably, however, the boosting effect by radiation was small relative to a strong T cell vaccine, *Listeria-SIY*, administered at a similar time. These results demonstrated that radiation can act as an *in situ* vaccine but that the systemic boost in tumor-reactive CD8+ T cells driven by this vaccination event was modest.

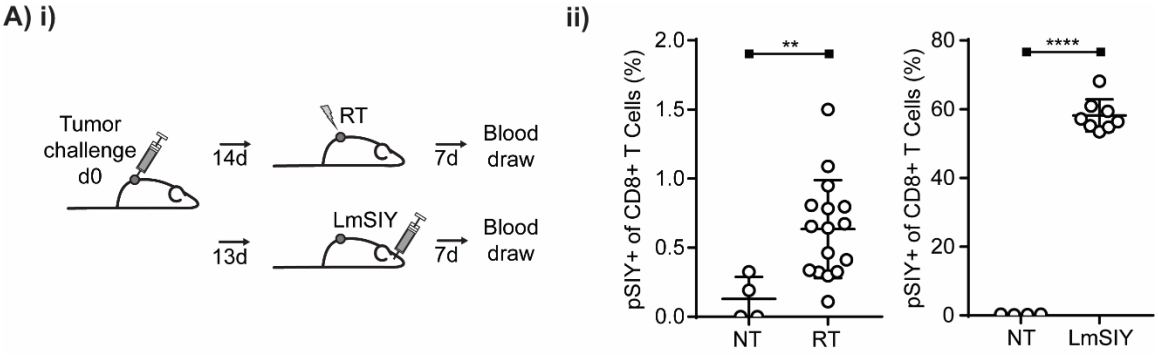

Fig. 2-1 Radiation boots tumor-reactive CD8+ T cells in circulation. A) i) Treatment schematic. C57Bl/6 mice were implanted with Panc02SIY tumors and then irradiated (12Gy) or vaccinated with *Listeria-SIY*. Peripheral blood was collected seven days after treatment from mice or matched controls. ii) Pentamer-SIY (pSIY) binding CD8+ T cells of total CD8+ T cells in peripheral blood seven days after treatment or matched in untreated (NT) controls.

2.4.2 Tumors are poorly controlled by therapeutic vaccination

In order to determine whether *Listeria*-induced CD8+ T cells were sufficient to control model-antigen expressing cancers, two subcutaneous murine tumor models were used. The first model, SCCVII-EGFRvIII, was derived from a spontaneously arising squamous cell carcinoma (285) transfected to express a rare constitutively active variant of the epidermal growth factor receptor (EGFR) found primarily in head and neck squamous cell carcinomas and glioblastomas (286, 287). Previously we had identified an $H2K^k$ restricted immunogenic peptide component of EGFRvIII, EEKKGNYV, recognized by CD8+ T cells in C3H mice (280) and transitioned a human version of this vaccine into a phase I clinical trial in patients with astrocytic brain tumors (ClinicalTrials.gov identifier: NCT01967758).

To determine whether *Listeria-EGFRvIII* (*LmEGFRvIII*)-induced T cells were able to control EGFRvIII-expressing cancer cells, mice were vaccinated with *LmEGFRvIII* prior to cancer cell inoculation and observed a significant increase in survival (Fig. 2-2A), indicating a protective antigen-specific vaccination response. In order to determine whether vaccine-induced protection was specific to SCCVII, we used a methylcholanthrene-induced murine pancreatic cancer model, Panc02 (288), transfected to express the $H2K^b$-restricted model antigen SIYRYYGL (SIY), Panc02SIY. When mice were similarly vaccinated prior to tumor challenge with *Listeria* expressing SIY (*LmSIY*), there was no significant enhancement in survival (Fig. 2-2B) or reduction in tumor establishment as there had been with the SCCVII-EGFRvIII cell line. In order to determine whether *LmSIY* failed to protect against tumor establishment due to lack of SIY expression by Panc02SIY, we examined cells for expression of the reporter protein GFP fused to the SIY peptide and determined only about 40% of cells in Panc02SIY expressed the model antigen (data not shown). To test whether *LmSIY* protected against

Panc02SIY cells homogenously expressing the model antigen, we derived a new cell line, Panc02SIY100, from a high-SIY-GFP-expressing Panc02SIY clone that was able to maintain SIY-GFP expression over multiple passages. Similarly, however, establishment of Panc02SIY100 tumors was not protected against by *LmSIY* vaccination (Fig. 2-2B), suggesting that some cancer cell lines are intrinsically resistant to control by *Listeria*-induced T cells.

In order to determine whether therapeutic vaccination is sufficient to control growth of model antigen expressing tumors, mice were challenged with SCCVII-EGFRvIII, Panc02SIY, or Panc02SIY100 and vaccinated before the tumors were palpable (Fig. 2-2C and Fig 2-2D). All three tumor types were equivalently controlled by vaccination against model antigens and control peptides. Interestingly, SCCVII-EGFRvIII growth was significantly inhibited by *Listeria* vaccination but was not differentially affected by the antigen, suggesting that SCCVII-EGFRvIII tumors are sensitive to other types of inflammation resulting from the vaccine rather than the antigen-specific T cells induced by it. Taken together, these experiments demonstrate that the tumors tested are poorly controlled by antigen-specific CD8+ T cells induced systemically by vaccination.

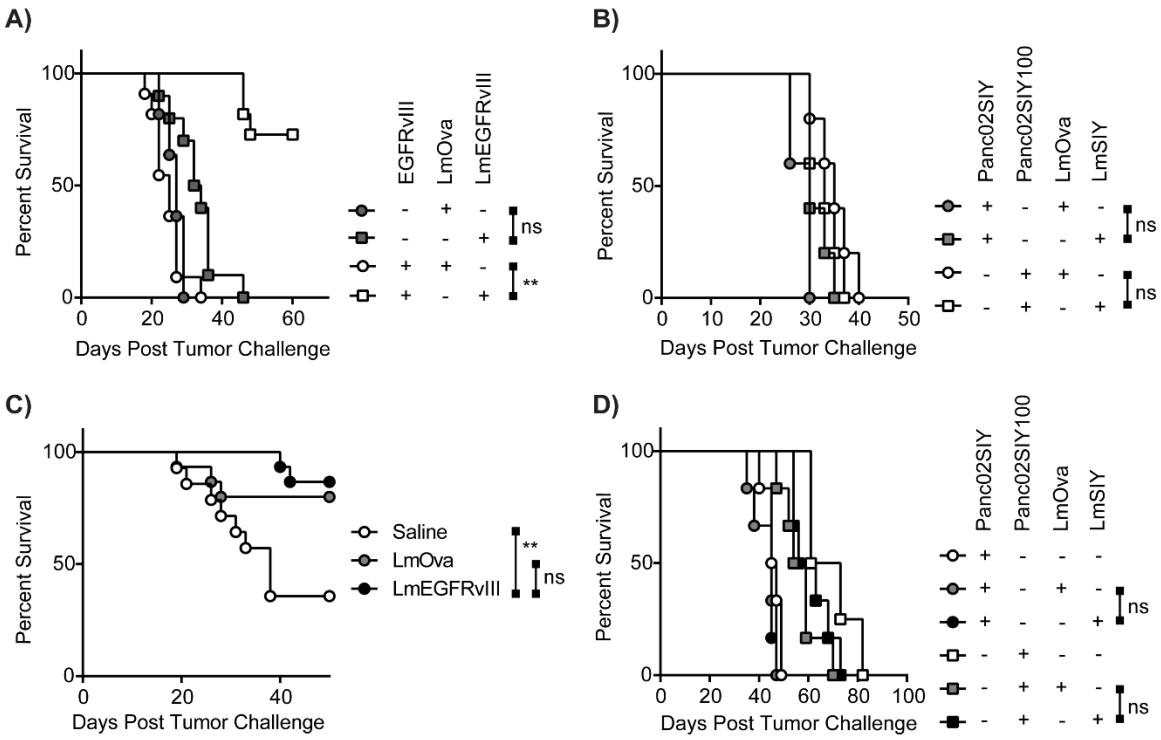

Fig. 2-2 *Listeria* vaccines can protect tumor establishment but are poorly effective therapeutically. A) Kaplan-Meier survival plot of mice vaccinated with *Listeria-EGFRvIII* or *Listeria-Ova* 21 days and 7 days prior to challenge with SCCVII cancer cells expressing EGFRvIII or control construct. B) Kaplan-Meier survival plot of mice vaccinated with *Listeria-SIY* or *Listeria-Ova* 7 days prior to challenge with Panc02SIY or Panc02SIY100 cancer cells. C) Kaplan-Meier survival plot of mice challenged with SCCVII-EGFRvIII cancer cells and treated with saline, *Listeria-EGFRvIII*, or *Listeria-Ova* 3 days after tumor challenge. D) Kaplan-Meier survival plot of mice challenged with Panc02SIY or Panc02SIY100 cancer cells and treated with saline, *Listeria-SIY* or *Listeria*-Ova 6 days after tumor challenge. Key: **p<0.01; ns = not significant.

2.4.3 Model antigen specific CD8+ T cells are necessary but not sufficient for therapeutic efficacy of combination radiation and checkpoint blockade

Previous studies have demonstrated that CD8+ T cells are required for the efficacy of radiation-induced therapeutic responses(20, 123-125) but sequencing of tumor-infiltrating T cell receptors (TCRs) following radiation has demonstrated an expansion in TCR diversity rather than clonality (210), indicating a broadening of potential tumor-reactive T cell clones and suggesting non-specific T cell recruitment. Thus, it was unclear whether the population of model antigen reactive CD8+ T cells generated by *Listeria* vaccination were analogous to the key T cell population generated by radiation. In order to determine whether the SIY-reactive CD8+ T cell population was necessary for therapeutic tumor control by radiation, we used PDX-Cre x B6.129S4-Gt(ROSA)26Sor$^{tm3(CAG-luc)Tyj}$/J mice (PDX-SIY mice) which express the SIY peptide under the PDX (pancreatic) promoter during thymic development and are unable to generate SIY-reactive CD8+ T cells (Fig. 2-3A). When wild-type (WT) and PDX-SIY mice were implanted with Panc02SIY100 tumors and treated with radiation and αPD-L1 checkpoint blockade, PDX-SIY mice were resistant to tumor control by combination therapy (Fig. 2-3B). This result indicates that SIY-reactive CD8+ T cells are necessary for the efficacy of radiation with PD-L1 blockade in this model. In order to determine whether a vaccination effect was sufficient to explain the therapeutic efficacy of radiation with checkpoint blockade, mice were implanted with Panc02SIY100 tumors and treated with radiation and αPD-L1 or *LmSIY* and αPD-L1. While a majority of mice were able to clear tumors with the radiation combination therapy, *LmSIY* and PD-L1 blockade did not perform better than PD-L1 blockade alone. These results suggest that vaccination is not sufficient to explain the ability of radiation to control these tumors in a SIY-reactive T cell dependent mechanism.

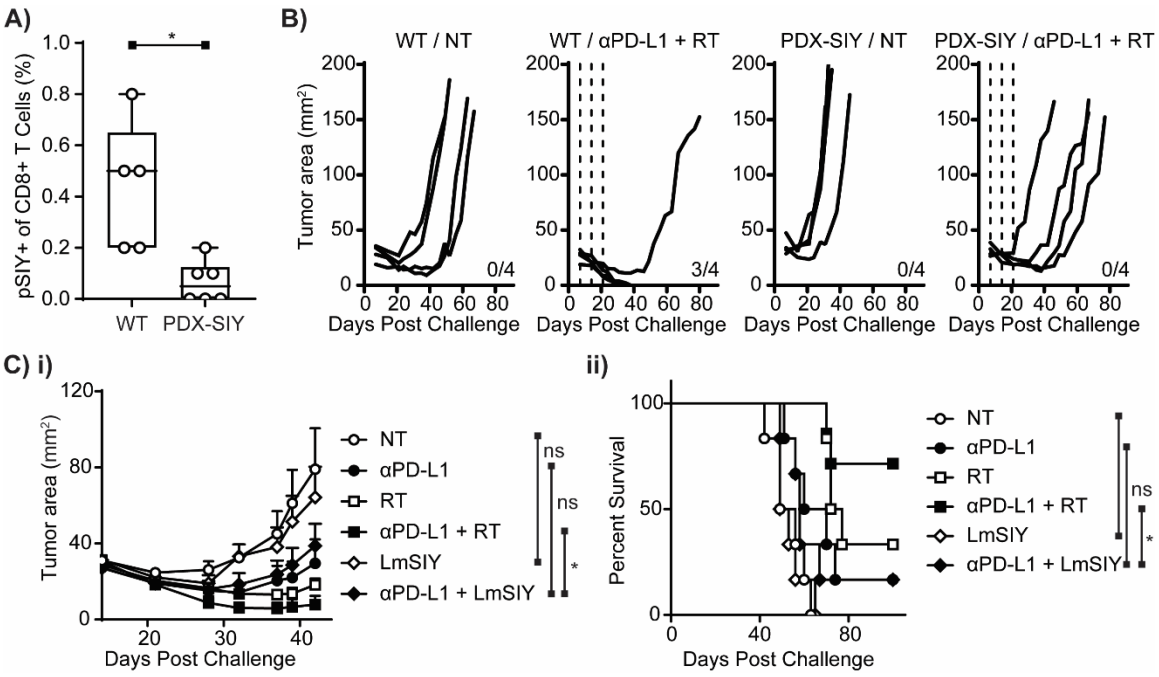

Fig. 2-3 Model antigen specific CD8+ T cells are necessary but not sufficient for therapeutic efficacy of combination radiation and checkpoint blockade. A) Pentamer-SIY binding CD8+ T cells of total CD8+ T cells in peripheral blood of wild type (WT) and PDX-SIY mice seven days after challenge with Panc02SIY cancer cells. B) Tumor growth curves of wild-type (WT) or SIY tolerant (PDX-SIY) mice implanted with Panc02SIY100 cancer cells and given no treatment (NT) or 12 Gy radiation at day 14 and αPD-L1 checkpoint blockade therapy at days 7, 14, and 21. Mice survival without tumors are listed as fractions in the lower right-hand side of each growth chart. C) *Listeria-SIY* does not replicate efficacy of radiation in treatment of Panc02SIY100 in combination therapy with αPD-L1 i) Tumor growth curves of wild-type mice given no treatment (NT), αPD-L1 at days 7, 14, and 21, 12 Gy radiation (RT) at day 14, combination αPD-L1 and RT, *Listeria-SIY* at day 14, or combination αPD-L1 and *Listeria-SIY*. ii) Kaplan-Meier survival plot of mice treated in ii). Key: *p<0.05; ns = not significant.

2.4.4 Tumors are resistant to tumor-specific CD8+ T cells generated by vaccination

Due to the inability of *LmSIY* to protect against Panc02SIY100 tumor establishment, we aimed to determine whether the inability of *LmSIY*-induced CD8+ T cells to control SIY-expressing cancer cells was specific to tumors or whether this population of T cells lacked cytotoxic capacity. To determine whether the failure of *Listeria*-induced CD8+ T cells to cure tumors was due to failure to traffic to tumors, we examined tumors by immunohistochemistry following *LmSIY* vaccination (Fig. 2-4Ai). Within SIY-expressing tumors, significant increases in T cell numbers compared to tumors from unvaccinated animals were observed (Fig. 2-4Aii) and found that T cells in more recently-vaccinated samples formed distinct clusters, suggestive of *in situ* activation (Fig 2-4Ai). To determine whether CD8+ T cells recognize antigen within tumors following vaccination we used transgenic Nur77^{GFP} reporter mice which rapidly express GFP following of TCR ligation (281). Nur77^{GFP} mice were implanted with SIY-expressing tumors and treated with radiation or *Listeria* vaccination (Fig. 2-4Bi) and tumors were harvested seven days later for analysis by flow cytometry. Tumors harvested from mice following *LmSIY* vaccination contained greater proportions of SIY-specific CD8+ T cells compared to vaccine controls (*LmOva*) and to irradiated tumors (Fig 2-4Biii), demonstrating that *LmSIY*-induced T cells effectively traffic to tumors. Across treatment groups, similar proportions of SIY-reactive CD8+ T cells expressed Nur77^{GFP}, indicating that recognition of tumor antigens was not improved by radiation compared to controls or *LmSIY* vaccination (Fig 2-4Biii). These results demonstrate that *LmSIY*-induced CD8+ T cells effective traffic to tumors in abundance and recognize tumor antigen *in situ* despite failure of therapy to reduce tumor burden.

In order to confirm that therapeutic failure of *LmSIY* was not due to the inability of *Listeria*-induced CD8+ T cells to kill antigen-presenting target cells, *in vivo* cytotoxicity was tested using peptide-pulsed, CFSE-labeled congenic splenocytes transferred into vaccinated animals. These transferred cells were found to be selectively depleted based on matched antigen (Fig 2C), indicating that *Listeria* vaccines generated functional antigen-specific CD8+ T cell cytotoxic immunity appropriately for SIYRYYGL- or SIINFEKL- presenting cells depending on vaccine specificity. Together, these results demonstrate that *Listeria* vaccines induce functional, antigen-specific CD8+ T cells that traffic to antigen-expressing tumors and indicate that the failure of *Listeria* vaccination to control antigen-expressing tumors is due to unknown mechanisms of tumor-intrinsic resistance. This suggests that the ability of radiation to potentiate CD8+ T cell responses is not due to its ability to enhance T cell numbers or activation (e.g. vaccination) but its ability to improve the intrinsic susceptibility of tumors to control by T cells.

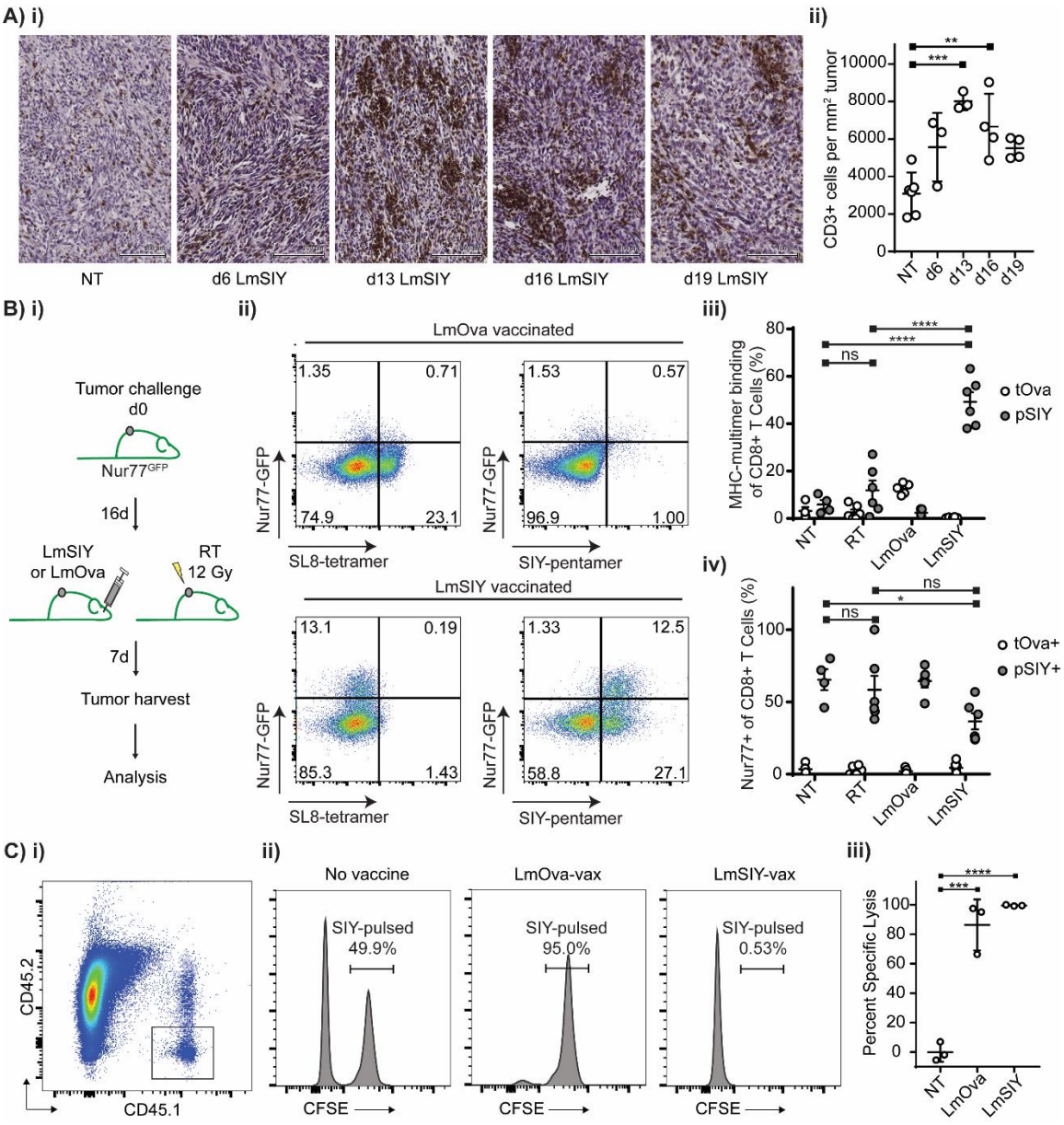

Fig. 2-4 *Listeria*-induced T cells are cytotoxic *in vivo* and traffic to tumors despite being unable to control tumor growth. A) i) Representative immunohistochemistry images from paraffin-embedded zinc-fixed Panc02SIY tumors harvested from mice treated with *Listeria-SIY* at indicated times following tumor challenge or given no treatment (NT). Tumors were harvested at 27 days following tumor challenge. 5μM tissue sections were stained with CD3-DAB (brown) and counterstained with hematoxylin and eosin ii) Quantification of images represented in i) where each dot represents a representative section from an individual animal. B) i) Treatment schematic. Nur77^{GFP} reporter mice were challenged with Panc02SIY tumors followed by treatment

with *Listeria-SIY*, *Listeria-Ova*, 12 Gy radiation (RT), or no treatment (NT). Tumors were harvested seven days after treatment and processed for analysis of tumor-infiltrating lymphocytes. ii) Representative flow cytometry dot plots of tumor-infiltrating CD8+ T cells described in i), quantification of iii) MHC-multimer binding or iv) Nur77-GFP expression of tumor-infiltrating CD8+ T cells. C) *in vivo* cytotoxicity of *Listeria*-induced CD8+ T cells. Briefly, $CD45.2^+$ mice were vaccinated with *Listeria-Ova* or *Listeria-SIY* and seven days later given CFSE-labelled, peptide-pulsed splenocytes from $CD45.1^+$ SJL mice i) representative dot plot of splenocytes harvested six hours after donor splenocyte challenge, ii) representative histograms of CFSE-labeled $CD45.1^+$ cells from unvaccinated, *Listeria-Ova* (*LmOva*) vaccinated or *Listeria-SIY* (*LmSIY*) vaccinated mice, iii) quantification of percent specific lysis in untreated (NT), *Listeria-Ova* (*LmOva*) vaccinated, or *Listeria-SIY* (*LmSIY*) vaccinated mice. Key: *p<0.05; **p<0.01; ***p<0.001; p<0.0001; ns = not significant.

2.4.5 Cancer cells are intrinsically resistant to killing but can be modified to increase susceptibility to control by CD8+ T cells

The ability of cancer cells to evade detection by the immune system is a hallmark of cancer (3) and has been discussed to some extent in chapter one of this thesis. Likewise, the ability of cancer therapies to disrupt cancer resistance to immunity is of significant interest to the field. To test whether Panc02SIY100 could be modified to improve susceptibility to control by *LmSIY*-induced CD8+ T cells, we developed an assay using an Incucyte (Sartorius) to monitor cancer cell growth microscopically *in vitro* over a time course (Fig. 2-5A). In order to monitor cancer cells separately from T cells, we used a confluence mask only over the portions of cells expressing GFP (Fig. 2-5Aii versus Fig. 2-5Aiii), which broadly tracked with overall growth confluence (Fig.2-5Aiv). This assay allowed visualization of killing of SIY-expressing cancer cells by T cells *in vitro* while quantitatively tracking cancer cell growth. To test whether *Listeria*-induced CD8+ T cells were capable of killing antigen-matched cancer cells and whether T killing could be enhanced by pretreatment of cancer cells with the potent pro-inflammatory cytokine interferon-gamma (IFNγ), cancer cells were cocultured with CD8+ T cells harvested from *LmSIY*- or *LmOva*- vaccinated mice. Strikingly, *LmSIY*-induced CD8+ T cells were no better at controlling cancer cells *in vitro* than control T cells in the absence of cytokine pretreatment (Fig. 2-5Bii). Additionally, incubating cancer cells with IFNγ for 24 hours and washing prior to administration of the T cells was sufficient to markedly improve control of tumor cells to control by antigen-specific CD8+ T cells (Fig. 2-5Bi and Fig 2-5Bii). Taken together, these results indicate that Panc02SIY100 cancer cells are intrinsically resistant to control by CD8+ T cells but can be modified to improve susceptibility to control. In Chapter 3, we discuss how radiation might drive these alterations to improve therapeutic control of cancer by T cells.

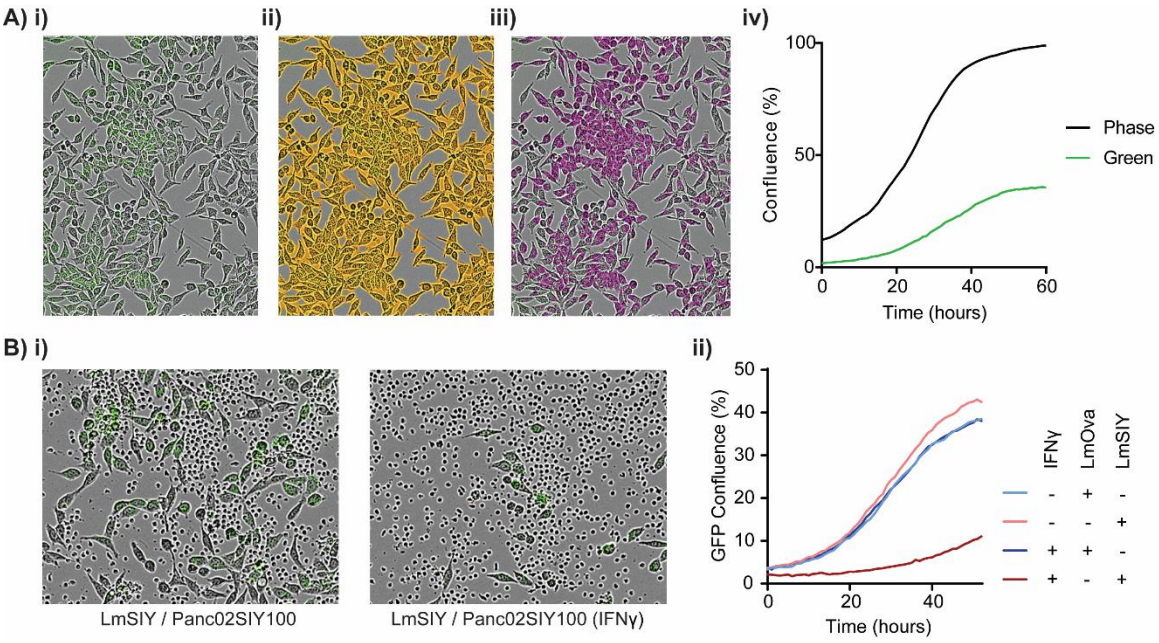

Fig. 2-5 Cancer cells can be modified by cytokines to increase susceptibility to killing by *Listeria*-induced CD8+ T cells. A) Characterization of assay to track *in vitro* cancer cell growth. i) Incucyte image of Panc02SIY100 cells expressing GFP. ii) Incucyte image in i) with confluence mask. iii) Incucyte image in i) with confluence mask only over GFP expression. iv) Quantification of cancer cell growth by phase (as in ii) or GFP (as in iii). B) Panc02SIY100 cell growth in coculture with *Listeria-SIY* induced CD8+ T cells i) Representative Incucyte images from Panc02SIY100 cells untreated (left) or treated with IFNγ (right) prior to addition of T cells at 25:1 effector:target ratio. ii) Panc02SIY100 growth chart following the addition of *Listeria-Ova* (*LmOva*) or *Listeria-SIY* (*LmSIY*) induced CD8+ T cells at 100:1 effector:target ratio.

2.5 Discussion

There are many challenges to creating effective therapeutic cancer vaccines including lack of known antigens, low expression of MHC-I on cancer cells, and failure of T cells in blood to penetrate the tumor (22, 61, 289-292). While there is no shortage of innovation in the field in designing novel vaccine strategies (61, 293), some of these barriers may be overcome by using radiation as an *in situ* vaccine: radiation-mediated vaccination uses dying tumor cells as an antigen source and radiotherapy can modulate the tumor environment to favor antigen presentation and trafficking of CD8+ T cells (127, 128, 229, 294). However, it is unclear whether control of tumors mediated by CD8+ T cells following irradiation is due to these vaccine effects or which other immune modulatory effects might permit T cell control. In this study, we used a live-attenuated *Listeria monocytogenes* vaccine expressing tumor antigen peptides in order to model vaccination in the absence of other radiation-mediated immune modulation and found that vaccination is not sufficient to explain how radiation enhances control of cancer cells.

Listeria-generated CD8+ T cells are capable of cytotoxicity and traffic to antigen-expressing tumors; however, the presence of these cells is not sufficient to control tumors. *In vitro*, we demonstrated that cancer cells are resistant to immune-mediated control, suggesting that the tumor microenvironment was not responsible for suppression of vaccine-generated T cells (Fig. 2-5). We found, however, that modulation of cancer cells using inflammatory cytokine (IFNγ) was sufficient to permit control by *Listeria*-induced CD8+ T cells (Fig. 2-5). These results suggest that radiation might alter cancer cell phenotypes in order to permit control by antigen-specific T cells.

The findings here concur with prior observations that tumor control by radiotherapy and checkpoint inhibitors depends on pre-existing immune responses rather than by vaccination by tumor irradiation (124). In preclinical models, implantation of cancer cells

can induce CD8+ T cell priming sufficient to establish T cell memory and can cause spontaneous rejection of immunogenic tumors (8, 124, 243, 295, 296); in models were pre-existing anti-tumor immunity is not present, additional vaccination is necessary to permit tumor control by radiation and checkpoint inhibition (297). While vaccination effects by radiation have been observed here and elsewhere (220), these data suggest that this response is inadequate to generate sufficiently effective T cells for tumor control.

While the traditional goal of radiotherapy is to induce cancer cell death, it is well established that sublethal irradiation can alter the ability of T cells to control cancer cells (26, 27, 127, 277, 298). Work by James Hodge and colleagues highlighted the upregulation of MHC-I by radiation as a potential means for augmenting these interactions (229). In this study, the authors proposed that increased MHC-I expression by radiation was due to the upregulation of mTOR and increased intracellular peptide pools resulting in enhanced peptide loading and cell surface expression of pMHC-I complexes; in order to inhibit this pathway, cells were treated with the mTOR inhibitor rapamycin, resulting in decreased MHC-I expression and attenuated control by T cells following radiation. These studies are confused, however, by the multifaceted role of mTOR signaling in both cancer cells and T cells, including regulating proliferation, cell cycle, metabolism, and protein synthesis (299-301). Studies isolating MHC-I modulation as a potential mechanism for enhanced T cell function following cancer irradiation are therefore lacking; we aim to address this deficit in Chapter 3.

Chapter 3: Induction of NLRC5 and upregulation of MHC-I induced by radiation as a key mechanism driving CD8+ T cell responses

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3.1 Abstract

Work by our group and others has demonstrated that cancer cells can be intrinsically resistant to control by CD8+ T cells and that radiation can alleviate some of this suppression. However, due to the broad capacity of radiation to modulate the immune responses, the precise means of how radiation can permit CD8+ T cell killing is unclear. Here, we aimed to explore the role of augmentation of MHC-I expression on cancer cells by radiation in permitting control by T cells. We found that radiation-mediated upregulation of MHC-I on cancer cells was not dependent on the ability of cancer cells to respond to STING ligands or type I interferon but found instead that radiation was able to upregulate the MHC-I transactivator, NLRC5, independent of these pathways. We found that NLRC5 expression correlated with basal MHC-I expression and that upregulation of NLRC5 was sufficient to enhance MHC-I expression on cancer cells. Significantly, we found that while NLRC5 upregulation did not improve CD8+ T cell recognition of MHC-I^{lo} cancer cells, antigen-matched T cells were better able to control NLRC5^{hi} cancer cells than NLRC5^{lo} controls. These results suggest that radiation might enhance the susceptibility of cancer cells to control by CD8+ T cells through upregulation of MHC-I via NLRC5.

3.2 Introduction

Broadly, the ability of the killer cells of the immune system to exert cytotoxic functions requires a signaling threshold within the cell to shift from a state where these functions are inhibited to a state where they are favored (i.e. where positive signaling overcomes negative signaling). For CD8+ T cells, a population of potentially potent cytotoxic cells with the ability to specifically control cancer cells while largely sparing healthy tissue, this threshold of activation has come under increasing scrutiny following the unprecedentedly wide success of checkpoint inhibitor therapies such as antibody blockade of CTLA-4 and PD-1/PD-L1 as discussed in Chapter 1. Blockade of the PD-1/PD-L1 signaling axis in particular directly favors positive signaling through the T cell receptor by alleviating suppressive signaling of CD28 via SHP-2 (94, 302). This understanding highlights our ability to augment T cell efficacy in cancer therapies by inhibiting negative signaling and, potentially, enhancing positive signaling.

The ability of radiation to augment the ability of CD8+ T cells to respond to cancer targets is well-established (229, 298, 303), although the mechanisms by which irradiation effects this change is unclear. Among the most compelling modifications to cancer cells induced by radiation is the upregulation of major histocompatibility complex class I (MHC-I) molecules. Work by Eric Reits and James Hodge proposed that radiation increases MHC-I expression on cancer cells by increasing intracellular peptide pools and thus increasing the favorability of peptide binding to MHC-I molecules in the endoplasmic reticulum (229), while others have proposed that MHC-I upregulation is mediated by type I interferons (IFN-I) downstream of activation of the cGAS-STING pathway (201). The functional consequences of MHC-I upregulation on target tumor cells is also unclear, given the theoretical ability of activated CD8+ T cells to kill target cells while engaging only 1-3 peptide-MHC-I (pMHC) complexes, although the impact of

epitope density on enhancing the magnitude and function of novel CD8+ T cell responses is clear (273).

In this chapter, we explore the ability of radiation to improve the susceptibility of normally resistant cancer cells to control by CD8+ T cells. We hypothesized that the ability of T cells to better control cancer cells is due to upregulation of MHC-I driven by radiation and explore the mechanism by which radiation increases MHC-I expression. Finally, we describe a novel mechanism for the induction of MHC-I by radiation through the transcriptional upregulation of NLRC5 independent of STING and IFN-I signaling.

3.3 Materials and Methods

Animals and Cell Lines

Animal protocols were approved by the Earle A. Chiles Research Institute IACUC (Animal Welfare Assurance No. A3913-01). All experiments were performed in accordance with relevant guidelines and regulations. 5-8 week old C57BL/6 mice (Stock #000664) and B6.129S7-Rag1^{tm1Mom}/J (Rag1$^{-/-}$) mice (Stock #002216) were purchased from the Jackson Laboratory (Bar Harbor, ME) for use in these experiments. Nur77^{GFP} reporter mice were kindly provided by Dr. Weinburg (Earle A. Chiles Research Institute, Portland, OR)(281). 2C transgenic mice were kindly provided by Dr. Gajewski (University of Chicago, Chicago, IL), bred in-house and crossed with Nur77^{GFP} reporter mice. OT-I transgenic mice were gifted by Dr. Redmond (Earle A. Chiles Research Institute). Survival experiments were performed with 6-8 mice per group.

Cell lines were cultured in RPMI-1640 (HyClone, Fisher Scientific, Hampton, NH) supplemented with 10% heat inactivated fetal bovine serum (Cat#10082147, Thermo Fisher Scientific, Waltham, MA), 2mM L-glutamine (Cat#SH3003401, HyClone, Fisher), 10mM HEPES (Cat#HOL06, Caisson Labs, Smithfield, UT), 100U/mL penicillin-streptomycin (Cat#PSL01, Caisson), 1X non-essential amino acids (Cat#SH3023801, Fisher), 1mM sodium pyruvate (Cat#PYL01, Caisson). The parental murine pancreatic adenocarcinoma cell line Panc02 was kindly provided by Dr. Woo (Mount Sinai School of Medicine, New York, NY). Panc02 expressing the model antigen SIY was kindly provided by Dr. Weishelbaum (University of Chicago, Chicago, IL), as used previously(124), and expresses GFP-SIY in approximately 40% of cells. Panc02SIY100 was derived and expanded from a high GFP expressing single clone within Panc02SIY on a BD FACSAria II cell sorter (Becton Dickinson, Franklin Lakes, NJ). Panc02SIYneg was similarly derived from a low GFP expressing clone sorted from Panc02SIY on a BD

FACSAria II cell sorter. PK5L1940 was generated from established spontaneous pancreatic tumors in Pdx-Cre$^{+/-}$Kras$^{(G12D)+/-}$Trp53$^{(R172H)+/-}$SIY^{+} as previously described(124).

Antibodies and Reagents

Viability staining was performed in PBS using Zombie Aqua Fixable Viability Kit (BioLegend, San Diego, CA) for 15 minutes prior to staining with fluorescently-conjugated antibodies for flow cytometry. Monoclonal antibodies were used against: CD3 [17A2], CD4 [RM4-5], CD8α [53-6.7], IFNAR1 [MAR1-5A3], IFNγ [XMG1.2], H-2Kb [AF6-88.5.5.3], and TNFα [MP6-XT22].

For *in vitro* stimulation of cell lines, recombinant mouse IFN-beta protein (Cat#8234-MB-101/CF, RND Systems, Minneapolis, MN) at a final concentration of 1 x 10^3 U/mL, recombinant mouse IFN-gamma protein (Cat#14-8311-63, Thermo Fisher) at a final concentration of 20ng/mL and mammalian 2'3'-cGAMP (Cat#tlrl-nacga23-02, InvivoGen, San Diego, CA) at a final concentration of 25µg/mL were used. Cancer cells were irradiated before plating by timed exposure to a Cs^{137} source in a Gammacell Elan 3000 (MDS Nordion, Ottawa, ON, CAN).

Gene Editing of Cancer Cell Lines

Panc02SIY100-NLRC5 was derived from Panc02SIY100 cells transfected with a plasmid harboring the sequence for hNLRC5 under the constitutive promoter eukaryotic translation elongation factor 1 alpha (EF-1α) (Cat#pUNO1-hNLRC5, Invivogen, San Diego, CA). Cells with constitutively high expression of MHC-I were isolated by cell sorting using a BD FACSAria II cell sorter.

IFNAR knockout Panc02SIY100 and PK5L1940 cell lines were generated using Alt-R S.p. Cas9 Nuclease 3NLS (Cat#192528883, Integrated DNA Technologies, Coralville,

IA), Alt-R CRISPR-Cas9 tracrRNA ATTO 550 (Cat#129528884, IDT), Opti-MEM I Reduced Serum Medium (Cat#31985062, Thermo Fisher), Lipofectamine CRISPRMAX Cas9 Transfection Reagent (Cat#CMAX00015, Thermo Fisher Scientific, Waltham, MA) and predesigned Alt-R CRISPR-Cas9 crRNA guide RNAs (IFNAR1 sequence: 5'-TCAGTTACACCATACGAATC-3'). Three gRNAs were tested for each target and pure populations were isolated from single cells (Panc02SIY100) or five cells (PK5L1940) based on ability to upregulate MHC-I in response to cytokine stimulation using a BD FACSAria II cell sorter.

Immunotherapy and Radiation Therapy of Tumors

Tumors were inoculated at a dose of 5 x 10^6 for Panc02SIY tumors, 10 x 10^6 for Panc02SIY100 and 10 x 10^6 for Panc02SIY100-IFNAR tumors. Tumor size was determined via caliper measurements of the longest length x the longest perpendicular width. Survival endpoint was defined as tumor size greater than or equal to 150mm^2 or when the mouse appeared moribund.

For *in vivo* experiments, 12 Gy of CT-guided radiation was administered to tumor isocenters using a Small Animal Research Radiation Platform (SARRP) (Xstrahl, Suwanee, GA) and Murislice software (Xstrahl), 14 days after tumor implantation. 250μg per dose αPD-L1 checkpoint blockade (Cat#BE101, BioXCell, West Lebanon, NH) was administered intraperitoneally at day 7, 14 and 21 post tumor implantation.

For adoptive T cell transfer, naïve CD8+ T cells were harvested as splenocytes from naïve 2C or OT-I mice and activated *in vitro* with αCD3ε (Cat#BE0001-1, BioXCell) and αCD28 (Cat#BE0015-1) at a final concentration of 10μg/mL each. After 48 hours, cells were rinsed with 10% complete RPMI supplemented with β-mercaptoethanol (βME) (Cat#21985023, Gibco, Thermo Fisher) and plated with 60IU/mL human recombinant IL-

2 (Chiron) for three days. Prior to retro-orbital intravenous injection, CD8+ T cells were purified from splenocytes using a CD8α+ T cell negative isolation kit (Cat#130-104-075, Miltenyi), rinsed in sterile PBS and diluted to 1e6 cells per 100µl injection volume.

In vitro T Cell Coculture Assays

Prior to coculture with cancer cells, CD8+ T cells were harvested as splenocytes from naïve animals and activated *in vitro* with αCD3ε (Cat#BE0001-1, BioXCell) and αCD28 (Cat#BE0015-1) at a final concentration of 10µg/mL each. After 48 hours, cells were rinsed with 10% complete RPMI supplemented with β-mercaptoethanol (βME) (Cat#21985023, Gibco, Thermo Fisher) and plated with 60IU/mL human recombinant IL-2 (Chiron) for three days. Prior to coculture, CD8+ T cells were purified from splenocytes using a CD8α+ T cell negative isolation kit (Cat#130-104-075, Miltenyi).

Cancer cell growth was monitored using an Incucyte (Sartorius, Goettingen, Germany) and Zoom software (Incucyte, Sartorius). Briefly, cancer cells were plated in the presence of cytokines or following *in vitro* irradiation. After 24 hours, adherent cells were rinsed twice in 10% complete RPMI supplemented with βME. Purified CD8+ T cells were added to cancer cell cultures at a 5:1 effector:target ratio. Non-adherent cells were allowed to settle for 15 minutes at room temperature prior to hourly tracking in the Incucyte, housed at 36.5°C/5%CO_2, until untreated cells reached confluence. In order to isolate cancer cells from T cells for analysis, endogenous green fluorescence was used as a proxy for cell confluence.

Cytokine production by T cells during coculture was determined after four-hour incubation with brefeldin A (Cat#B7450, Thermo Fisher). Non-adherent cells were harvested, surface stained and permeablized using BD cytofix/cytoperm fixation and permeabilization solution (Cat#554655, Becton Dickinson), then frozen at -80°C

overnight. The next day, cells were thawed and rinsed twice with perm/wash buffer (Cat#554723, Becton Dickinson) and stained for intracellular cytokines.

Immunoblotting

Briefly, cells from pancreatic cell lines were washed twice with PBS and lysed in Pierce RIPA Buffer (Cat#8990, Thermo) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail 100X (Cat#78440, Thermo). Protein concentrations were quantified using Pierce BCA Protein Assay Kit (Cat#23225, Thermo). Samples were denatured at 95°C in XT Sample Buffer 4X (Cat#1610791, Bio-Rad, Hercules, CA) and loaded onto 4-12% Criterion XT Bis-Tris Protein Gels (Cat#345-0124, Bio-Rad). Proteins were transferred onto PVDF Transfer Membrane (Cat#88518, Thermo) and probed for STING (Cat#13647S, Cell Signaling Technology, Danvers, MA), IRF3 (Cat#4302S, Cell Signaling) and GAPDH (Cat#2118S, Cell Signaling). HRP-conjugated goat anti-rabbit IgG (Cat#31460, Invitrogen, Carlsbad, CA) was used as a secondary antibody. Proteins were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Cat#34580, Thermo).

Statistics

Data was analyzed and graphed using FlowJo (Tree Star, Ashland, OR) and Prism (GraphPad Software, La Jolla, CA). Individual data sets were compared using Welch's T tests. Growth curves and analysis across multiple groups were analyzed using ANOVA (two-way and one-way, respectively).

3.4 Results

3.4.1 Radiation improves the susceptibility of cancer to control by antigen-specific CD8+ T cells

In order to determine whether tumor irradiation improves the ability of antigen-specific CD8+ T cells to control tumor growth, mice bearing dual flank Panc02SIY100 tumors were adoptively transferred with *in vitro* activated CD8+ T cells from antigen matched (SIYRYYGL-reactive, 2C) or control (SIINFEKL-reactive, OT-I) TCR transgenic mice and treated with 12 Gy radiation to one flank tumor (Fig. 3-1Ai). Unirradiated tumors in both 2C- and OT-I-bearing mice grew at the same rate, indicating that Panc02SIY100 tumors *in vivo* are resistant to killing by antigen-specific CD8+ T cells (Fig. 3-1Aii), in agreement with experiments with *Listeria monocytogenes* vaccines discussed in Chapter 2. Conversely, irradiated tumors were controlled significantly better by antigen-specific T cells than by control T cells (Fig. 3-1Aii), suggesting that radiation improved the ability of CD8+ T cells to react to cognate antigen-expressing tumors *in vivo*. In order to determine whether radiation improved the intrinsic susceptibility of cancer cells to control by T cells irradiated cancer cells were cocultured with *in vitro* activated CD8+ T cells derived from 2C or OT-I mice and the growth of the cancer cells was tracked with an Incucyte (Sartorius). While unirradiated cancer cells were similarly controlled by both antigen-specific and control T cells, irradiated cancer cells were significantly better controlled by the antigen-specific 2C cells (Fig. 3-1B). These results demonstrate that radiation augments the susceptibility of irradiated cancer cells to killing by antigen-specific CD8+ T cells.

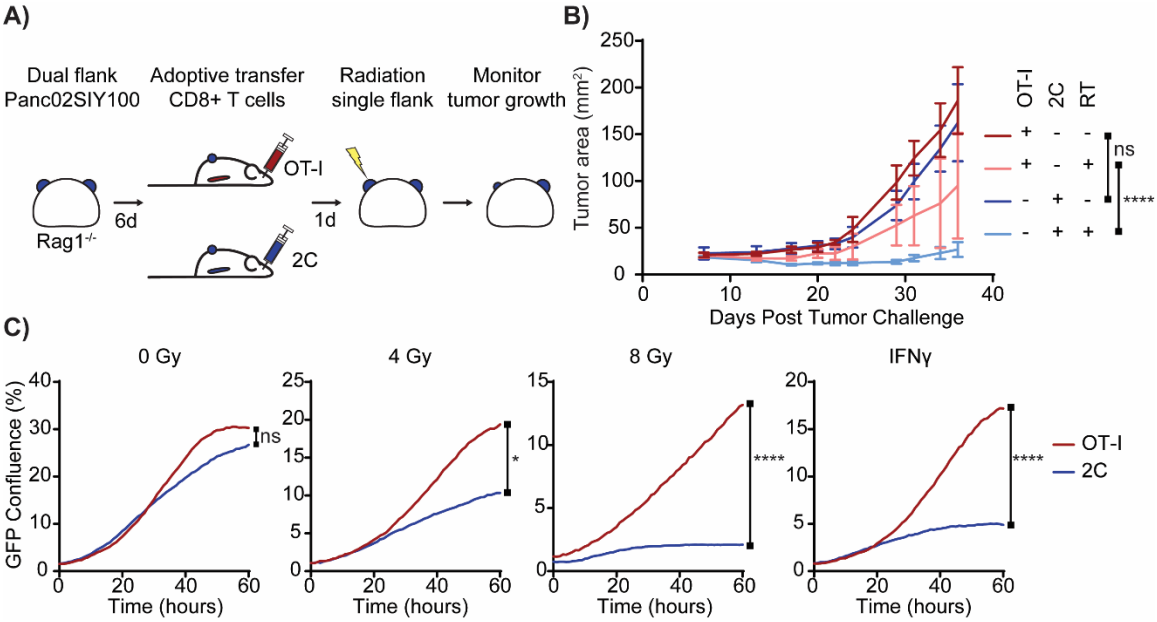

Fig. 3-1 Radiation enhances the ability of CD8+ T cells to control Panc02SIY100. A) Treatment schematic. Rag1$^{-/-}$ mice were implanted with dual flank Panc02SIY100 tumors followed by adoptive transfer of *in vitro* activated 2C or OT-I CD8+ T cells. One day after adoptive transfer tumors were irradiated with 12 Gy on one flank. B) Tumor growth curves for animals treated as in A. Lines represent average tumor area of each tumor group, n=4. Blue lines represent tumors areas in mice bearing 2C T cells and red lines represent tumor areas in mice bearing OT-I T cells. C) Panc02SIY100 growth curves measured with an Incucyte using GFP as a proxy for confluence. Cancer cells were irradiated or pretreated with IFNγ and plated 24 hours prior to coculture with *in vitro* activated CD8+ T cells derived from 2C or OT-I mice. Key: *p<0.05; **p<0.01; ****p<0.0001; ns = not significant.

3.4.2 Radiation increases cancer cell expression of major histocompatibility complex class I (MHC-I)

Previous studies have examined how radiation alters the phenotype of cancer cells to improve control by immune cells, as discussed in Chapter 1. Among these potential alterations, upregulation of major histocompatibility class I (MHC-I) by cancer cells was of significant interest due to the essential role of MHC-I in permitting recognition of target cells by CD8+ T cells. The murine pancreatic cancer cell lines Panc02SIY100 and PK5L1940 express very low levels of MHC-I at baseline but expression can be augmented by interferon-gamma (IFNγ) cytokine stimulation (Fig. 3-2Ai and 3-2Aii), demonstrating the potential for therapeutic modulation. Accordingly, we demonstrated that MHC-I expression can be enhanced by *in vitro* irradiation over 24 to 72 hours (Fig. 3-2Bi and 3-2Bii). Considering these results, we hypothesized that upregulation of MHC-I by radiation improved the susceptibility of cancer cells to control by CD8+ T cells.

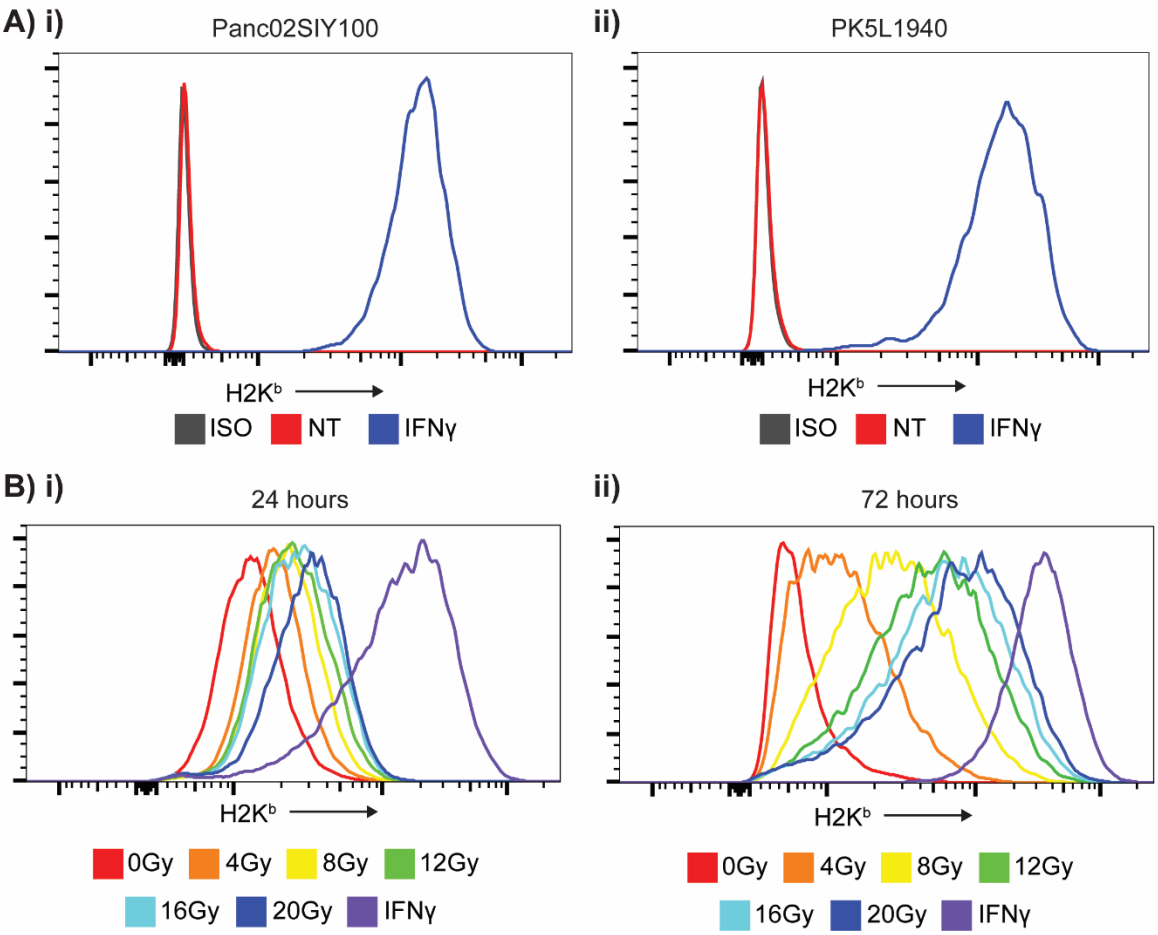

Fig. 3-2 Radiation and IFNγ stimulation upregulate MHC-I on cancer cells. A) Representative histograms of MHC-I (H2K^b) Panc02SIY100 (i) and PK5L1940 (ii) cancer cells 72 hours following IFNγ stimulation *in vitro*. ISO refers to isotype control antibody, NT to cells not treated, and IFNγ to cells cultured in the presence of gamma-interferon. B) Representative histograms of MHC-I (H2K^b) expression on Panc02SIY100 24 hours (i) or 72 hours (ii) after *in vitro* irradiation at indicated doses or treatment with IFNγ.

3.4.3 Upregulation of MHC-I on cancer cells by radiation is independent of STING and IFNAR signaling

In order to determine whether MHC-I upregulation was necessary for radiation-induced augmentation of T cell mediated control of cancer cells, we attempted to engineer cancer cells to disrupt signaling pathways proposed to drive MHC-I upregulation by radiation. Studies have demonstrated that the cGAS-STING pathway plays an essential role in radiation-induced tumor clearance by T cells (125, 215) and it has been proposed that the production of type I interferons (IFN-I) downstream of STING signaling leads to upregulation of MHC-I downstream of the type I interferon receptor (IFNAR) (201, 275). While attempting to engineer STING knockout cancer cells, however, we observed that several pancreatic cell lines, including Panc02SIY100, have very low expression of STING protein (Fig. 3-3A) and are insensitive to upregulation of MHC-I by exogenous application of the endogenous STING ligand 2'3'-cGAMP (Fig. 3-3B). These results concur with work suggesting that STING is not uniformly expressed in cancer cells and that deregulation of STING signaling can correlate with immune evasion and cancer progression (304-306). Notably, however, cell lines which have low expression of STING and are insensitive to STING ligand are still able to upregulation MHC-I in response to radiation. These results suggest that upregulation of MHC-I is not dependent on the STING pathway.

In order to determine whether MHC-I upregulation is dependent on IFN-I signaling, activated through other nucleic acid sensors, we engineered murine pancreatic cancer cell lines to disrupt expression of IFNAR1, an essential component of the IFNAR heterodimer. We demonstrated that IFNAR1^{ko} cell lines have significantly reduced capacity for upregulation of MHC-I by interferon-beta (IFNβ) (Fig 3-4A). Importantly, however, cancer cells which cannot respond to IFN-I still upregulate MHC-I

equivalently to control cells (Fig. 3-4B). These results demonstrate that IFNAR signaling is not necessary for the upregulation of MHC-I by radiation in these cancer cells. To determine whether the loss of IFN-I signaling in cancer cells affects the therapeutic response to radiotherapy, we treated mice bearing Panc02SIY100-IFNAR1^{ko} tumors with radiation and PD-1/PD-L1 checkpoint blockade. *In vivo*, IFNAR1^{ko} tumors grow faster than controls but are cured by single-agent radiotherapy at similar rates and synergy between radiotherapy and αPD-L1 is similarly observed (Fig. 3-4C); it is unclear whether the reduction in survival for IFNAR^{ko} tumor bearing mice treated with combination radiation and αPD-L1 blockade is due to the increased growth rate of tumors outpacing therapy or if these cells produce tumors which are intrinsically more resistant to the combination therapy. Taken together, these experiments demonstrate that cGAS-STING/IFNAR signaling is not necessary for the upregulation of MHC-I in our pancreatic cancer cell lines and that IFN-I signaling through cancer cells is not necessary for tumor cure by radiotherapy with PD-1/PD-L1 checkpoint blockade.

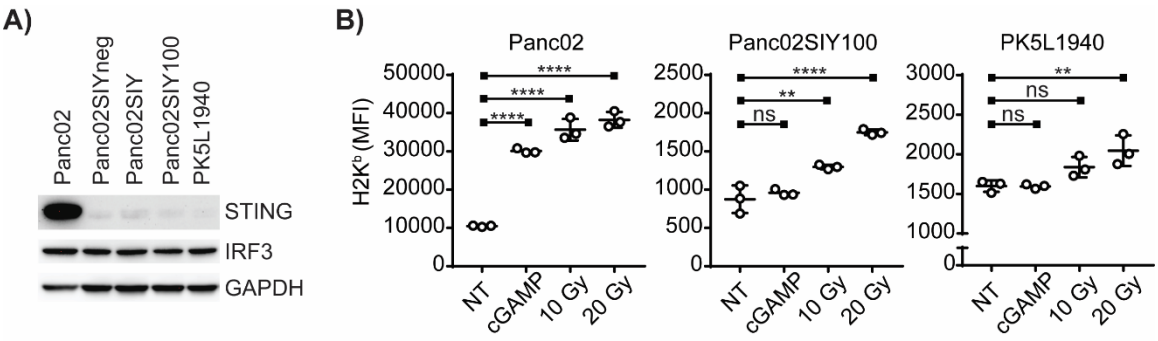

Fig. 3-3 Pancreatic cancer cell lines unable to respond to exogenous STING ligand are able to upregulate MHC-I in response to radiation. A) Pancreatic cancer cell lines processed for protein and immunoblotted against STING, IRF3 and GAPDH. B) MHC-I ($H2K^b$) mean fluorescent intensity (MFI) of pancreatic cancer cell lines 72 hours following irradiation or culture in the presence of 2'3'-cGAMP, measured by flow cytometry. Key: *p<0.05; **p<0.01; ****p<0.0001; ns = not significant.

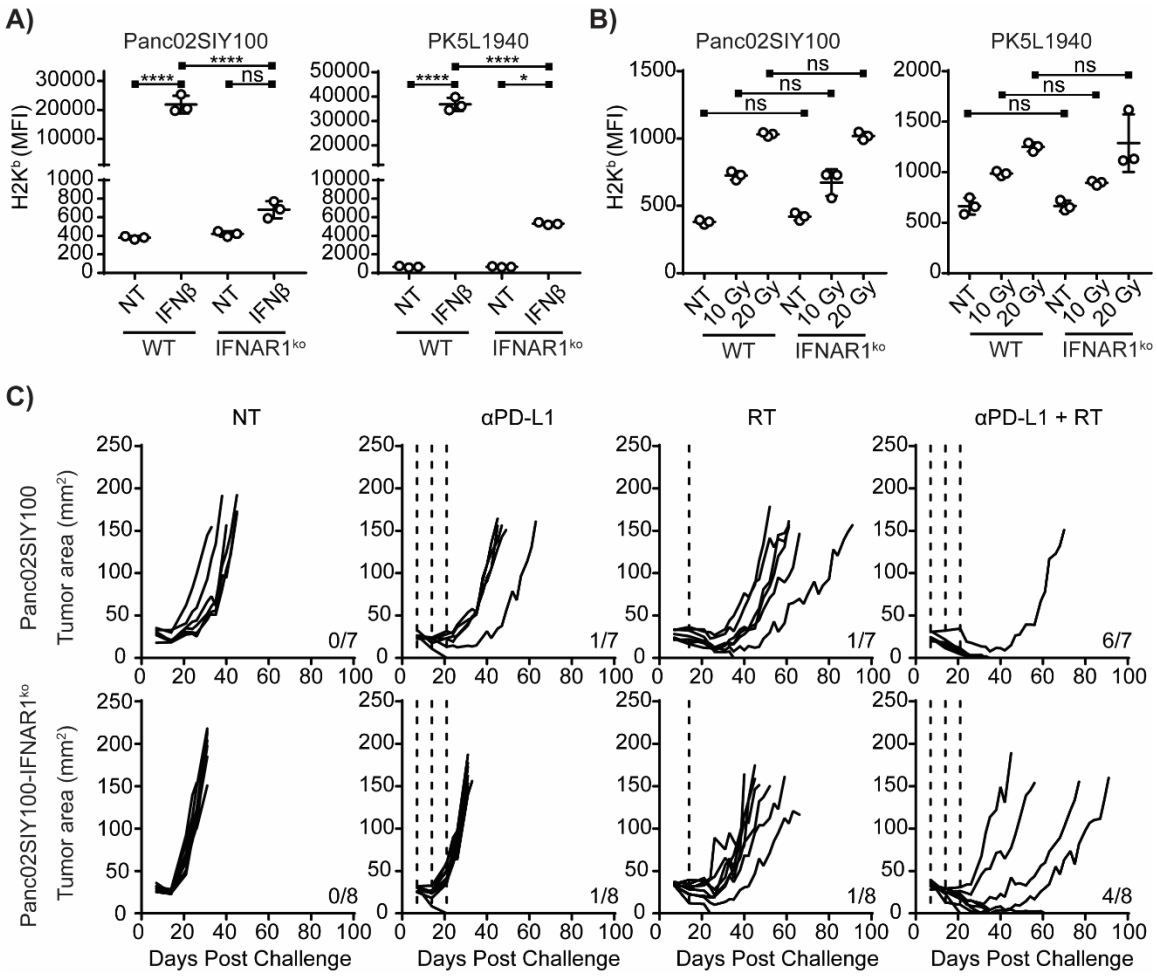

Fig. 3-4 Upregulation of MHC-I by radiation on pancreatic cancer cells does not require IFN-I signaling. Pancreatic cancer cell lines were edited by CRISPR/Cas9 to knock out the type I interferon (IFN-I) receptor subunit IFNAR1. A) MHC-I (H2K^b) mean fluorescent intensity (MFI) of pancreatic cancer cell lines 72 hours following culture in the presence of IFNβ, measured by flow cytometry. B) MHC-I (H2K^b) mean fluorescent intensity (MFI) of pancreatic cancer cell lines 72 hours following irradiation or culture in the presence of 2'3'-cGAMP measured by flow cytometry. C) Tumor growth curves of mice implanted with Panc02SIY100 or Panc02SIY100-IFNAR1^{ko} tumor cells, given no treatment (NT), αPD-L1 as indicated by dashed lines, 12Gy radiotherapy (RT) or combination αPD-L1 and radiotherapy (αPD-L1 + RT). Each line represents one mouse. Fractions indicate tumor-free mice at 100 days following tumor challenge. Key: *p<0.05; ****p<0.0001; ns = not significant.

3.4.4 NLRC5 expression correlates with MHC-I expression in murine pancreatic cancer cell lines

As discussed in Chapter 1, a key genetic driver of MHC-I expression is the MHC Class I Transactivator (CITA, or NLRC5) (250, 259), a reported target of immune evasion in human cancers (265). We observed that NLRC5 expression by pancreatic cancer cells correlated with basal MHC-I expression (Fig. 3-5A) in accordance with previous studies (307), and questioned whether modulation of NLRC5 expression might play a role in MHC-I induction by radiotherapy. In order to determine if radiation altered expression of NLRC5, cancer cells were treated with radiation *in vitro* and mRNA transcripts were quantified by qRT-PCR. Radiation significantly upregulated expression of NLRC5 along with genes associated with antigen presentation, B2M and TAP1, which can be regulated by the NLRC5 transactivation complex (Fig. 3-5B). Notably, radiation was similarly able to upregulate NLRC5 and B2M in Panc02SIY100-IFNAR1^{ko} cells (Fig. 3-5C), but did not upregulate NLRC5 in Panc02 cells (Fig. 3-5D), which have high basal expression of the gene (Fig. 3-5Ai). These results suggest that radiation can augment antigen presentation through genetic regulation of MHC-I associated genes, including by upregulation of NLRC5, in an IFNAR-independent manner. These results also suggest that NLRC5 upregulation is not responsible for augmentation of MHC-I expression in cells with high basal expression of NLRC5.

In order to determine if induction of NLRC5 is sufficient to augment expression of MHC-I in cancer cells, we engineered Panc02SIY100 cells to express NLRC5 under the constitutive eukaryotic promoter, EF-1α (Fig. 3-5Ei), generating a new cell line, Panc02SIY100-NLRC5. Panc02SIY100-NLRC5 expressed constitutively high levels of MHC-I, demonstrating that augmenting NLRC5 expression is sufficient to increase expression of MHC-I in these cancer cells.

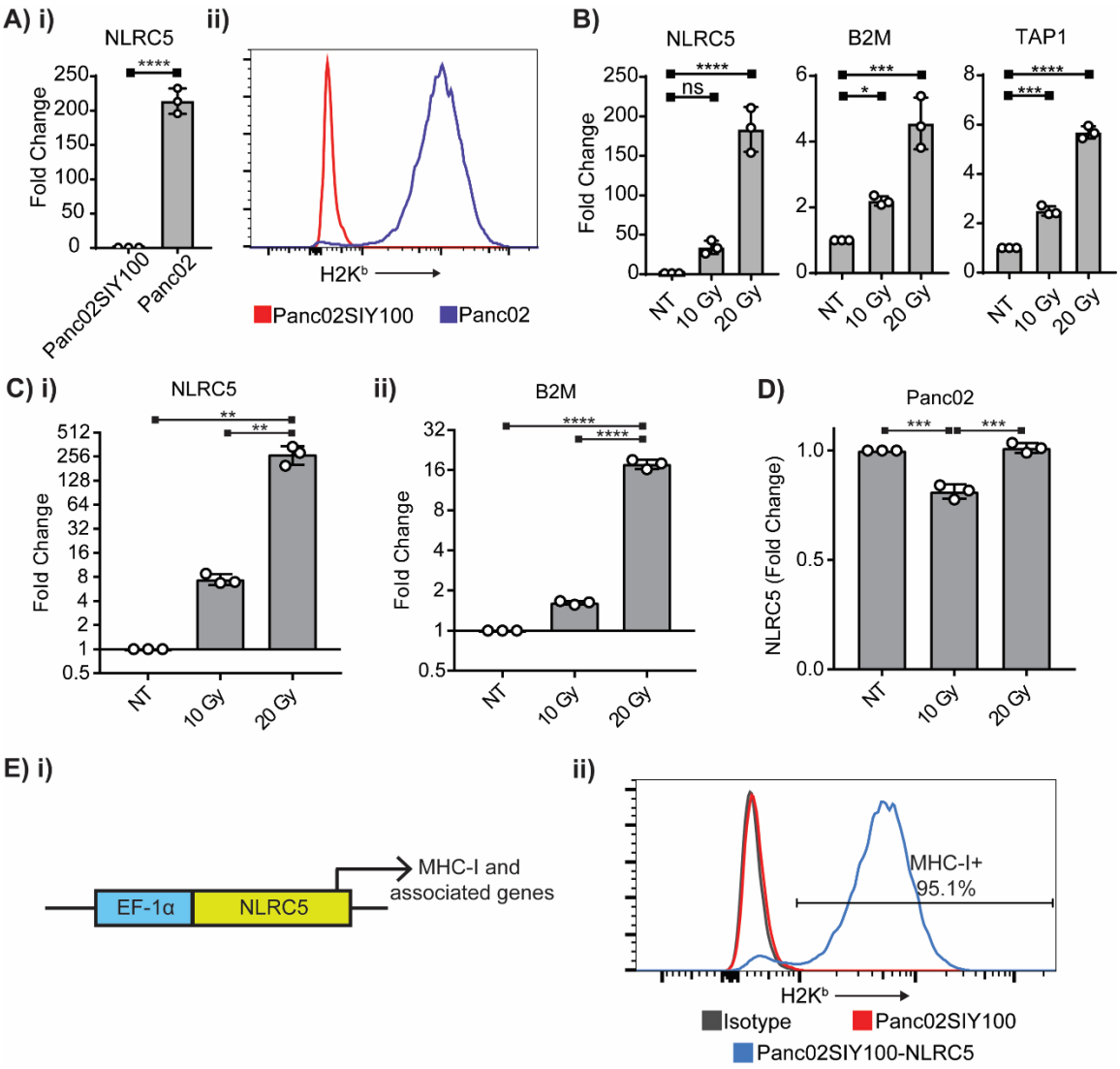

Fig. 3-5 NLRC5 expression correlates with MHC-I expression in murine pancreatic cancer cell lines. A) i) qRT-PCR analysis of basal NLRC5 transcription of pancreatic cancer cell lines. ii) Representative flow cytometry histograms of basal MHC-I ($H2K^b$) expression by pancreatic cancer cell lines. B) qRT-PCR analysis of radiation-induced transcription of NLRC5, B2M, and TAP1 normalized to untreated Panc02SIY100 cells. Cancer cells were analyzed 72 hours following irradiation *in vitro*. C) qRT-PCR analysis of radiation-induced transcription of NLRC5 and B2M normalized to untreated Panc02SIY100-IFNAR1^{ko} cells. Cancer cells were analyzed 72 hours following irradiation *in vitro*. D) qRT-PCR analysis of radiation-induced transcription of NLRC5 normalized to untreated Panc02 cells. Cancer cells were analyzed 72 hours following irradiation *in vitro*. E) i) Panc02SIY100 cells were transfected with NLRC5 under a constitutive EF-1α promoter. ii) Representative flow cytometry histograms of basal MHC-

I ($H2K^b$) expression by pancreatic cancer cell lines including cells transfected to express basal NLRC5, Panc02SIY100-NLRC5. Key: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns = not significant.

3.4.5 Cancer cell expression of NLRC5 is sufficient to enhance MHC-I expression and control by antigen-specific CD8+ T cells

Reduction of MHC-I expression by cancer is a well-established immune evasion strategy which is thought to reduce ability of CD8+ T cells to recognize tumor targets (308). I hypothesized that NLRC5 expression would therefore increase CD8+ T cell recognition of tumor antigen by upregulating MHC-I. To determine if constitutive expression of NLRC5 increased the ability of CD8+ T cells to recognize cognate antigen, I cocultured cancer cells with naïve T cells from 2C TCR transgenic mice crossed with Nur77^{GFP} reporter mice, which express GFP when the T cell receptor (TCR) engages with cognate pMHC. Interestingly, recognition of cancer cells was very high by 2C cells cocultured with control Panc02SIY100 cancer cells expressing low basal MHC-I and, accordingly, enhanced NLRC5 expression did not improve this level of recognition (Fig. 3-6A), indicating that recognition of antigen was intact despite low levels of MHC-I expression. In order to test whether enhanced NLRC5 expression improved the functional responses of T cells to antigen, we examined cytokine secretion by 2C cells cocultured with Panc02SIY100 and Panc02SIY100-NLRC5 and determined that NLRC5 expression was sufficient to enhance the ability of T cells to secrete effector cytokines (IFNγ and TNFα) (Fig. 3-6B), suggesting that there are different thresholds for recognition of antigen via the TCR, indicated by Nur77-GFP expression, and exertion of effector function by CD8+ T cells.

In order to confirm that enhanced expression of NLRC5 is sufficient to enhance control of cancer cells by antigen-specific CD8+ T cells, Panc02SIY100 and Panc02SIY100-NLRC5 were cocultured with *in vitro* activated 2C and OT-I control CD8+ T cells. While Panc02SIY100 cells were resistant to control by 2C cells as before, Panc02SIY100-NLRC5 were significantly more susceptible to control by antigen-specific

CD8+ T cells. These results indicate that expression of NLRC5 is sufficient to permit control of antigen-matched cancer cells by CD8+ T cells through enhanced effector functions.

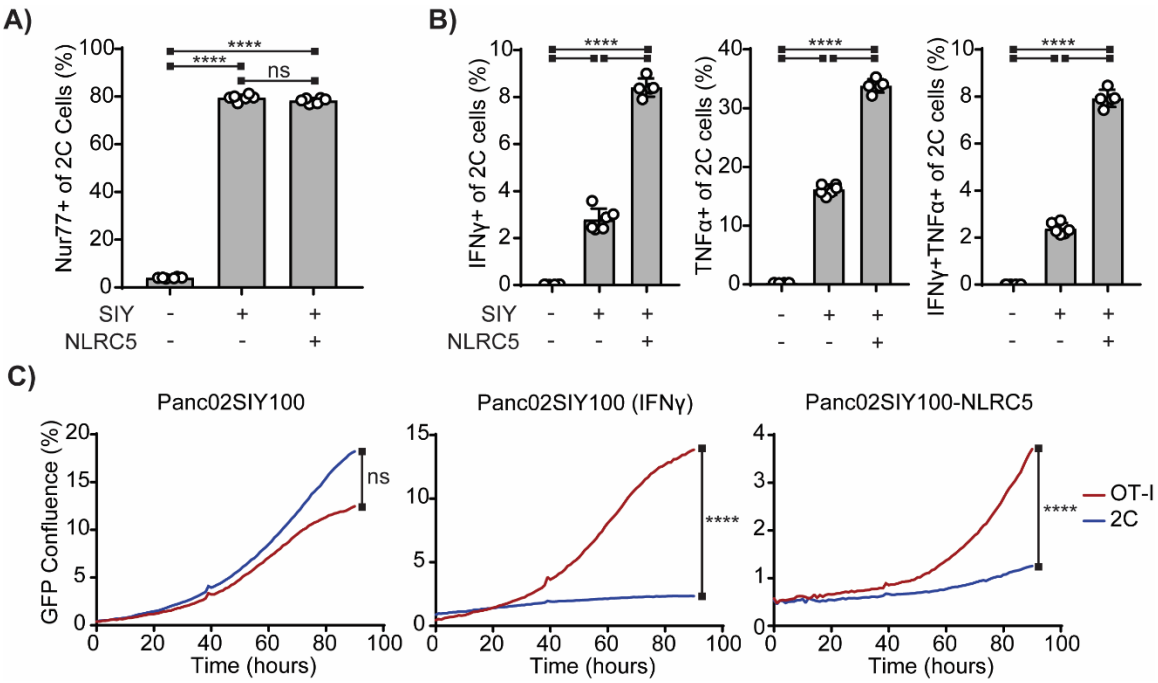

Fig. 3-6 NLRC5 expression by pancreatic cancer cells is sufficient to enhance control by antigen-specific CD8+ T cells. A) Naïve CD8+ T cells from Nur77^{GFP} x 2C mice were plated on top of Panc02SIYneg, Panc02SIY100, and Panc02SIY100-NLRC5 cancer cells. Analysis of Nur77^{GFP} expression by 2C CD8+ cells was measured following 24 hours of coculture by flow cytometry. B) *In vitro* activated 2C CD8+ T cells were plated on top of Panc02SIYneg, Panc02SIY100, and Panc02SIY100-NLRC5. Cytokine production (IFNγ and TNFα) was measured by intracellular cytokine staining and flow cytometry following 24 hours of coculture. C) Pancreatic cancer cell line growth curves measured with an Incucyte using GFP as a proxy for confluence. Cancer cells were pretreated with IFNγ and plated 24 hours prior to coculture with *in vitro* activated CD8+ T cells derived from 2C or OT-I mice. Key: *p<0.05; ****p<0.0001; ns = not significant.

3.5 Discussion

Downregulation of major histocompatibility complex class I (MHC-I) molecules on cancer cells represents a major immune evasion tactic of murine and human cancers (309-312) and, accordingly, increasing MHC-I expression has been identified as a key target for effective immunotherapy (271, 308, 313). In the present study we have demonstrated that radiation increases MHC-I expression on cancer cell surfaces, enhances susceptibility of normally resistant cancer cells to control by antigen-specific CD8+ T cells and identified a novel mechanism of MHC-I regulation by induction of NLRC5 gene expression.

Differential responses of CD8+ T cells to varying pMHC (peptide-MHC complex) concentration have been observed previously, wherein increased epitope density corresponds with greater responsiveness to IL-2, enhanced proliferation and increased cytotoxic function including cytokine production (273, 314, 315). This phenomenon is better understood in naïve T cells, where high levels of antigen presentation in combination with costimulation and integrin stabilization are required to generate a stable immunological synapse and to cross an activation threshold of T cell receptor (TCR) signaling (273, 316). In activated T cells, pMHC:TCR interactions at the kinapse are much shorter: a single pMHC complex can serially engage with rapidly internalizing TCRs and a CD8+ T cell can exert cytotoxic functions after engaging with as few as 1-3 pMHC complexes per target cell (317, 318). It is clear that higher concentrations of pMHC can engage more TCRs and it has been proposed that serial engagement of the TCR allows increased stability and enhanced signaling within the TCR/pMHC-I/CD8 molecular complex (315). Functionally, downregulation of MHC-I induced by viral infection can significantly attenuate the ability of CD8+ T cells to kill infected targets (319). Interestingly, expression of the early activation marker CD69 appears to be independent of epitope density (320), corresponding with the data presented here

demonstrating equivalent expression of Nur77 by naïve T cells cultured with MHC-I^{lo} Panc02SIY100 versus MHC-I^{hi} Panc02SIY100-NLRC5. However, we observed improved IFNγ and TNFα production by activated CD8+ T cells cultured with MHC-I^{hi} Panc02SIY100-NLRC5 cells in line with previous observations that increased epitope density is associated with increased T cell functional activation. Cumulatively, these data suggest that different densities of pMHC can activate different thresholds in T cells for expression of early activation markers, cytolytic degranulation and cytotoxic cytokine release (321). These experiments support the hypothesis that increased engagement of T cell receptors of effector CD8+ T cells enhances cytotoxic effector functions over those with minimally engaged TCRs. This work agrees with findings that tumor downregulation of antigen presentation via MHC-I correlates with checkpoint blockade resistance in human patients (271, 313) and highlights that total loss of MHC-I is not necessary for resistance to T cells.

In this study, we propose a novel mechanism for increasing MHC-I density on cancer cell surfaces induced by radiation. Previous studies of MHC-I regulation by radiation have primarily focused on two distinct mechanisms for induction. One mechanism has highlighted tumor-derived type I interferon (IFN-I) signaling following irradiation as a mechanism of MHC-I induction (201). IFN-I secretion has been observed following cancer cell irradiation downstream of activation of cGAS/STING signaling (194), but we found that MHC-I expression on cancer cells following irradiation was not attenuated by knockdown of the IFN-I receptor (IFNAR). Additionally, we observed very little expression of STING in pancreatic cell lines that upregulated MHC-I in response to radiation. Together, these data suggest that in these models MHC-I upregulation is not dependent on IFN-I or STING signaling. Alternatively, another mechanism has proposed that RT-induced protein damage and enhanced mTOR signaling, as a means to facilitate damage repair responses, leads to increased abundance of intracellular peptide pools

and enhanced MHC-I loading to the cell surface (229). We found that genes involved in antigen presentation on MHC-I, including the MHC-I transactivator NLRC5, were upregulated following treatment, but surprisingly that this was not mediated via STING-mediated innate sensing leading to type I IFN signaling. To our knowledge this represents a novel mechanism of MHC-I induction on cancer cells by radiation. Together with data showing increased cytokine release by CD8+ T cells cultured with NLRC5-expressing Panc02SIY100, these data support our proposal that induction of NLRC5 by radiation enhances the ability of T cells to functionally respond to cancer cells expressing cognate antigen allowing for tumor regression and clearance. Further experiments are required, however, to determine whether induction of NLRC5 is necessary for the induction of MHC-I by radiation or increased susceptibility of cancer cells to control by CD8+ T cells.

Chapter 4: Concluding Remarks

4.1 Summary and Conclusions

The traditional role of radiotherapy in cancer treatment is to maximize direct killing of cancer cells while preserving normal tissue function; however, in part due to restrictions in radiation regimes due to normal tissue toxicity, tumor irradiation can leave behind viable cancer cells capable of leading to disease recurrence. In this manuscript, we have aimed to explore the way that sublethal radiation, that is radiation which does not entirely ablate the tumor on its own, can enhance CD8+ T cell responses to clear residual disease.

We first aimed to establish whether vaccination by radiotherapy, also called *in situ* vaccination, was sufficient to explain immune-mediated tumor clearance by radiation. Previous reports by our lab demonstrated that T cell-mediated regression of tumors by radiation requires pre-existing CD8+ T cell immunity (124), suggesting that radiotherapy functioned poorly to prime/initiate novel T cell responses. This study, however, did not address whether T cell boosting, or reactivation and expansion of T cells, by radiation was sufficient to explain therapeutic efficacy. In order to model T cell boosting, we administered an exogenous vaccine capable of generating an order of magnitude higher tumor-specific CD8+ T cell in circulation and demonstrated that these cells had cytotoxic capacity, trafficked to tumors, and recognized antigen within tumors. Despite treatment-induced influx of T cells, however, tumors were refractory to vaccination alone or in combination with PD-1/PD-L1 checkpoint blockade therapy. Taken together, these results suggest that vaccination effects are not sufficient to explain the enhanced responses of CD8+ T cells driven by radiation, and raise questions

about whether radiotherapy might otherwise modulate the susceptibility of tumors to control by T cells.

Sublethal doses of radiation can alter cancer cell expression of proteins in a number of ways, but for these studies we were primarily interested in the role of augmentation of MHC-I expression (229) due to the integral role of antigen presentation in CD8+ T cell reactivity. In order to determine the role of radiation-mediated upregulation of MHC-I in enhancing CD8+ T cell responses, we examined candidate pathways which might play a role in cytokine-driven regulation of antigen presentation initiated by cancer cell irradiation, STING and type I interferon (IFN-I) (125, 201). We found, however, that our cancer cell lines express very low levels of STING protein and are insensitive to MHC-I induction by administration of exogenous STING ligands, suggesting that MHC-I upregulation occurs independently of the STING pathway. In order to determine whether, through means of alternate signaling, radiation induced the production of IFN-I and triggered MHC-I presentation through IFNAR, we used CRISPR/Cas9-mediated gene editing to disrupt IFNAR and render cancer cells insensitive to IFN-I signaling. Here, however, we determined that radiation-mediated upregulation of MHC-I and that therapeutic efficacy of radiation do not require cancer cell signaling through IFNAR. These results suggest that radiotherapy augments antigen presentation on MHC-I through activation of alternate pathways not yet elucidated.

In order to address the question of whether upregulation of MHC-I was sufficient to enhance the ability of CD8+ T cells to control, we genetically engineered cancer cells to express the MHC-I class I transactivator, NLRC5. We found that radiotherapy was sufficient to induce upregulation of NLRC5 in MHC-I^{lo} cancer cells and found that expression of NLRC5 was sufficient to increase expression of NLRC5. These findings suggest that induction of NLRC5 expression may represent a novel mechanism of

upregulation of MHC-I in response to radiotherapy. We found that enhancement of NLRC5 expression by cancer cells did not improve recognition by CD8+ T cells, but was able to enhance the ability of T cells to control cancer cells *in vitro* and produce cytokines in response to cancer cells in culture. These results suggest that different amounts or densities of pMHC presentation on target cells, controlled in part by NLRC5 expression, can trigger different thresholds of activation in effector CD8+ T cells and prompt different therapeutic outcomes.

Taken together, the data presented here support a model in which radiotherapy augments MHC-I expression by cancer cells and the resulting increase in antigen presentation can enhance the ability of cytotoxic CD8+ T cells to control these cells (Fig. 4-1). In this model, radiotherapy functions primarily to potentiate pre-existing immune responses by altering the phenotype of target cancer cells, rather than by enhancing systemic immunity, in tumor models which are resistant to control by CD8+ T cells at baseline. Significantly for this work, studies have demonstrated that the gene for NLRC5 in human cancers is frequently epigenetically suppressed rather than genetically disrupted (263, 265), suggesting that NLRC5 expression can be therapeutically induced, possibly by radiotherapy, in these tumors. Taken together, the experiments presented here expand our understanding of how radiotherapy can be used to control local disease but suggest, unfortunately, that radiation of a primary tumor is unlikely to aid in the control of tumors at distal sites. Accordingly, the research presented here has, in part, provided justification for a clinical trial examining the efficacy of neoadjuvant immunoradiotherapy with PD-1/PD-L1 checkpoint blockade in head and neck squamous cell carcinomas (HNSCC) in downstaging tumors prior to surgical resection (ClinicalTrials.gov Identifier: NCT03247712).

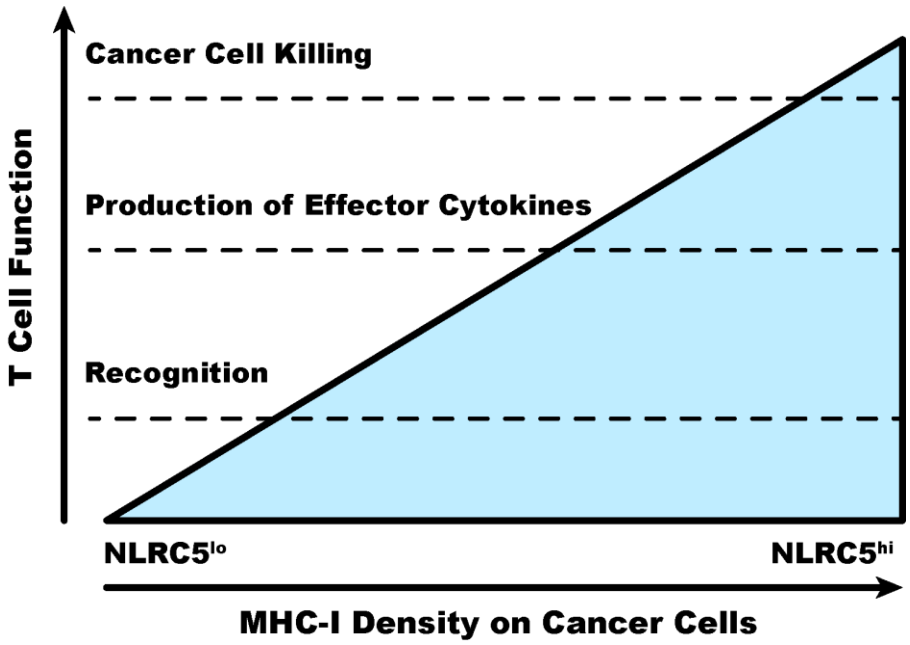

Fig. 4-1 Radiation-mediated induction of NLRC5 in cancer cells as a mechanism to enhance control by CD8+ T cells. The data presented in this manuscript support a model where effector CD8+ T cells differentially respond to target cells depending on expression of MHC-I on the cell surface, with different thresholds of activation for recognition (measured by Nur77 expression), cytokine production, and control/killing of target cells.

4.2 Future Directions

In this thesis, we have presented evidence to support a model where radiotherapy can upregulate antigen presentation on MHC-I on cancer cells by induction of NLRC5. These data add complexity to our previous understanding of MHC-I regulation by radiation, wherein augmentation of MHC-I expression might occur either downstream of mTOR activation and without modulation of gene transcription (229) or mediated by cancer cell intrinsic secretion of IFN-I and ligation of IFNAR, triggering transcription of interferon-stimulated genes and genetic upregulation of MHC-I (201, 275). Here we have shown that radiation can increase transcription of genes related to antigen presentation on MHC-I including NLRC5 in the absence of IFNAR signaling, demonstrating that alternative signaling pathways can play a role in MHC-I induction by cellular irradiation. While these insights aid in our understanding of how radiation can modulate cancer cell phenotypes to enhance anti-tumor immunity, significant gaps remain in our understanding of how the damage initiated by radiation triggers transcription of relevant genes in the absence of IFN-I as a cytokine mediator.

It is clear, based on the work presented here and elsewhere (229, 322, 323), that gene transcription and protein expression are altered following cellular irradiation, but it is unclear what cellular machinery and pathways facilitate these changes. Due to the wide-ranging roles of DNA repair apical kinases, which are activated downstream of radiation, in regulating chromatin structure, cell cycle, and gene expression (discussed in Chapter 1), we question whether activation of these protein kinases might mediate the transition of cancer cells from an immunologically silent phenotype to one which is capable of stimulating CD8+ T cells (e.g. through upregulation of antigen presentation). To our knowledge, a potential linkage between these two phenomena has not yet been elucidated and has the potential to directly tie the sensing of DNA damage within cancer

cells to enhancement of immunogenicity potentially selected for due to the potential for generation of neoantigens following incorporation of mutations by radiation-induced DNA damage. The question of whether ATR, ATM, or other members of DNA damage sensing pathways are essential to immune modulation of cancer may additionally be therapeutically relevant, considering that agents designed to sensitize tumors to radiotherapy by inhibition of DNA repair kinases including ATR and poly(ADP-ribose) polymerase (PARP) are being tested (324-328) and that therapeutically effective radiation regimes can require CD8+ T cells to control residual disease (20, 21). Thus, disruption of critical pathways required to allow CD8+ T cell sensing of cancer cells may increase susceptibility of patients treated with radiotherapy and DNA repair kinase inhibitors to disease recurrence.

While little is known about the link between DNA damage sensing and upregulation of MHC-I/NLRC5, studies indicate that ATR/ATM may be involved in regulation of expression of the immune inhibitory receptor PD-L1[5] (329-331). The gene encoding PD-L1, CD274, is located on human chromosome 9p.24.1 and is frequently upregulated by copy number expansion in human disease (332-335). Expression of PD-L1 in human cancers appears to be regulated by a variety of factors including STAT3, HIF1, MYC, AP-1, and NF-κB (335-339). Cursory studies using shRNA or pharmaceutical inhibitors of ATR suggest that expression of PD-L1 in cancer cells can additionally be regulated by phosphorylated ATR via Chk1 and IRF1 (329, 340), although the precise signaling cascade is yet to be elucidated. Similar work with pharmaceutical inhibition of ATM demonstrated that blocking signaling through this pathway decreased PD-L1 expression by cancer cells following irradiation (329),

[5] We and others have demonstrated that upregulation of PD-L1 and MHC-I are correlated following cancer cell irradiation or stimulation with interferon-gamma, suggesting that expression of these molecules are similarly regulated.

suggesting activated ATM kinase may also play a role in PD-L1 regulation. Together, this work suggests that expression of immune modulatory proteins such as PD-L1 and potentially MHC-I can be mediated by ATM/ATR/Chk1 kinases downstream of DNA damage induced by radiation.

It is important to note that the current paradigm regarding how radiation stimulates anti-tumor immunity is heavily focused on the role of cGAS/STING/IFNAR signaling and it is unclear to what degree this pathway mediates upregulation of PD-L1 downstream of radiotherapy. In the studies presented in this thesis, we disrupted IFNAR signaling and found MHC-I upregulation by radiation remained the same, but ATM/ATR signaling has been observed to enhance immunogenic signaling in cancer cells and antitumor immunity though cGAS/STING/IFNAR (275, 325, 329, 341). Interestingly, these effects are not necessarily dependent on induced damage by radiation and may be a result of chromosomal instability within cancer cells (342, 343). Due to the variable expression of STING pathway members in cancer models and human cancers (304, 306, 344, 345), it is of interest to determine the degree to which STING signaling is necessary for radiation-induced control of tumors by immune cells.

There are significant practical challenges to studying the role of DNA repair kinases in protein expression modulation following cellular irradiation. ATM, ATR, and Chk1 have integral roles in mediating DNA double-strand break (DSB) repair during mitosis (discussed in Chapter 1) and inhibition of these pathways results in significant sensitization of cancer cells to radiation-induced cell death. Accordingly, ATM/ATR deficiencies in cancer cells, which are commonly found in certain types human cancers and are associated with genomic instability, can are associated with greater sensitivity to radiation (346-350). While expression of ATM is important, it appears to be largely redundant in the presence of ATR, while a lack of functional ATR appears to be crucial

for dividing cells and $ATR^{-/-}$ mice demonstrate early embryonic lethality (351). It is unclear to what degree enhancing cancer cell death signaling may affect the results of studies aimed to elucidate the significance of pathways controlled by these kinases in phenotype modulation.

Once potential solution to alleviate some of the impact of enhanced sensitization of cancer cells to radiation-induced cell death may be to replace the randomized and catastrophic DNA damage triggered by cellular irradiation with induction of genetic damage in known and non-essential segments of DNA. Experimental methods have been developed to generate controlled DSBs through genetic engineering of cell lines to express Cas9 or restriction enzymes, triggering chromatin remodeling and activation of DNA repair pathways (322, 352). These methods can activate ATM/ATR signaling in the absence of radiation and may be useful in determining the phenotypic consequences of activating these pathways while avoiding significant activation of cell death pathway signaling. Notably, it is unclear to what extent the genetic damage initiated by these methods replicates radiation-induced damage and it would be necessary to quantify and compare the extent to which they activate relevant signaling.

In this thesis, we have focused on a model where radiation acts on cancer cells predominantly through the induction of DSBs in genomic DNA, it should not be ignored that radiation can cause significant damage to cancer cells through additional means and it is unclear to what extent these other types of damage, including single-stranded breaks (SSBs) and oxidative damage by ROS generation, play in modulation of cancer cell inflammatory phenotypes. *Roux et al* suggest that oxidative damage, as is initiated by cellular irradiation, can trigger PD-L1 expression by initiating NF-κB signaling (338); it is unclear whether NF-κB signaling downstream of oxidative damage can similarly augment expression of MHC-I and whether this signaling may occur in parallel with

NLRC5-mediated MHC-I upregulation (251). It may be of interest to determine which aspects of radiation-initiated damage signaling, including those stimulated by oxidative damage, are responsible for immune phenotype modulation of cancer cells. This may have additional implications for immune modulation by a wide range of chemotherapeutic agents, some of which have been shown to enhance anti-tumor immunity (201, 353, 354).

Taken together, the findings presented in this thesis complement existing work demonstrating that cellular irradiation can lead to transcriptional alterations within and suggest that these previously underappreciated alterations might have significant impact on the interactions between treated cancer cells and immune cells. It will be important to elucidate the signaling pathways involved in order to better target treatments to enhance therapeutic benefits and avoid deleterious effects. The studies proposed here provide a jumping-off point from which basic biological pathways linking radiation-induced cellular damage to inflammatory cancer cell phenotypes, but it will additionally be important to better understand how these treatments affect immune cell function in *in vivo* settings.

Appendix A: Radiation and Vaccine Combination Therapy

The ability of radiotherapy to modulate anti-tumor CD8+ T cell responses was well-established prior to the beginning of this thesis (26, 123, 126, 229, 298, 303), but we questioned whether radiation responses were limited by a lack of tumor-reactive CD8+ T cells able to respond to the newly altered tumor environment. Studies have demonstrated that radiotherapy can synergize with *Listeria* vaccines generating tumor model antigen-reactive T cell populations to control B16 melanoma and TRAMPC1 prostate tumors (355-357), suggesting that radiation can permit or enhance the activity of activated tumor-reactive CD8+ T cells in circulation.

In order to test whether radiation and *Listeria* vaccine synergized in our tumor models, we implanted mice with SCCVII-EGFRvIII tumors and treated the tumors with radiation and live-attenuated *Listeria monocytogenes* vaccines expressing a peptide antigen derived from EGFRvIII, EEKKGNYV (*LmEGFRvIII*) or a expressing an unrelated peptide (*LmOva*) (Fig. A-1A). In this model, radiation significantly extended the survival of mice inoculated with tumors but this effect was not enhanced by vaccination. In order to determine whether vaccination could improve the ability of radiation to control tumors in a model where radiation was poorly effective as a single therapy, we implanted mice with Panc02SIY tumors, which express the model antigen SIYRYYGL (SIY), and treated tumors similarly with radiation and *Listeria* vaccines expressing the model antigen peptide SIY (*LmSIY*) or an unrelated control (*LmOva*) (Fig. A-1B). In this case, none of the given therapies or combinations improved the responses of mice bearing Panc02SIY tumors.

In published reports where radiation and *Listeria* combinations were effective (355-357), the vaccine was given after radiation as in the experiments described in Figures A-1A and A-1B. We questioned whether establishing a large population of tumor-reactive, activated CD8+ T cells prior to tumor irradiation might improve the ability of radiotherapy to mediate T cell control. In order to determine if vaccination prior to radiation might improve therapeutic control, we inoculated mice with Panc02SIY tumors and administered *LmSIY* or control *LmOva* 14 days (Fig. A-1Ci) or 3 days (Fig. A-1Cii) prior to tumor irradiation. However, these tumors were similarly unaffected by single agent or combination therapies.

These studies suggest that our tumor models are intrinsically resistant to control by vaccination as by radiation or exogenous agents, unlike the B16 and TRAMPC1 models previously published. In at least two of reports where vaccination with *Listeria* improved radiation responses, survival of tumor bearing mice was improved by antigen-specific vaccination alone (355, 357), suggesting that the tumors were already susceptible to T cell mediated control and that radiation enhanced these effects through unknown mechanisms. In tumor models where single agent *Listeria* vaccination did not improve survival, such as SCCVII-EGFRvIII and Panc02SIY, radiation was not able to alleviate tumor suppression of vaccine-induced T cells. These studies emphasize that certain tumor types are more resistant to control by T cells and further studies are required to determine the mechanisms of model-specific T cell suppression.

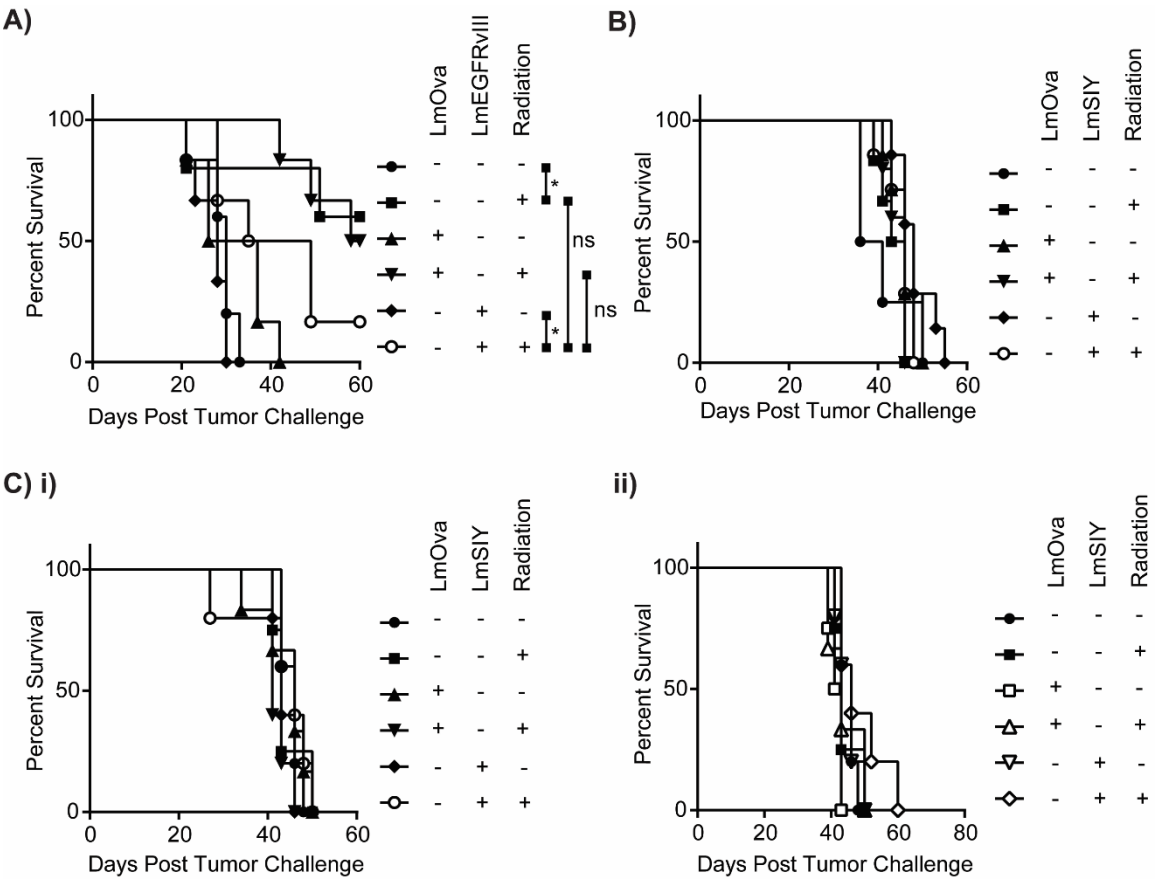

Fig. A-1 Radiation efficacy does not improve when combined with antigen-matched *Listeria* vaccination. A) Kaplan-Meier survival plot of mice implanted with SCCVII cancer cells expressing EGFRvIII and treated 6 Gy radiation 14 days later and/or with *Listeria-EGFRvIII* (*LmEGFRvIII*) or *Listeria-Ova* (*LmOva*) 17 days after tumor inoculation. B) Kaplan-Meier survival plot of mice implanted with Panc02SIY cancer cells and treated with 12 Gy radiation 20 days later and/or with *Listeria-SIY* (*LmSIY*) or *Listeria-Ova* (*LmOva*) 23 days after tumor inoculation. C) i) Kaplan-Meier survival plot of mice implanted with Panc02SIY cancer cells and treated with *LmSIY* or *LmOva* 6 days later and/or radiation 20 days after tumor inoculation ii) Kaplan-Meier survival plot of mice implanted with Panc02SIY cancer cells and treated with *LmSIY* or *LmOva* 17 days later and/or radiation 20 days after tumor inoculation. Key: *p<0.05; **p<0.01.

Materials and Methods

Animals and Cell Lines

Animal protocols were approved by the Earle A. Chiles Research Institute IACUC (Animal Welfare Assurance No. A3913-01). All experiments were performed in accordance with relevant guidelines and regulations. 5-8 week old C3H/HeJ mice (Stock #00659) and 5-8 week old C57BL/6 mice (Stock #000664) were purchased from the Jackson Laboratory (Bar Harbor, ME) for use in these experiments. Survival experiments were performed with 5-6 mice per group.

Cell lines were cultured in RPMI-1640 (HyClone, Fisher Scientific, Hampton, NH) supplemented with 10% heat inactivated fetal bovine serum (Cat#10082147, Thermo Fisher Scientific, Waltham, MA), 2mM L-glutamine (Cat#SH3003401, HyClone, Fisher), 10mM HEPES (Cat#HOL06, Caisson Labs, Smithfield, UT), 100U/mL penicillin-streptomycin (Cat#PSL01, Caisson), 1X non-essential amino acids (Cat#SH3023801, Fisher), 1mM sodium pyruvate (Cat#PYL01, Caisson).

The parental squamous cell carcinoma line SCCVII was kindly provided by Walter T. Lee (Duke Cancer Center Institute, Durham, NC). To generate SCCVII cells expressing EGFRvIII, cancer cells were transfected using Lipofectamine 2000 Transfection Reagent (Life Technologies, Carlsbad, CA) with MSCV-loxp-dsRed-loxp-eGFP-Puro-WPRE (Addgene plasmid #32702, gifted from Hans Clevers) and MSCV-XZ066-EGFRvIII (Addgene plasmid #20737, gifted from Alonzo Ross) and sorted on a BD FACSAria II cell sorter (Becton Dickinson, Franklin Lakes, NJ) for high expression of GFP (SCCVII-EGFRvIII) (280). The parental murine pancreatic adenocarcinoma cell line Panc02 was kindly provided by Dr. Woo (Mount Sinai School of Medicine, New York, NY). Panc02 expressing the model antigen SIY was kindly provided by Dr. Weishelbaum (University

of Chicago, Chicago, IL), as used previously(124), and expresses GFP-SIY in approximately 40% of cells. Panc02SIY100 was derived and expanded from a high GFP expressing single clone within Panc02SIY on a BD FACSAria II cell sorter.

Immunotherapy and Radiation Therapy of Tumors

Tumors were inoculated at a dose of 2 x 10^6 for SCCVII-EGFRvIII tumors, 5 x 10^6 for Panc02SIY tumors and 10 x 10^6 for Panc02SIY100. Tumor size was determined via caliper measurements of the longest length x the longest perpendicular width. Survival endpoint was defined as tumor size greater than or equal to 150mm^2 or when the mouse appeared moribund.

For *in vivo* irradiation, a Small Animal Research Radiation Platform (SARRP) (Xstrahl, Suwanee, GA) and Murislice software (Xstrahl) were used. For treatment, 6 Gy radiation was administered to SCCVII-EGFRvIII isocenters 14 days following tumor inoculation and 12 Gy of radiation was administered to Panc02-derived tumor isocenters 20 days following tumor inoculation.

ActA deleted (***ΔactA***) *Listeria monocytogenes* (*Lm*) strains used for vaccination were engineered to express the EGFRvIII peptide EEKKGNYV (*LmEGFRvIII*) as described previously (280), the ovalbumin peptide SIINFEKL (*LmOva*), or SIYRYYGL peptide (*LmSIY*) cloned in-frame with the *actA* N-terminal fragment. Bacteria were grown in brain-heart infusion broth, washed twice in PBS and administered by retro-orbital injection at a dose of 1 x 10^5 (C3H mice*)* or 1 x 10^7 (C57BL/6 mice) CFU in 100µL total volume. Effective vaccination was confirmed seven days later by MHC-multimer binding of peripheral blood.

Statistics

Data was analyzed and graphed using FlowJo (Tree Star, Ashland, OR) and Prism (GraphPad Software, La Jolla, CA). Kaplan Meier survival curves were compared using log-rank tests.

Appendix B: Enhancement of T Cell Trafficking by Tumor Irradiation

Lack of infiltration of T cells into tumors represents a significant barrier to the efficacy of immunotherapies including checkpoint inhibitors, and strategies to enhance T cell trafficking, turning “cold” tumors into “hot” tumors, are of keen interest to researchers and clinicians (358). Studies have suggested that radiotherapy can trigger the production of intratumoral T cell chemoattractants including CXCL9 (MIG), CXCL10 (IP-10) and CXCL16 (127, 128, 359), and can increase the expression of cellular adhesion molecules on tumor vasculature required for T cell migration from the blood into tumors (26, 126, 359). These studies led us to question whether radiation might function in our model to enhance T cell trafficking.

In order to determine whether radiation improved T cell trafficking into tumors, we irradiated Panc02SIY tumors implanted subcutaneously in mice and harvested tumors a week later. By immunohistochemistry, we observed significantly higher numbers of CD3+ cells within the tumors (Fig. B-1Ai and B-1Aii), suggesting an increase in T cell trafficking, in agreement with previous studies (20, 126). Previous studies have demonstrated that radiotherapy can increase representation of CD4+ regulatory T cells within tumors (360), but further characterization of infiltrating T cell subsets is required to determine whether this is the case here. In order to determine whether the infiltrating T cells were CD8+ T cells, we similarly treated tumors with radiation harvested the tumors for analysis by flow cytometry (Fig. B-1Bi). We observed that while radiation did not increase the proportion of CD8+ T cells of total live cells within tumors (Fig. B-1Bii), there was a significant increase in the proportion of T cells expressing CD8 (Fig. B-1Biii). Combined with the immunohistochemistry data, these results strongly suggest that

radiation can increase CD8+ T cell infiltration into tumors. In Chapter 2, we discussed whether increased numbers of T cells in tumors are sufficient to cause regression of tumors in this model.

Recruitment of T cells to inflamed tissue by chemokines is not antigen-specific; however, following activation of CD8+ T cells within the tissue, expansion of T cells can be observed. Previously we had observed no significant expansion in endogenous model antigen-specific CD8+ T cells in tumors treated by radiotherapy compared to untreated controls (Fig. 2-4Biii), but did not determine whether antigen-specific CD8+ T cells were preferentially retained or expanded intratumorally after radiotherapy. In order to determine whether tumor irradiation favored enrichment of antigen-specific CD8+ T cells over a fixed number of control T cells, we adoptively transferred *in vitro* activated 2C T cells with equivalent numbers of control OT-I T cells into $Rag1^{-/-}$ animals bearing dual flank Panc02SIY100 tumors and irradiated the tumor on one flank (Fig. B-1C). Seven days following radiation, irradiated tumors were significantly smaller than controls (Fig. B-1Ci), but there was equivalent enrichment of antigen-specific 2C cells in both tumors (Fig. B-1Ciii), suggesting that antigen-specific T cell trafficking or retention was not enhanced by radiotherapy.

Taken together, these studies suggest that in our model, radiotherapy does not significantly alter antigen-specific or bulk CD8+ T cell numbers to improve therapeutic outcomes, and support our hypothesis that here, other immune modulatory functions are responsible for enhanced CD8+ T cell responses driven by tumor irradiation.

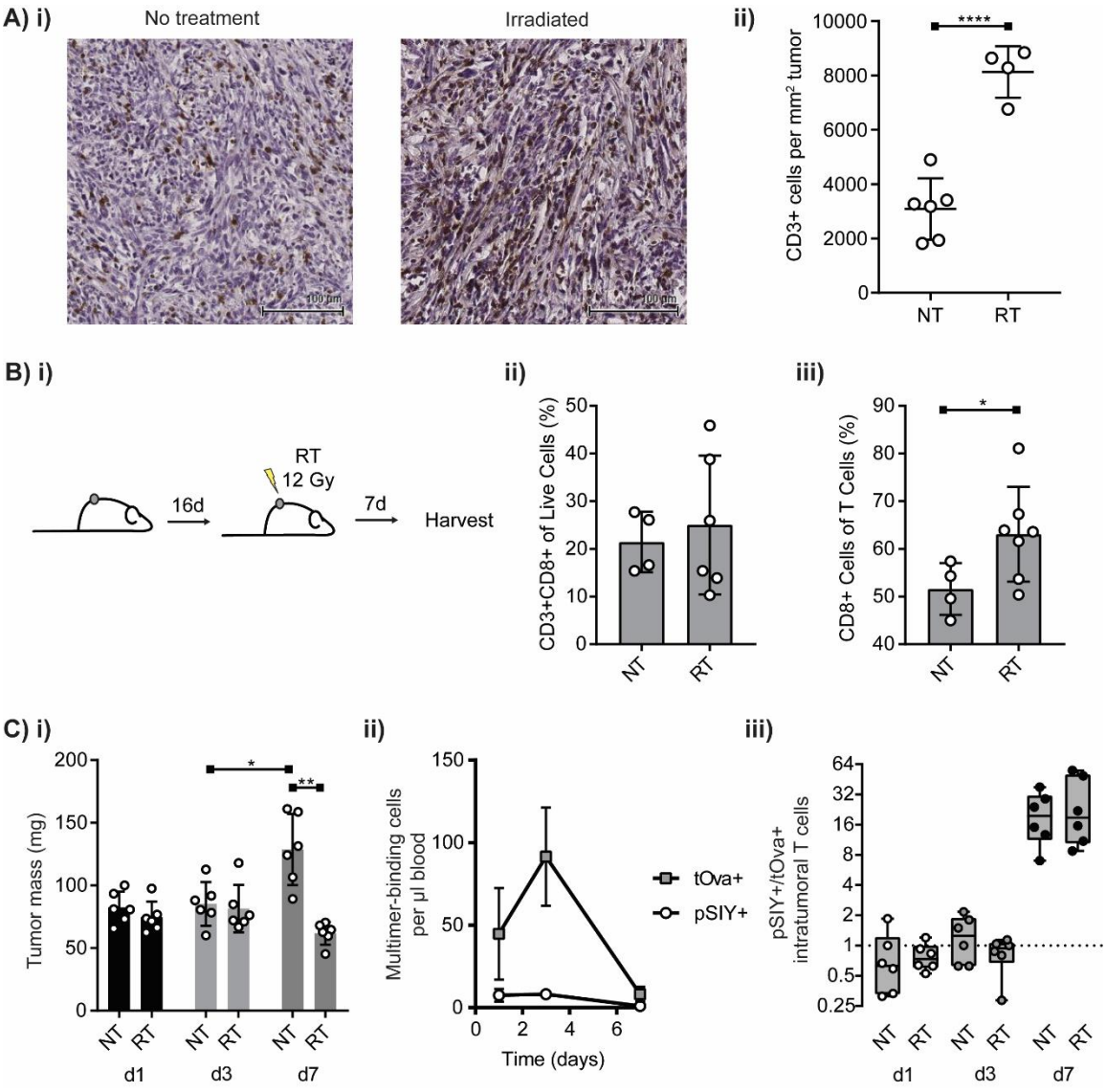

Fig. B-1 Radiation does not improve antigen-specific CD8+ T cell trafficking into SIYRYYGL-expressing tumors. A) i) Representative immunohistochemistry images from paraffin-embedded zinc-fixed Panc02SIY tumors harvested from mice treated with 12 Gy radiation (RT) or given no treatment (NT). Tumors were harvested at 27 days following tumor challenge. 5µM tissue sections were stained with CD3-DAB (brown) and counterstained with hematoxylin and eosin ii) Quantification of images represented in i) where each dot represents a representative section from an individual animal. B) i) Treatment schematic. C57Bl/6 mice were implanted with Panc02SIY tumors and irradiated (12Gy). Tumors were harvested and processed for flow cytometry ii) Proportion of tumor-infiltrating CD3+CD8+ T cells out of total live cells and iii) Proportion of CD3+CD8+ of total CD3+ cells. Each point represents one mouse. C) Rag1$^{-/-}$ mice

implanted with dual flank Panc02SIY100 tumors and were adoptively transferred with *in vitro* activated OT-I or 2C T cells 13 days following tumor challenge. At day 14, 12 Gy radiation was administered to tumors on one flank. Tumors and peripheral blood were harvested at day 1, day 3, and day 7 following irradiation. i) Mass of tumors not treated (NT) or giving radiotherapy (RT) harvested at indicated timepoints. Each point represents an individual tumor. ii) MHC-multimer (SIINFEKL-tetramer (tOva) or SIYRYYGL-pentamer (pSIY)) binding CD8+ T cells in peripheral blood at indicated timepoints. iii) pSIY-binding CD8+ T cells normalized to tOva-binding T cells in tumors receiving indicated treatments and harvested at indicated timepoints determined by flow cytometry. Each point represents an individual tumor. Key: *$p<0.05$; **$p<0.01$, ****$p<0.0001$.

Materials and Methods

Animals and Cell Lines

Animal protocols were approved by the Earle A. Chiles Research Institute IACUC (Animal Welfare Assurance No. A3913-01). All experiments were performed in accordance with relevant guidelines and regulations. 5-8 week old C57BL/6 mice (Stock #000664) and B6.129S7-Rag1^{tm1Mom}/J (Rag1$^{-/-}$) mice (Stock #002216) were purchased from the Jackson Laboratory (Bar Harbor, ME) for use in these experiments. Nur77^{GFP} reporter mice were kindly provided by Dr. Weinberg (Earle A. Chiles Research Institute, Portland, OR)(281). 2C transgenic mice were kindly provided by Dr. Gajewski (University of Chicago, Chicago, IL). OT-I transgenic mice were gifted by Dr. Redmond (Earle A. Chiles Research Institute).

Immunotherapy and Radiation Therapy of Tumors

Tumors were inoculated at a dose of 5 x 10^6 for Panc02SIY tumors and 10 x 10^6 for Panc02SIY100 tumors. Tumor size was determined via caliper measurements of the longest length x the longest perpendicular width. Survival endpoint was defined as tumor size greater than or equal to 150mm^2 or when the mouse appeared moribund.

For *in vivo* experiments, 12 Gy of CT-guided radiation was administered to tumor isocenters using a Small Animal Research Radiation Platform (SARRP) (Xstrahl, Suwanee, GA) and Murislice software (Xstrahl), 14 days after tumor implantation. 250μg per dose αPD-L1 checkpoint blockade (Cat#BE101, BioXCell, West Lebanon, NH) was administered intraperitoneally at day 7, 14 and 21 post tumor implantation.

For adoptive T cell transfer, naïve CD8+ T cells were harvested as splenocytes from naïve 2C or OT-I mice and activated *in vitro* with αCD3ε (Cat#BE0001-1, BioXCell) and αCD28 (Cat#BE0015-1) at a final concentration of 10μg/mL each. After 48 hours, cells

were rinsed with 10% complete RPMI supplemented with β-mercaptoethanol (βME) (Cat#21985023, Gibco, Thermo Fisher) and plated with 60IU/mL human recombinant IL-2 (Chiron) for three days. Prior to retro-orbital intravenous injection, CD8+ T cells were purified from splenocytes using a CD8α+ T cell negative isolation kit (Cat#130-104-075, Miltenyi), rinsed in sterile PBS and diluted to 1e6 cells per 100µl injection volume.

Tumor analysis

For flow cytometric analysis of tumor-infiltrating cells, tumors harvested seven days after treatment were chopped into small fragments and dissociated in a solution of 250 U/mL collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ) and 30 U/mL DNase (Millipore Sigma, St Louis, MO) using a GentleMACS tissue dissociator (Miltenyi Biotec, Auburn, CA). After 30 minutes incubation at 37°C, the digest was quenched in RPMI-1640 (Cat#SH30027LS, Fisher) supplemented with 10% FBS (Cat#16000069, Thermo Fisher) and 2mM EDTA (Cat#324504, Millipore Sigma) and strained through 100µM and 40µM cell strainers. Filtered cells were rinsed in cold PBS twice prior to counting and staining for analysis on a BD LSR II flow cytometer (Becton Dickinson).

Immunohistochemistry

Immunohistochemistry was performed on Zinc-fixed tumors embedded in paraffin preserved as described previously (282). Five micron sections were stained with primary αCD3 (SP7, Cat#ab16669, Abcam, Burlingame, CA) diluted in blocking buffer, secondary goat anti-rabbit IgG conjugated to HRP (Cat #AP1879, EMD Millipore, Burlingame, MA), and ImmPACT DAB Peroxidase (HRP) Substrate (Cat#SK-4105, Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin 7211 (Cat#S7439-1, Cardinal Health, Dublin, OH). CD3 infiltration was quantified using Aperio ImageScope (Aperio, Sausalito, CA).

Antibodies and Reagents

Viability staining was performed in PBS using Zombie Aqua Fixable Viability Kit (BioLegend, San Diego, CA) for 15 minutes prior to staining with fluorescently-conjugated antibodies for flow cytometry. Monoclonal antibodies were used against: CD3 [17A2], CD4 [RM4-5], and CD8α [53-6.7]. Fluorescently-conjugated MHC-multimer complexes were used as follows: tetramer-SIINFEKL (tOva, NIH Tetramer Core, Atlanta, CA), and pentamer-SIYRYYGL (pSIY, ProImmune, Sarasota, FL).

Statistics

Data was analyzed and graphed using FlowJo (Tree Star, Ashland, OR) and Prism (GraphPad Software, La Jolla, CA). Individual data sets were compared using Welch's T tests. Growth curves and analysis across multiple groups were analyzed using ANOVA (two-way and one-way, respectively).

Appendix C: NLRC5 Correlates in Human Pancreatic Tumors

In Chapter 3, we discussed the role of NLRC5 in regulating expression of genes related to antigen processing and presentation on MHC-I complexes (250, 361) and proposed a novel role for NLRC5 in augmenting MHC-I expression on cancer cells following radiotherapy. The role of NLRC5 in therapeutic responses in human cancer patients is currently under investigation (264, 265, 313), as well as mechanisms of tumor suppression of NLRC5 expression (263), with the hope of gaining insight into how to turn cancer cells into better targets for T cell killing (362). The potential role of radiotherapy in enhancing expression of NLRC5 in MHC-I^{lo} tumors is therefore of significant scientific and clinical interest.

Accordingly, we questioned whether NLRC5 expression in human pancreatic tumors could predict therapeutic outcomes. Using the open source resource cBioPortal, we obtained RNAseq data from surgical samples from the TCGA human pancreatic adenocarcinoma cohort. In order to determine if NLRC5 might play a role in enhancing antigen processing and presentation on MHC-I in accordance with previous studies, we plotted and identified positive correlations between gene expression values of NLRC5 and components of this antigen presentation pathway, including classical MHC-I subunits (HLA-A, HLA-B, HLA-C, and B2M), TAP1, and PSMB8 (gene for the immunoproteasome component LMP2) (Fig. C-1). Interestingly, we also identified positive correlation between NLRC5 and CIITA, the MHC Class II Transactivator, suggesting that these pathways might be similarly regulated.

In order to determine whether NLRC5 expression in pancreatic tumors correlated with prognosis, we divided patients into NLRC5-hi and NLRC5-lo cohorts and plotted survival. In this cohort, we found no significant differences in overall survival (Fig. C-2A)

or progression free survival (Fig. C-2B) between NLRC5-hi and NLRC5-lo expressing tumors. We questioned whether NLRC5 expression within the tumor could predict therapeutic responses to radiotherapy, but also found no significant difference in overall survival based on NLRC5 status (Fig. C-2C).

In the model presented in this document (Fig. 4-1), we proposed that radiotherapy could act on NLRC5^{lo} expressing tumors to enhance antigen presentation. The findings here demonstrate that low NLRC5 expression within tumors correlates with low expression of antigen presentation machinery (Fig. C-1), but the lack of predictive value of NLRC5 status in radiotherapy responses (Fig. C-2C) suggest that our simplistic model tells an incomplete story. In our murine model, radiotherapy requires alleviation of tumor immune suppression with αPD-L1 checkpoint inhibitors (Fig. 2-3) in order to efficiently clear tumors, suggesting there could be a role for immune suppressive signaling through known checkpoints within the tumor microenvironment. However, human pancreatic cancers are largely resistant to systemic therapies including checkpoint blockade (363), in part due the tendency of pancreatic cancer cells to encapsulate in desmoplastic stroma, composed largely of fibroblasts, extracellular matrix proteins, immune cells such as macrophages, and pancreatic stellate cells. This dense microenvironment is largely considered immune suppressive and additionally reduces perfusion of systemic drug therapies within the tumor (364, 365). Therefore, without addressing the differences in tumor microenvironment between subcutaneous murine models and human pancreatic tumors, NLRC5 status might still be poorly predictive of survival in patients even with the inclusion of checkpoint blockade therapy in treatment regimes.

Additionally, while we have focused in this thesis on the potential of radiation to induce anti-tumor immunity mediated by CD8+ T cells, it is well-established that radiation

can also have immune suppressive effects. In Chapter 4 we discussed the ability of radiation to upregulate PD-L1 expression by tumor cells, which is capable of dampening the ability of T cells and NK cells to response to disease (329, 339, 366). Additionally, radiation can trigger apoptosis in sensitive cell populations such as lymphocytes, including CD8+ T cells, at relatively low doses (129, 367, 368). Typical radiotherapy regimes, in which tumors are irradiated daily for several weeks, can thus dampen anti-tumor immunity (20, 117). Significantly, fractionated radiation is also linked to increased immunosuppressive macrophage infiltration into tumors, further dampening the ability of the adaptive immune system to respond to and control cancer cells (10, 20, 369). These tumor-associated suppressive phenotype macrophages are associated with poor prognosis in human pancreatic cancer (370). Together, these phenomena suggest that *in vivo* treatment of pancreatic cancer with radiation may have opposing effects in initiating anti-tumor immunity and further study is required to determine how the scales might be tipped in favor of tumor eradication.

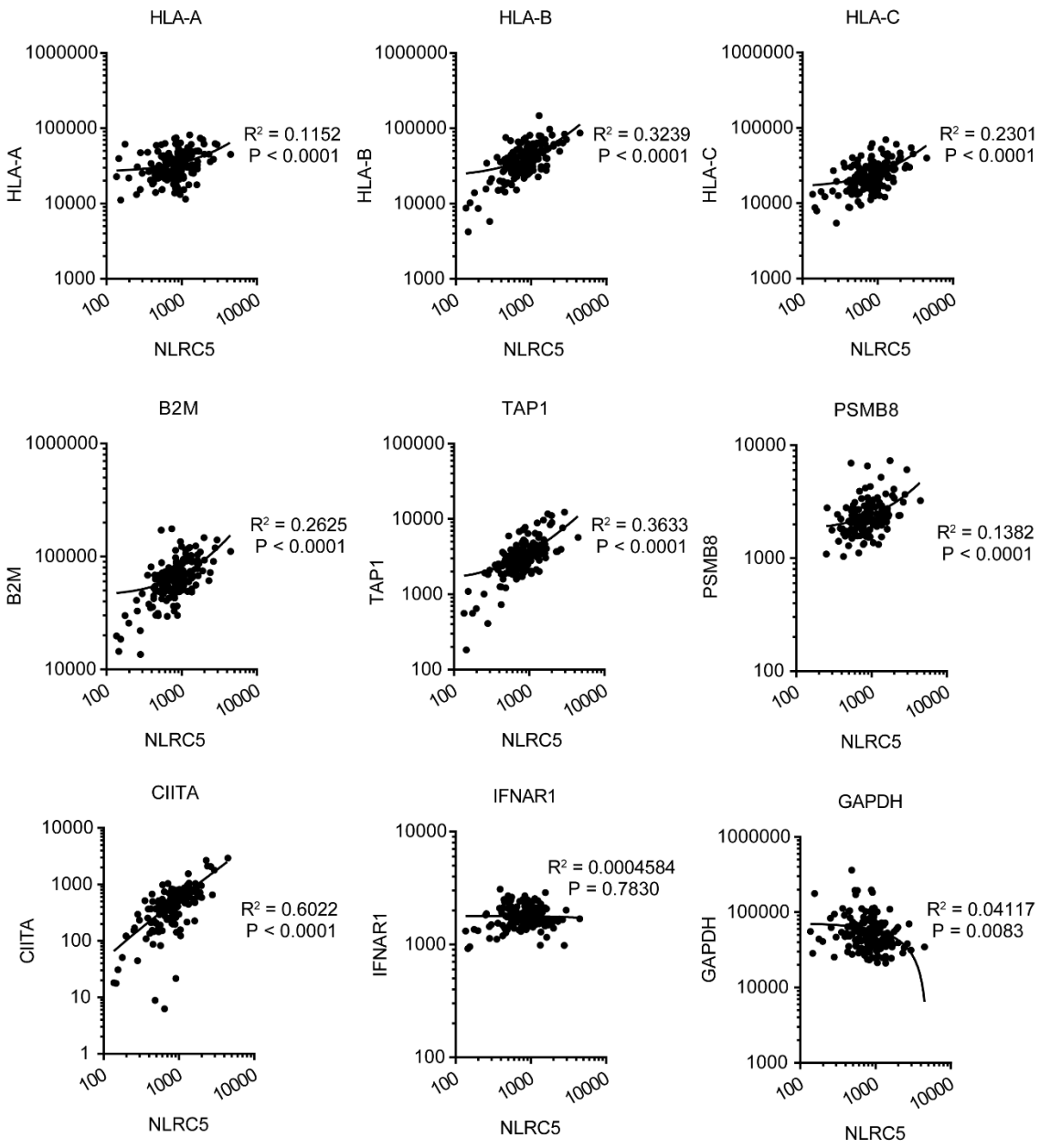

Fig. C-1 Correlation of NLRC5 gene expression with antigen presentation machinery in human pancreatic tumors. Correlation between NLRC5 gene expression and expression of genes related to antigen processing and presentation. RNAseq data obtained from TCGA PanCancer Atlas cohort for pancreatic adenocarcinomas. Pearson correlation coefficients and P values are given.

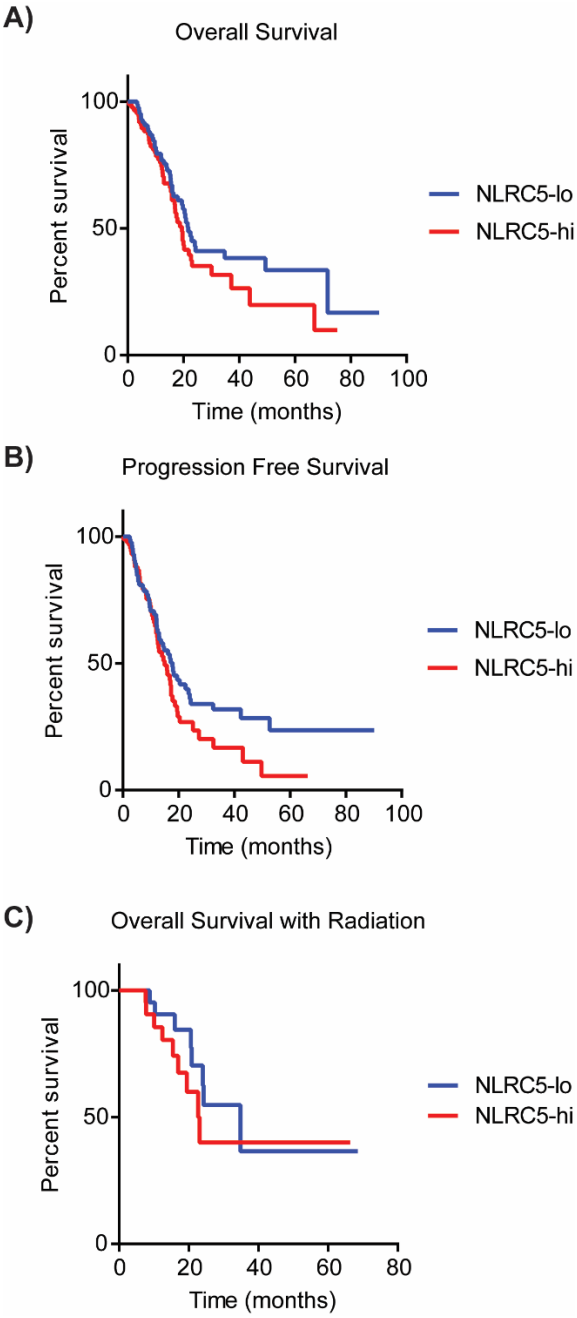

Fig. C-2 High expression of NLRC5 does not correlate with increased patient survival. A) Overall survival of patients having undergone surgical resection for pancreatic adenocarcinoma from the TCGA PanCancer Atlas cohort, segmented by high or low expression of NLRC5 quantified by RNAseq. B) Progression free survival for cohort as described in A. C) Overall survival for patients described in A which underwent post-surgical radiotherapy.

Materials and Methods

RNAseq of Human Pancreatic Tumors

RNAseq data from primary human pancreatic adenocarcinoma tumors (TCGA, PanCancer Atlas) for the TCGA dataset were downloaded from cBioPortal as previously described (371, 372). Of 184 patients in the cohort (35-88 years of age at diagnosis), RNAseq data was provided for 177 of which 167 included data about treatment with radiotherapy (43 received radiation, 123 did not). Patients did not receive neoadjuvant therapy prior to surgery. NLRC5-hi and NLRC5-lo groups were segmented by expression above or below the median expression for the cohort.

Statistics

Data was analyzed and graphed using FlowJo (Tree Star, Ashland, OR) and Prism (GraphPad Software, La Jolla, CA). Correlation was determined by linear regression analysis. Kaplan Meier survival curves were compared using log-rank tests.

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