The Role of Dendritic Cells in Radiation Mediated Tumor Regression

Tiffany C. Blair

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Certificate of Approval

This is to certify that the PhD dissertation of

Tiffany C. Blair

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Has been approved

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Mentor: Michael Gough, PhD

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Member/Chair: Timothy Nice, PhD

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Member: Andrew Weinberg, PhD

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Member: Evan Lind, PhD

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Member: Megan Ruhland, PhD

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List of Abbreviations

AhR	Aryl hydrocarbon receptor
APC	Antigen presenting cells
BM	Bone marrow
CD62L	L-selectin
cDC	Conventional dendritic cells
cDC1	Conventional type 1 dendritic cells
cDC2	Conventional type 2 dendritic cells
CDPs	Common dendritic cell progenitor
CLPs	Common lymphoid progenitors
CLRs	C-type lectin receptors
CMPs	Common myeloid progenitors
DC	Dendritic cell
DGE	Differential gene expression
dLN	Draining lymph node
ER	Endoplasmic reticulum
FLT3	FMS-like tyrosine kinase 3
FLT3L	FMS-like tyrosine kinase 3 ligand
HEVs	High endothelial venules
HMGB1	High mobility group box 1
HSP70	Heat shock protein 70
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferons
IPA	Ingenuity Pathway Analysis
Ii	Non-polymorphic invariant chain
LN	Lymph node
LXR	Liver X receptor
MDPs	Macrophage dendritic cell progenitor
MHC	Major histocompatibility complex
NLRs	NOD like receptors
pDC	Plasmacytoid dendritic cells
PGE_2	Prostaglandin E2
PRRs	Pattern recognition receptors
RIG-I	Retinoic-acid inducible gene-I
STING	Stimulator of interferon genes
TAP	Transporter associated with antigen processing
TdLN	Tumor draining lymph node
TLRs	Toll-like receptors

TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor

Contents

Abstract

Radiation therapy has been used for over a century to treat malignancies, but the mechanisms underlying its effectiveness as a cancer therapeutic remain poorly understood. Immune cells are capable of sensing and responding to signals released from dying tumor cells following radiation, and it is now appreciated that the immune system plays an important role in contributing to the overall response to treatment. Dendritic cells (DCs) in particular are critical for the generation of cytotoxic $CD8^+$ T cells that are capable of killing tumor cells. It is unclear how radiation therapy impacts the function of intratumoral DCs across different cancer pathologies. In our studies, we tested the hypothesis that DC maturation and migration is required for the immunological efficacy of radiation therapy. In tumors that utilize the adaptive immune system to enhance the baseline cytotoxic response to radiation, termed “radio-immunogenic” tumors, we demonstrate that radiation therapy successfully drives intratumoral DC maturation. Conversely, we show that DCs fail to mature in tumors that poorly mobilize the immune system in response to radiation. In these unresponsive tumors, administration of exogenous adjuvants that drive DC maturation leads to T cell- and DC-dependent tumor control. To test the hypothesis that DC migration to the lymph node (LN) is also required for the efficacy of radiation therapy, we used a mixed bone marrow chimera approach to block DC migration during treatment. We found that DC migration is required for radio-immunogenic tumor cure following radiation. Using photoconvertible mice to specifically track tumor migratory DC populations, we show that radiation therapy reduces the number of DCs migrating from the tumor to the tumor draining LN (TdLN) in poorly radio-immunogenic tumors. Finally, using transcriptional-based approaches, we identify that DCs in radio-immunogenic tumors upregulate genes associated with pattern recognition receptor activation by nucleic

acid sensors, and this fails to occur in poorly radio-immunogenic tumors. Taken together, our data demonstrate that DCs play a critical role in radiation mediated tumor regression and their capacity to signal through pattern recognitions receptors dictates their contribution to treatment response.

Chapter 1:
Introduction

Dendritic Cell Overview

BASIC FUNCTION IN IMMUNITY

Dendritic cells (DCs) were first discovered by Ralph Steinman and Zanvil Cohn in the early 1970s. Using microscopy Steinman and Cohn noted that within adherent splenocytes there was a rare cell type with stellate morphology, leading the two scientists to call this population “dendritic” cells [1, 2]. Steinman went on to demonstrate that DCs express high levels of major histocompatibility complex (MHC) proteins and showed that DCs are potent at initiating mixed leukocyte reactions [3, 4]. It was work from this group and others that determined that DCs were critical for the generation of cytotoxic T cells, providing the first evidence for the instrumental role of DCs in stimulating T cell responses within the immune system [5]. This group later discovered that DCs take up antigens and undergo a process termed “maturation” that significantly enhances their capacity to stimulate T cells [6]. Steinman’s pioneering research opened the door to a new field in immunology, the study of DCs, and as a result he was awarded the Nobel Prize in Physiology or Medicine in 2011. Since the initial discovery of DCs, many of the developmental pathways and processes that enable DCs to orchestrate T cell immunity to pathogens have been characterized. Beyond activating T cells, DCs also play an

important role in inducing peripheral tolerance to self-antigens [7]. Thus, DCs are critical gatekeepers of the immune system, capable of sensing cues in their environment to determine whether to initiate an adaptive immune response towards foreign pathogens or enforce tolerance to self-antigens.

Given DCs superior ability to prime naïve T cells, they are referred to as professional antigen presenting cells (APCs) [8]. Successful T cell priming requires that three signals be provided to the T cells by DCs in a coordinated succession [9]. Signal 1 occurs when DCs present cognate antigen loaded onto MHC molecules to T cells. For $CD4^+$ T cells, antigens are presented on MHC-II molecules, while $CD8^+$ T cells antigens are loaded onto MHC-I molecules. In order for effective priming to occur, T cells need additional signals from DCs in the form of co-stimulatory molecules which act as signal 2 to T cells [10, 11]. Cognate antigens presented on MHC molecules to T cells will fail to trigger naïve T cell activation in the absence of co-stimulation and instead results in T cell tolerance to those antigens [12]. DCs also produce cytokines that serve as signal 3 to further support T cell activation and proliferation [13]. To be proficient at providing each of these signals to T cells, DCs must go through a developmental process termed “maturation.” In the following sections we discuss the process by which DCs develop and how they become efficient at inducing T cell immunity. We will then focus on how these steps are disrupted in the context of tumor immunity and explore how radiation therapy can overcome some of these failures.

DEVELOPMENT & SUBSETS

DCs are a heterogenous group of cells consisting of multiple subsets, each with their own specialized function in immunity. The majority of DC subsets are relatively short lived, and as a result they are continuously replenished by precursors that develop in the bone

marrow (BM) during hematopoiesis (**Figure 1-1**) [14]. Adoptive transfer studies using common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) into irradiated mice have suggested that while both lineages are capable of giving rise to DCs, CMPs are significantly more efficient at generating DCs on a per cell basis [15-18]. The next step of DC development occurs when macrophage dendritic cell progenitors (MDPs) differentiate from CMPs and ultimately give rise to the common DC progenitors (CDPs) and monocytes [19-21]. Studies have demonstrated that CDPs express high levels of the receptor FMS-like tyrosine kinase 3 (FLT3) and these progenitors depend on the cytokine FMS-like tyrosine kinase 3 ligand (FLT3L) to support their expansion and development [22-24]. The majority of DC subsets differentiate from CDPs, including both conventional

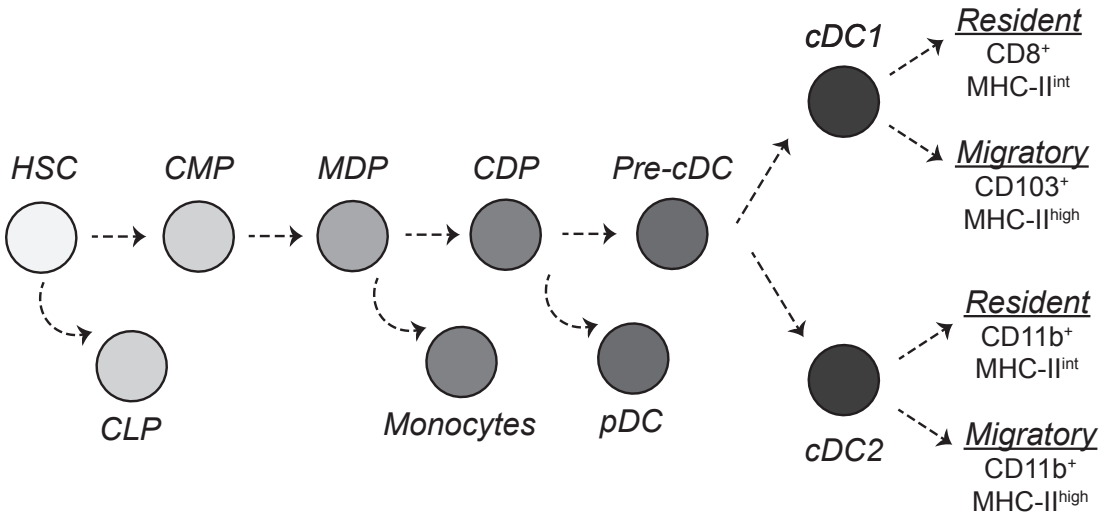

Figure 1-1: DC development. In the bone marrow DCs develop from hematopoietic stem cells (HSC) that first give rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CMPs then branch into monocytes and common DC progenitors (CDP). The CDP lineage is then split into plasmacytoid DCs (pDCs) and pre conventional DC (pre-cDC) progenitors. Both conventional type I DCs (cDC1) and conventional type II DCs (cDC2) derive from the pre-DC progenitors. Within cDC1s, there is a subset that is resident to lymphoid tissues ($CD8a^+$ $MHC\text{-}II^{int}$) and a subset with the capacity to migrate from the tissues ($CD103^+MHC\text{-}II^{high}$). cDC2s can be further divided into a subset that is resident to lymphoid tissues ($CD11b^+$ $MHC\text{-}II^{int}$) and a subset with the capacity to migrate from the tissues ($CD11b^+MHC\text{-}II^{high}$).

DCs (cDCs) and plasmacytoid DCs (pDCs) populations [20, 21]. In steady-state both cDC and pDC precursors circulate in the blood and lymphoid tissues, but only cDC precursors populate the non-lymphoid tissues [25].

There are multiple subsets of DCs and each have distinct functions in the immune system. pDCs are known for their unique plasma cell like morphology and their ability to secrete large quantities of type I interferons (IFNs) following viral infection, though there is limited evidence for their role as professional APCs in the tissues [26, 27]. cDCs on the other hand, are found in peripheral tissues where they excel as professional APCs and as a result are considered the classical DC type discovered by Steinman [28]. cDCs differentiate from pre-DC precursors that circulate in the blood, independent of monocytes, and can be defined by their expression of the zinc finger transcription factor ZBTB46, though this gene is not required for their development [29, 30]. There are two primary subsets of cDC, conventional type 1 DCs (cDC1s) and conventional type 2 DCs (cDC2s).

The cDC1 subset has a superior ability to prime $CD8^+$ T cell responses. This is due to cDC1s capacity to cross-present antigens, whereby they take up exogenous antigens and present them loaded on MHC-I molecules to $CD8^+$ T cells [31, 32]. *In vivo* DCs have been shown to be the primary cell type capable of cross-presenting antigens to $CD8^+$ T cells [33]. cDC1s are often defined by their expression of the transcription factors BATF3, IRF8, and ID2, all of which are critical for their development [34-36]. For example, mice lacking Batf3 have an impaired ability to cross-present antigens and as a result are unable to generate virus specific T cells or clear immunogenic tumors [34]. The surface markers CLEC9A, XCR1, CD8α and CD103 are useful when identifying cDC1 populations [37]. Using these markers, cDC1s can be further divided based on their location. Migratory cDC1s expressing $CD103^+$ are continuously sampling antigens in peripheral tissues and

have the capacity to traffic to the draining lymph node (dLN) via lymphatics [38]. Migratory CD103$^+$ cDC1s deliver antigens from the tissues to T cell zones in the LN, where they either directly present these antigens to CD8$^+$ T cells or share the antigens with other LN-resident DC populations [38-41]. Lymphoid organs also contain a population of non-migratory cDC1s that spend their entire lifespan in tissues such as the LN [42]. These resident cDC1s are often defined by the expression of CD8α^+ and have been shown to be capable of taking up antigens that either drain directly to from the tissues or antigens that are transferred to them from other migratory DCs populations [42, 43].

The other major cDC subset consists of cDC2s, which excel at presenting antigen loaded on MHC-II, and as a result they have been shown to be potent at priming CD4$^+$ T cell responses [37, 44]. In general, the cDC2 subset is significantly more heterogenous than cDC1s. However, the transcription factors that appear to be important for cDC2 development are IRF4, RBP-J and NOTCH2 [45-48]. Surface markers that are useful in differentiating cDC2s from other DCs subsets include the integrin CD11b and SIRPα [49]. Similar to cDC1s, there are cDC2s with migratory capacity that are constantly surveying peripheral tissues and those that are resident to lymphoid tissues, although markers differentiating the two subsets are lacking. Tissue migratory cDC2s are often delineated as being MHC-II high in lymphoid organs, whereas LN-resident cDC2s are typically identified as being MHC-II intermediate [25]. Differentiating resident versus migratory cDC2s becomes more difficult during inflammation as both subsets become mature and express high levels of MHC-II.

ANTIGEN PROCESSING & PRESENTATION

DCs are phagocytes that use a range of receptors to take up antigens in their environment. Within the tissues, immature DCs are constantly surveying tissues by

sampling antigens in their surroundings and its only when they receive inflammatory signals do they become mature and begin to process the antigens they have acquired for presentation to T cells [50-52]. By shutting down antigen uptake this ensures that DCs will only present antigens that were captured in the vicinity of inflammatory stimuli, such as viruses and bacteria, thus preventing them from taking up self-antigens on the way to lymphoid tissues that could potentially trigger autoimmunity. As DCs switch from antigen uptake to antigen processing and presentation, they undergo morphological changes whereby their dendrites become elongated and extend outward from the cell body. These dendrites are thought to promote interactions with T cells during antigen presentation. Exogenous antigens that are taken up by DCs are processed and are diverted to one of two paths depending on whether they are going to be loaded onto MHC-I molecules versus MHC-II molecules for presentation to T cells.

Internalized antigens that are processed for MHC-II restricted presentation to $CD4^+$ T cells are targeted to endo/lysosomal compartments where they are degraded by proteolysis [53]. DC maturation signals have been shown to result in increased acidification of lysosomes which activates proteases that degrade the antigens into peptide fragments [54, 55]. While the decreased pH in these compartments favors antigen degradation, the pH in the endo/lysosomes of DCs is still higher than the same compartment in macrophages which completely destroy antigens, suggesting that there is a balance in DCs between antigenic proteolysis and complete destruction of proteins that can no longer be loaded on MHC-II molecules [56]. The decreased pH in these compartments also facilitates the removal of the non-polymorphic invariant chain (Ii) protein from Ii-MHC-II complexes in endo/lysosomal compartments [54]. Removal of Ii from MHC-II enables antigen peptides to be loaded onto the now free MHC-II complexes and trafficked to the plasma membrane for presentation to $CD4^+$ T cells [57]. Maturation

signals have been shown to increase both the transcription of MHC-II and its translocation to surface of cells [58, 59].

Antigens taken up by DCs for MHC-I-restricted presentation to $CD8^+$ T cells via cross-presentation go through a different process than MHC-II restricted antigen processing. Most cells are capable of processing proteins and loading them on MHC-I molecules using the classical MHC-I antigen presentation pathway. In this pathway intracellular proteins are degraded by the proteasome in the cytosol into peptide fragments, and then translocated by transporter associated with antigen processing (TAP) protein into the endoplasmic reticulum (ER), where they are then loaded onto MHC-I molecules [60, 61]. However, cross-presentation of antigens derived from outside of the cell requires a different process. Two pathways have been proposed to contribute to exogenous peptide loading on MHC-I molecules: the vacuolar pathway and the cytosolic pathway. In the vacuolar pathway, antigens are processed and loaded onto MHC-I molecules directly in endo/lysosomes and this process does not require proteasomal degradation of peptides or TAP [62-64]. The cytosolic pathway requires antigens first be transferred from the lumen of phagocytic compartments into the cytosol [65]. Once these antigens are in the cytosol, they are then processed similarly to endogenous intracellular proteins. This process includes proteasomal degradation, transport into the ER via TAP proteins and loading onto MHC-I molecules to be carried to the surface of DC [66, 67]. Antigens presented on MHC molecules by DCs serve as signal 1 to initiate T priming.

MATURATION

For DCs to successfully prime T cell responses they must first receive signals from their environment that induce their maturation (**Figure 1-2**). DCs that seed both lymphoid and peripheral tissues exist in an immature state, whereby they excel at capturing

antigens, but they don't efficiently present these antigens to T cells. However, in inflammatory settings such as infection, pathogen or cell damage associated products are released into the environment and these can serve as signals that induce DC maturation. DCs express various types of pattern recognition receptors (PRRs) capable of sensing pathogens and other types of molecular damage signals. There are several families of PRRs that can be expressed by DCs, including toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD like receptors (NLRs) and cytosolic nucleic acid sensors such as stimulator of interferon genes (STING) or retinoic-acid inducible gene-I (RIG-I) [68]. Each DC subset expresses a unique set of PRRs, enabling them to detect different types of pathogens or damage throughout the body. For example, cDC1s are known to express CLEC9A, a CLR which enables them to phagocytose dying cells and the toll-like receptor

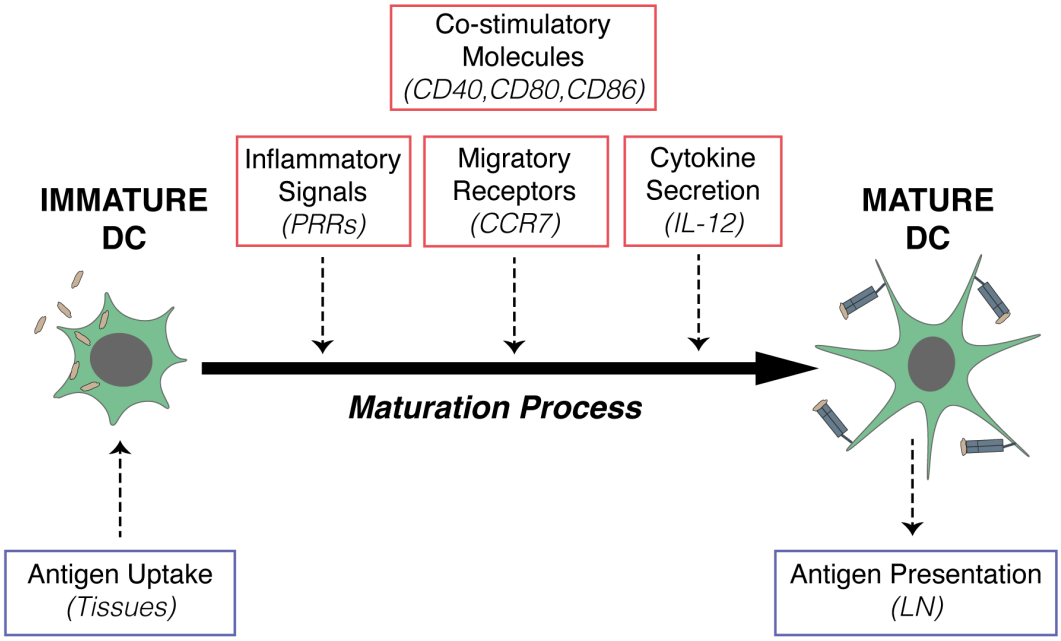

Figure 1-2: DC maturation. Immature DCs excel at taking up antigens in the tissues. When inflammatory signals trigger signaling through PRRs this initiates the start of the DC maturation process. DCs begin to upregulate expression of co-stimulatory molecules (CD40, CD80 & CD86), receptors (CCR7) that are important for migrating to LN and cytokines (IL-12) that further support the activation and proliferation of T cells. Mature DCs excel at presenting antigens to T cells.

3 (TLR3) which recognizes double stranded RNA that is often associated with viral infection [69, 70]. Signaling through either one of these PRRs has been shown to promote the cross-presentation of captured antigens by cDC1 subsets [71-74]. Additionally, signaling through IL-1R and tumor necrosis factor receptor (TNFR) family members can also induce DC maturation [75, 76]. Many of the DC maturation signals described above trigger the activation of NF-kB and IRF3/7 signaling pathways, resulting in the transcription of cytokines such as TNFα, IL-1β and type I IFN that can signal back on DCs to further promote their maturation [77].

After receiving maturation signals, DCs then begin to upregulate co-stimulatory molecules that further support T cell activation, and these serve as signal 2 during the priming process. Some of these co-stimulatory molecules include the immunoglobulin superfamily members, CD80 and CD86, and the tumor necrosis factor (TNF) superfamily member, CD40 [58, 78, 79] . The co-stimulatory molecules CD80 and CD86 which are expressed by mature DCs binds to CD28 that is expressed on T cells and this interaction further amplifies peptide-MHC signaling through the T cell receptor. While CD80 and CD86 provide signals to the T cells themselves, CD40 actually signals within the DCs and facilitates a process termed licensing. Licensing requires that cognate CD4 T cells are first primed by DCs which then causes the T cells to upregulate CD40L. CD40L expression on $CD4^+$ T cells then enables them to signal through CD40 expressed on DCs [80]. Signaling through CD40 on DCs leads to increased cytokine production by DCs, including the cytokine IL-12, which supports the activation of cognate $CD8^+$ T cells [81, 82]. Signaling through CD40 can also function to further promote the upregulation of CD80, CD86 and MHC-II on DCs [83]. The cytokines released by licensed DCs act as signal 3 to T cells and without this signal the T cells don't become fully activated and functional. Finally,

maturation signals cause DCs to upregulate receptors that are important for trafficking to T cell zones in the dLN, including the chemokine receptor CCR7, as we will discuss below [84]. Fully mature DCs are capable of priming productive T cell responses.

TRAFFICKING

DC progenitors that exit the BM into the circulation must express adhesion molecules and chemokine receptors that enable them to enter and seed both lymphoid and non-lymphoid tissues throughout the body. Pre-cDC progenitors are thought to enter lymphoid tissues like the LN through high endothelial venules (HEVs) and antibodies that block L-selectin (CD62L) on these precursors prevents their accumulation around HEVs [85] . In steady state conditions it is unclear what signals drive DC progenitors to seed non-lymphoid tissues. However, under inflammatory conditions the chemokine receptors CCR2, CCR5 and CCR6 have been shown to play an important role attracting DC progenitors that are circulating in blood into non-lymphoid tissues [86-88]. Moreover, DC migration from the non-lymphoid tissues to LN is important for co-localizing T cells and DC together to initiate T priming. The chemokine receptor CCR7 has been shown to be essential for the migration of DCs from the tissue to the dLN [84, 89]. DC maturation signals cause tissue migratory DCs to upregulate CCR7 and this enables DCs to follow chemotactic gradient of CCL19/CCL21 through the lymphatics to T cell zones within the dLN, and mice lacking CCR7 have impaired migration of DCs from the tissues [90-93]. In steady state conditions where there is an absence of DC maturation signals, there can still be some low level of DC migration from the tissues to dLN [84]. However, these DCs are only semi-mature, expressing low levels of co-stimulatory markers, making them poor stimulators of naïve T cells and instead proficient at inducing T cell tolerance to antigens [84, 94, 95]. CXCR4 is another chemokine receptor is also involved in DC migration to the

LN by binding its ligand CXCL12 [96]. Maturation signals significantly enhance the number of DCs migrating from the tissue and enables them to prime antigen-specific T cells in the dLN.

Initiation of Tumor Immunity

CANCER-IMMUNITY CYCLE

What makes a tumor susceptible to being destroyed by the immune system? If we take the knowledge acquired from immunity to pathogens and apply it to cancer, we end up with the cancer-immunity cycle proposed by Chen and Mellman (**Figure 1-3**) [97]. Thus, in order to generate tumor-specific T cells capable of killing tumors the following steps need to occur in succession; 1) tumor antigens and PRR signals must be released; 2) APC need take up these antigens and mature; 3) APC need to migrate to the dLN; 4) APC must to present antigens to T cells; 5) T cells traffic to the tumor; and 6) T cells then initiate tumor cell killing [97]. Thus, completion of this cycle would lead to successful recognition and rejection of tumor cells by the immune system. These rejected tumors would then be considered immunogenic as they are effectively cleared by the immune system. The classic definition of tumor immunogenicity comes from murine tumor models, where mice are injected with irradiated cancer cells, given a sublethal dose, or given a lethal dose followed by surgical resection, and evaluated for their ability to reject a subsequent challenge with a normally lethal dose of the same tumor [98-101]. If the tumor fails to grow upon secondary tumor challenge, then it is classified as immunogenic. However, this classic definition of immunogenicity does not break down the mechanisms of immune rejection, which may result from a failure to successfully initiate the cancer-immunity cycle in the first place, tumors themselves being resistant to effector destruction

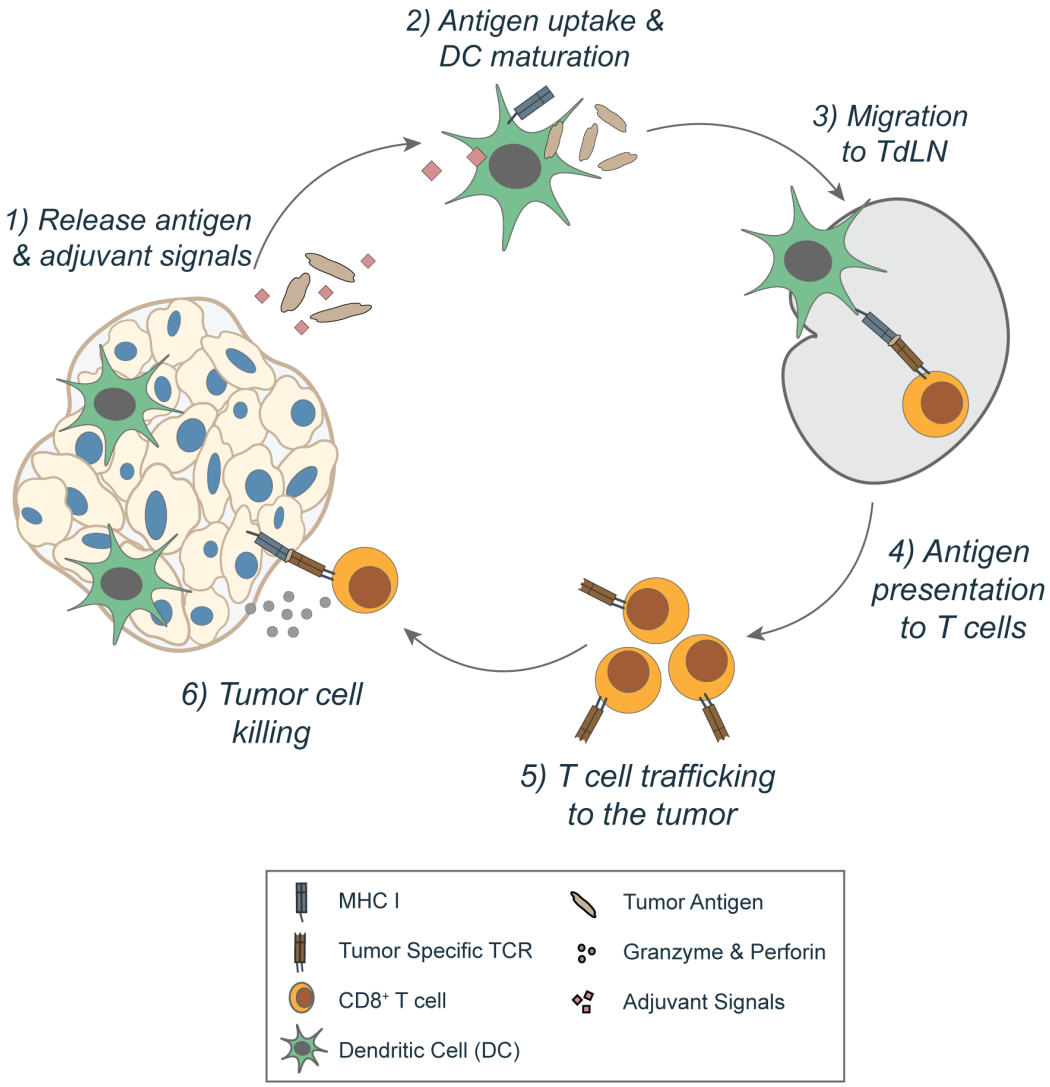

Figure 1-3: The cancer-immunity cycle. To generate a productive anti-tumor immune response the following events need to occur; 1) tumor antigens and adjuvant signals need to be released from the tumor; 2) tumor antigens must be taken up by DCs and the adjuvant like signals must induce DC maturation; 3) DCs need to migrate to the TdLN; 4) DCs must present tumor antigens to $CD8^+$ T cells; 5) these T cells have to traffic to the tumor; and 6) the $CD8^+$ T cells then directly kill the tumor cells.

or some combination of the two. While multiple mechanisms have been documented detailing tumor resistance to effector destruction, below we will focus on the events that lead to the initial priming of tumor-reactive T cells.

In the absence of therapy how do tumors initiate priming of $CD8^+$ T cells? Given DCs superior ability to prime T cells it would make sense that DCs are critical to development

of tumor-reactive T cells and thus the cancer-immunity cycle. As described above, cDC1s in particular excel at cross-priming $CD8^+$ T cells and thus this subset likely plays an important role in generating T cells capable of destroying tumors. This is evident in mice that lack the transcription factor Batf3 which is important for cDC1 development, and as a result are unable to clear highly immunogenic tumors. Moreover, $CD8^+$ T cell responses are most efficiently generated via coordinated $CD4^+$ T cell help, which indicates that cDC2s potentially play an important role in a comprehensive T cell response to tumor-associated antigens [102-104]. Beyond the actual presence of cDCs in the tumor, other components to consider when evaluating the successful priming of tumor-reactive T cells include whether tumor antigens are available in the first place and how these antigens become accessible to DCs. Commonly, antigen release is discussed as a part of cancer therapies, such as following chemotherapy or radiation therapy that result in cancer cell death; however, this does not explain how immune responses develop in untreated tumors in the absence of therapy [105]. Finally, immunostimulatory signals must be present after DCs have taken up tumor antigens to induce their maturation. In the following sections we will explore how each of these components can contribute to the immunogenicity of tumors (**Figure 1-4**).

MECHANISMS OF ANTIGEN RELEASE

To evaluate how tumor antigens are released, we will first consider commonly used preclinical models whereby a bolus of syngeneic murine cancer cell lines is implanted into immune competent mice. One of the artifacts of this system is that implanting a bolus of cells into immune competent murine models can function as an immunological vaccine event, resulting in an immune response directed towards cancer cells [106-110]. This immune response generated following tumor implantation can initiate the development of

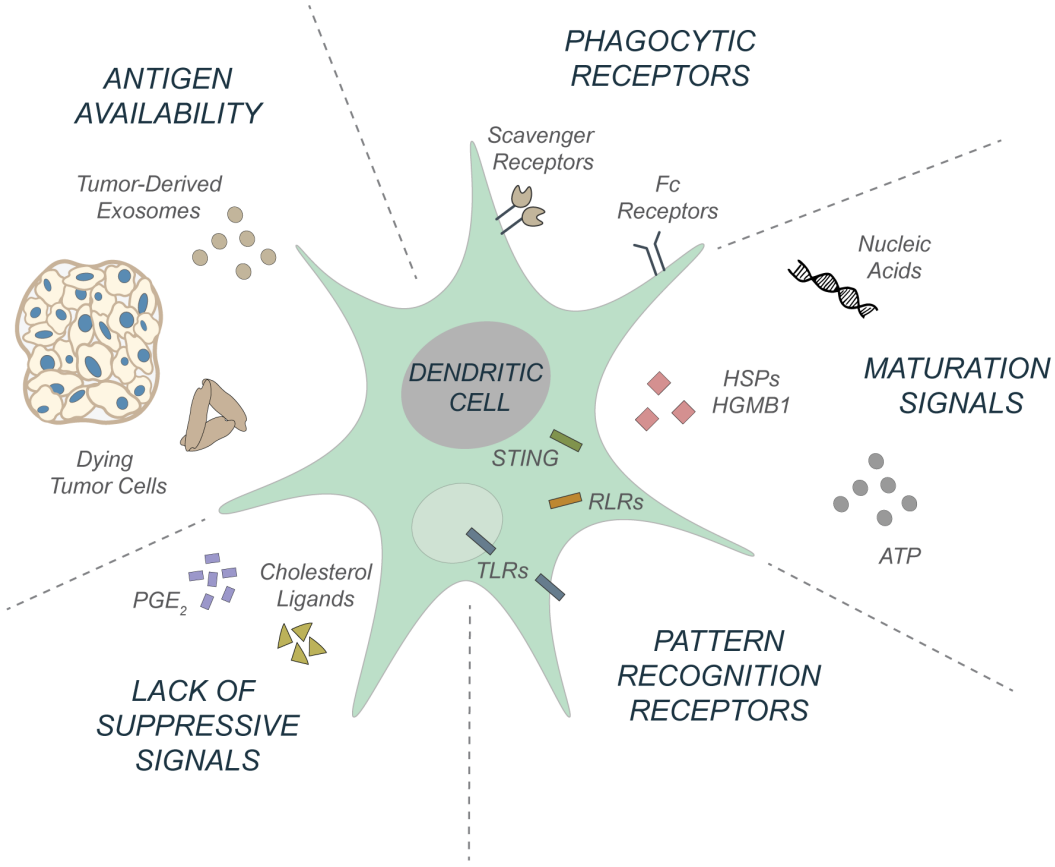

Figure 1-4: Early requirements for spontaneous priming of tumor-reactive T cells. To prime T cells capable of killing tumor cells antigens need to be present and available in tumors. DCs need to be present and express phagocytic receptors that enable them to take up antigens. After DCs take up antigens, maturation signals need to be present, and DCs must express the proper pattern recognition receptors (PRRs) to respond to maturation signals. In addition, there needs to be a lack of suppressive signals that prevent DCs from maturing. HSPs = heat shock proteins. RLRs = RIG-I-like receptors. TLRs = toll-like receptors.

tumor-specific $CD8^+$ T cells capable of driving the development of a tumor-specific immune response [106-110]. The underlying mechanism for this response requires dead or dying cancer cells to be presented by DCs, as tumors that are normally spontaneously rejected in mice are able to grow in $Batf3^{-/-}$ mice that lack cross-presenting DCs [34]. Thus, cancer cell injection into immune competent mice can serve as a vaccine event that generates an initial $CD8^+$ T cell response via cross-presenting DCs.

In patients, or in mouse models of progressive tumorigenesis that occur without cancer cell injection, how are antigens released? Without the initial bolus of cancer cells to provide cellular debris that may serve as a vaccine event, other mechanisms are

required to generate immunity. In such cases, to generate T cell responses, cancer cells must transfer antigenic material to DCs in another manner. Soluble cancer-associated antigens can be released from tumor cells – for example PSA is secreted from prostate cancer cells and can be a T cell target for immunotherapy, and mesothelin can be released from pancreatic cancer cells and also serves as a T cell target [111, 112]. However, the majority of cellular proteins are not secreted, and therefore will require transfer of cellular material for their uptake by DCs. Cancer cells have been shown to release exosomes, which can deliver tumor-associated antigens directly to DCs [113-116]. Engineering a tumor to direct model tumor antigens to exosomes resulted in increased tumor immunogenicity, with significantly slower tumor growth than matched tumors directed to secrete the same antigen, and this growth delay was dependent on an intact immune system [115]. Tumors with antigens directed to exosomes were also more immunogenic than those with antigens directed to non-secretory components [117], indicating that the subcellular localization of antigens may be a critical feature of immunogenicity or immunodominance of an individual neoantigen. Importantly, redirection of potential antigens to autophagosomes can increase the immunogenicity of the tumor by generating vesiculated particles that are efficiently cross-presented, which may provide an option to increase the immunogenicity of tumors where the potential antigens are not generally directed to exosomes [118, 119].

Tumor cell death is an alternative mechanism for antigens to be released and taken up by DCs. Despite common resistance to cell death in cancer cells, DNA damage and metabolic stressors can result in cancer cell death and is particularly pronounced as their growth outstrips the supply nutrients in their environment [120, 121]. Multiple types of cell death have been described; however, the two most extensively studied forms are apoptosis and necrosis [122]. Apoptotic cell death is typically thought of as being

immunologically silent [123, 124], as compared to necrotic cell death which results in the release of inflammatory signals [125, 126]. However, recent work has suggested these pathways are more nuanced and depending on the circumstances both pathways can lead to the release inflammatory signals [127-130]. Thus, some tumors might be classified as poorly immunogenic because they are more resistant to cell death, resulting in a failure to release adequate tumor antigens for T cell priming.

Alternatively, a cancer cell that is proportionally more resistant to apoptosis may still die if the environment is sufficiently toxic, but through non-apoptotic mechanisms [131]. A high rate of cell death, in a region of the tumor can overwhelm local phagocytic capacity and result in necrosis. Necrotic material includes a range of endogenous adjuvants with varying ability to stimulate immune responses to associated proteins [126]. However, in patients, the presence of pathological necrosis in their tumor is generally associated with poor outcomes across a range of malignancies [132-135]. There are likely a wide range of conflicting mechanisms at work in a tumor with extensive pathological necrosis, since a high level of cancer cell death is often correlated with a high rate of cancer cell proliferation [136], and necrotic regions are enriched for macrophages [137] that drive biological pathways to repair necrotic damage and have been shown secrete factors capable of suppressing DCs [138, 139]. These data suggest that the most efficient means of antigen transfer to DCs is not necessarily related to high rates of cancer cell death but may depend on the specific mode of cell death, the means of antigen transfer to DCs and the types of signals present during antigen capture.

PRESENCE OF DC SUBSETS

Released tumor antigens will ultimately fail to trigger an immune response unless professional APCs are around to take up these antigens. As discussed above, DCs excel

as professional APCs and multiple DC subsets exist, each with their own specialized function in immunity [25]. Thus, in addition to considering the availability of suitable antigens in tumors, the appropriate type of DC still needs to be localized in the vicinity of these released antigens to initiate T cell priming. cDC1s are particularly potent at priming cytotoxic $CD8^+$ T cell responses [140]. Mice entirely lacking cross-presenting DCs via deletion of the cDC1-specific transcription factor Batf3 fail to develop anti-tumor T cell responses and even highly immunogenic tumors that are ordinarily rejected can grow out in these mice [34]. Increased cDC1s signatures in patient tumors often correlates with improved survival [141-143]. Moreover, in tumors with very few cDC1s at baseline, administration of drugs that expand cDC1s numbers in the tumor results in improved responses to therapy in murine models [144, 145]. This raises the question, why do some tumors have more cDC1s than others?

cDC1s have been shown to have some limited proliferative capacity in peripheral sites, but they are typically short lived and need to be continuously replaced in the tissues by cDCs precursors from the blood [85, 146, 147]. The chemokine receptors CCR1, CCR5 and CCR6 have been implicated in the recruitment of cDC precursors from blood into tissues, though these requirements likely change during tissue inflammation [86-88]. Spranger et al. reported that in their melanoma model, tumor intrinsic β-catenin signaling leads to decreased CCL4 production by tumor cells and impaired recruitment of CCR5 expressing cDC1s into the tumor, ultimately resulting in a failure to prime anti-tumor $CD8^+$ T cell responses [148]. NK cell derived XCL1 has also been shown to promote the mobilization of XCR1 expressing cDC1s into tumors and this recruitment is inhibited in tumors that secrete prostaglandin E_2 (PGE_2) [142]. These data suggest that different tumors may actively secrete factors that either promote or suppress the recruitment of

cDCs to the tumor, and this regulation determines whether successful T cell priming occurs.

ANTIGEN CAPTURE

To take up antigens cDC1s must express receptors that enable them to phagocytose dead or dying cells. These include some of the key markers of the DC lineage, such as DC-SIGN, CLEC9A, DEC-205, FC receptors and DCIR [149-153]. CLEC9A for example is a CLR that binds to F-actin filaments that are exposed on dying cells and diverts these antigens be processed in the cross-presentation pathway [151, 154]. AXL is another receptor expressed by DCs that is capable of indirectly recognizing apoptotic cells through Gas6 which is bound to phosphatidylserine on the outside of dying cells [155]. Moreover, tumor cells themselves have been known to express signals that might prevent them from being recognized and phagocytosed by DCs in the first place, including the “don’t eat me” signal CD47 [156]. Elimination of CD47 on tumor cells enhances the development of anti-tumor immune responses in preclinical models via DC-dependent mechanisms [157]. Taken together these data suggest that there are multiple signals that can promote or suppress the uptake of dying cells by DCs and crosstalk between these pathways has important implications for whether or not tumor antigens are taken up by DCs to prime tumor-reactive T cell responses.

MATURATION SIGNALS

While many types of materials released from dying cells are likely capable of being phagocytosed by DCs, additional immunostimulatory signals released from these dying cells are critical to determining whether successful priming occurs. As described above DCs are the primary professional APC uniquely capable of sensing and integrating signals

in their environment to determine whether to initiate an adaptive immune response. When DCs receive signals from their environment that trigger signaling through PRRs this can result in the release of type IFN that can further signal back on DCs to promote their maturation. In the absence of infection, dying cells must trigger DCs maturation by releasing endogenous activators of these innate signaling pathways [158]. In support of this concept, DCs have been shown to produce type I IFN following tumor implantation in murine models [159] and additional work has demonstrated that when type I IFN is blocked with neutralizing antibodies [160] or DCs lack type I IFN receptors, mice ultimately fail to reject highly immunogenic tumors [161]. These data suggest that innate signaling pathways are required for the development of spontaneous tumor-reactive T cells.

This then raises the question, what are the upstream pathways triggering type I IFN in the absence of infection or therapy? Recent work has suggested that following injection of cancer cells into mice, DCs can detect tumor cell derived DNA through the cGAS/STING pathway [162]. Woo et al demonstrated that signaling through the STING pathway resulted in increased expression type I IFN and blocking components of this pathway led to diminished tumor-specific T cell priming and a failure to reject highly immunogenic tumors [162]. It's also plausible that nucleic acid sensors, such as MDA5, RIG-I or TLR3, function to detect various forms of RNA released by dying tumor cells to trigger interferon pathways. Endogenous retroviral elements are embedded throughout the genome and though their expression is typically silenced, some tumors might be better than others at suppressing the expression of these potentially immunostimulatory RNAs [163, 164]. Other signals include, high mobility group box 1 (HMGB1), a danger signal that signals through TLR4 and has been shown to be released from dying tumor cells that is capable of inducing DC maturation and tumor regression [165]. These data suggest tumors lacking

signals that promote DC maturation may give off the appearance of being poorly immunogenic, despite effectively transferring antigen to DCs.

Another point to consider are tumor derived metabolites that can function to inhibit DC maturation. Tumors that successfully release antigens and maturation signals, but also secrete factors that inhibit DC maturation will ultimately result in a failure for these DCs to prime tumor-specific T cell responses. This is illustrated by work from Villablanca et al, which showed that tumors can produce and secrete oxidized cholesterol ligands that bind to the liver X receptor (LXR) and signaling through this pathway in DCs suppresses the expression of CCR7 on maturing DCs [166]. As a result, signaling through LXR impaired DCs ability to migrate to the LN to prime $CD8^+$ T cells and knock out of LXR in DCs reversed these effects [166]. Other metabolites and signaling pathways that have been shown to suppress DC function in the tumor, including adenosine and PGE_2 [142, 167-169]. For example, tumors grown in mice that cannot synthesize PGE_2 are spontaneously rejected, indicating that PGE_2 is a critical suppressor of immunogenicity in mice [142]. These data suggest that DCs are capable of sensing both activating and inhibitory signals within tumors and the integration of these signals is critical to determining whether a productive anti-tumor immune response is generated.

RESTARTING THE CANCER-IMMUNITY CYCLE

As outlined above, multiple factors contribute to determining whether DCs are capable of spontaneously priming tumor-reactive T cells. These include the presence of tumor-associated antigens, localization of DCs subsets to tumors and how these DCs interact with dying tumor cells. For tumors that fail to initiate the cancer-immunity cycle, and thus are thought of as poorly immunogenic, is there a way to initiate the cancer-immunity cycle and convert these tumors into immunogenic tumors? Therapies that drive

the release of tumor-associated antigens and adjuvant signals capable of driving DC maturation would seem to be ideal for generating new tumor-specific T cell responses. Chemotherapy and radiation therapy are well known for their ability to generate widespread tumor cell death. Radiation is particularly useful as it can be directly targeted to the tumor itself, limiting the off-target toxicities that are often associated with other systemic therapies that generate tumor cell death. In the next section we will highlight the ways in which tumor cell death generated by radiation therapy has the potential to restart the cancer-immunity cycle.

Tumor Response to Radiation Therapy

RADIATION OVERVIEW

Radiation therapy was first used to treat malignancies over a century ago [170]. It is now estimated that over half of all cancer patients will receive radiation at some point during the course of their treatment [171]. Treatment consists of targeting high energy particles directly to a tumor to generate widespread DNA damage in cancer cells [172]. These high energy particles create double stranded breaks in the tumor cell DNA [173-176]. *In vitro* studies have shown that 1Gy of radiation can generate up to 40 double stranded breaks in cellular DNA [177]. Highly proliferative cancer cells are more sensitive to this DNA damage than surrounding healthy tissues and treatment results in tumor cell death [178]. Historically the efficacy of radiation therapy was attributed to its ability to directly kill tumor cells through irreparable DNA damage, and the varying responsiveness to treatment across cancers was thought to be due to the combination of tumor cell intrinsic radiosensitivity, cell cycle status and the degree of hypoxia within the tumor [179-181].

More recently, the role that the immune system plays in contributing to the responsiveness of different tumor types to radiation has been appreciated.

INTERACTION WITH THE IMMUNE SYSTEM

Early evidence supporting the immune systems role in the response to radiation came from early preclinical models which demonstrated the efficacy of radiation therapy is significantly diminished when administered to tumor bearing mice lacking an intact immune system [182]. Additional work has gone on to shown that $CD8^+$ T cell depletion prior to radiation significantly impairs the efficacy of treatment, suggesting the adaptive immune response contributes to treatment response [183]. The immune system's role in the response to treatment is further supported by the rare observation of the abscopal effects following radiation. This occurs when a primary tumor is treated with radiation and this leads to tumor shrinkage of both the treated tumor and a distant untreated tumor that is outside of the field of radiation [184-188]. In preclinical tumor models that recapitulate the abscopal effect following radiation therapy, the distant untreated tumor no longer shrinks when tumors are treated in athymic nude mice lacking mature T cells [189]. Taken together these data suggest not only does radiation directly kill cancer cells, but treatment is also capable of activating immune responses directed against tumors.

A long-standing question within the radiation field is how exactly does radiation promote the development of immune responses against tumors? One possibility is that radiation functions to remodel the tumor microenvironment by removing or repolarizing existing immunosuppressive cells, rendering the remaining tumor cells more accessible and susceptible to immune mediated destruction by pre-existing tumor-specific T cells. Extensive tumor cells death may also function to slow down tumor growth enough to give pre-existing T cells the opportunity to eliminate the remaining tumor cells. Other studies

have provided evidence for radiations capacity to remodel the vasculature that exists within tumors while increasing the expression of adhesion molecules, cytokines and chemokines that would help to draw immune cells into the tumor [190]. Alternatively, the tumor cell death initiated by radiation may result in the release of tumor-associated antigens and immunostimulatory signals that drive the development of new tumor-specific T cells responses. While many of these mechanisms are likely interrelated, below we will focus on the ways in which radiation has the potential to initiate the cancer-immunity cycle by activating DC capable of priming new tumor-specific T cell responses.

INFLUENCE ON TUMOR DCs

As outlined above in the cancer-immunity cycle, the first requirement for generating tumor-specific $CD8^+$ T cells is that tumor antigens need to be released and available to the cDC1s within the tumor (Fig 1-3). This raises the question; how might radiation influence the capacity of DCs to initiate the cancer-immunity cycle? One possibility is that radiation increases the quantity of antigens that are available for DCs to take up. Radiation excels at killing tumor cells and this extensive cell death provides DC with plenty of tumor associated antigens. Work from Strome et al, demonstrated that irradiated tumor cells are taken up and presented by DCs more efficiently than non-irradiated tumor cells and these DCs that had taken up irradiated tumor cells were more proficient at priming antigen-specific T cells capable of killing tumor cells [191]. Thus, radiation may function to increase the number of dying tumors cells that are available for DCs to phagocytose and process for presentation to tumor-reactive T cells.

Tumor cell death induced by radiation may also function to increase DCs capacity to take up dying tumor cells in part by modifying the type and density of “eat me” signals on the cancer cells themselves. In order to phagocytose potential tumor antigens, DCs must

recognize molecules on dying tumor cells that trigger them to engulf these cells. Radiation has been shown to drive the translocation of the ER resident protein, calreticulin, from inside the cell to outside of the tumor cell [192]. When calreticulin is exposed on the outside of dying cells this serves as an “eat me” signal that can be sensed by the scavenger receptors C1q and LRP expressed by phagocytes such as DCs [193, 194]. Recognition of these ligands by their receptor’s triggers uptake by phagocytic cells. The heat shock protein 70 (HSP70) is another signal which has been shown to be translocated to the cell surface following radiation in stressed tumor cells, and HSP70 can also serve as a ligand for the scavenger receptor LRP [195, 196]. Interestingly, it’s been shown that different tumor lines expressed varying levels of HSP70 on the cell surface following radiation therapy [196]. Another possibility is that radiation causes cytoskeletal F-actin filaments to be exposed by dying tumors cells, facilitating their recognition and uptake by the cDC1-specific scavenger receptor CLEC9A [151]. While the role of radiation specifically mediating antigen uptake by CLEC9A has yet to be explored, recent work has suggested that phagocytic uptake by this receptor directs dead cell-associated antigens to be processed specifically by the cross-presentation pathway in DCs [74]. Collectively these data indicate that radiation is capable of increasing the expression of ligands that target and facilitate tumor cell uptake by DCs.

Another point to consider is that antigens taken up by DCs following radiation will ultimately fail to drive tumor-specific T cell immunity unless adjuvant signals are present to drive DCs maturation following antigen capture. Thus, it is plausible that treatment with radiation functions to drive the release of endogenous adjuvant signals that promote intratumoral DC maturation and their subsequent migration to the TdLN. For example, radiation has been shown to increase the expression of molecules that are downstream of DC maturation, including TNFα and type I IFN [197, 198]. What are the factors released

by dying tumor cells that are capable of triggering DC maturation? Reports have suggested that radiation promotes the release of HMGB1 from irradiated tumor cells which then signals through TLR4 on DCs to promote their capacity to prime antigen-specific T cell responses [199]. In these studies TLR4 was required for the radiation mediated tumor regression, although the investigators used total $TLR4^{-/-}$ mice, as opposed to conditional $TLR4^{-/-}$ in DCs only [199]. Extracellular ATP is another compound has been shown to be released from dying tumors cells *in vitro* following radiation and it can serve as an activating signal for DCs [200]. Other potential PRR signals involve different forms of nucleic acids, including both DNA and RNA [201, 202]. In support of this concept, the cGAS/STING pathway, which senses cytosolic DNA has been shown to be critical to the response to radiation in certain tumor models and signaling through this pathway induces DCs maturation and expression of type I IFN [201]. Thus, radiation can promote the release of compounds that have to capacity to promote DC maturation within the tumor.

HYPOTHESIS: DENDRITIC CELL MATURATION AND MIGRATION IS REQUIRED FOR THE IMMUNOLOGICAL EFFICACY OF RADIATION THERAPY

While multiple studies have suggested that radiation therapy is capable of promoting DC maturation, it remains unclear how radiation therapy specifically impacts the function of intratumoral cross-presenting DCs and whether radiation is capable of promoting DC migration to the TdLN to prime new tumor-reactive $CD8^+$ T cell responses [183, 203, 204]. Cross-presenting DCs would be predicted to be important for the generation of tumor-specific T cells following treatment, but to date there is limited evidence to date to support this. Previous work has demonstrated that $Batf3^{-/-}$ mice which lack cross-presenting DCs, have impaired responses to radiation therapy [205, 206]. However, these models are suboptimal for studying the role of cDC1s in radiation mediated tumor regression as the

animals completely lack cross-presenting DCs throughout development, including at tumor implantation. Therefore, it has been difficult to isolate whether cDC1s are required to prime initial tumor-reactive T cells responses that might be generated at tumor implantation versus the requirement of cDC1s to prime new T cells response following treatment with radiation. It also remains unclear whether cDC1s can function locally within the tumor to recruit and re-prime tumor-reactive $CD8^+$ T cells or if cDC1s are required to migrate to the dLN to initiate priming of new tumor-specific $CD8^+$ T cells. Actively growing tumors have managed to escape or hide from the immune system, and thus it is plausible that they lack immunostimulatory adjuvant signals that would typically be present during infection to drive DC maturation. While tumor antigens may be available to DCs in the absence of therapy, deficiency in maturation signals would prevent DCs from activating T cells and might instead drive tolerance. Thus, it is possible that tumors will fail to induce DC maturation unless therapeutic interventions such as radiation therapy are administered to drive the release of adjuvant like signals within the tumor. The ability of radiation to promote anti-tumor immunity likely depends on its ability of treatment to promote intratumoral DC maturation. Therefore, we hypothesize that DC maturation and migration is required for the immunological efficacy of radiation therapy. In the following sections we test this hypothesis by examining how radiation therapy directly impacts intratumoral DCs.

Chapter 2:

Dendritic Cell Maturation Defines Immunological Responsiveness of Tumors to Radiation Therapy

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Introduction

Radiation therapy is used to treat over half of all cancer patients at some point during the course of their treatment [171, 207]. However, the treatment response varies significantly across cancer pathologies and mechanisms describing why particular cancers respond poorly to radiation are lacking. Traditionally, the efficacy of radiation has been attributed to direct killing of cancer cells following radiation induced DNA damage [208]. Recently this paradigm has shifted, as studies have demonstrated that radiation can trigger immunogenic cancer cell death capable of igniting tumor-specific immunity [183, 209, 210]. Treatment with radiation leads to the release of endogenous adjuvants and tumor associated antigens that can be recognized by the immune system to direct anti-tumor immune responses [211-213]. Conversely, it has also been reported that radiation therapy can promote upregulation of molecules that foster immunosuppression following treatment [214-218]. Thus, the cumulative integration of these signals within individual

tumors likely plays a significant role in determining whether a successful anti-tumor immune response is generated following radiation. A better understanding for how individual tumor microenvironment shape the immune response following radiation is needed to improve patient outcomes following treatment.

Dendritic cells (DCs) are key sentinels of the immune system, capable of processing and presenting antigens, sensing innate danger signals and integrating microenvironmental cues to regulate whether an adaptive immune response is mounted towards foreign invaders. In particular, conventional type 1 DCs (cDC1s) have the specialized ability to uptake exogenous cell-associated antigens and potently cross-prime antigen-specific $CD8^+$ T cell responses [34, 219-221]. Cross-presenting cDC1s are defined by their expression of the transcription factors BATF3, ZBTB46, ID2 and IRF8 [37]. cDC1s can be further divided into those capable of migrating from tissues ($CD103^+$ cDC1s) and those resident to lymphoid organs ($CD8\alpha^+$ cDC1s) [222, 223]. $CD103^+$ cDC1s are present in many murine tumors, and are thought to be the predominant cell type capable of trafficking intact tumor-associated antigens to the draining lymph node (dLN) to initiate cross-priming of tumor-reactive $CD8^+$ T cells [141, 224].

In preclinical models, cDC1s are required for the rejection of immunogenic tumors and they are known to play an important role in promoting anti-tumor immune responses following treatment with many immunotherapies [34, 144, 159, 225]. Moreover, it has been reported that increased cDC1 signatures in patient tumors correlates with improved outcomes in a range of cancers [141-143]. Activation of intratumoral cDC1s is proposed to support the development of anti-tumor immunity through two key mechanisms; 1) cDC1s migrate to the dLN to deliver tumor-associated antigen and initiate priming of tumor-reactive $CD8^+$ T cells, and 2) cDC1s function within the tumor to recruit and re-

prime tumor-reactive $CD8^+$ T cells locally. The role of cDC1 activation and migration in radiation mediated tumor regression remains to be determined. In certain tumor models the efficacy of radiation has been shown to depend on the presence of cDC1s [205, 206]. However, these studies utilized mice that lack cDC1 ($Batf3^{-/-}$) throughout the course of tumor development, as opposed to only during therapy, making it difficult to draw conclusions regarding the mechanism. Thus, the question remains whether cDC1 activation and migration is required to successfully promote anti-tumor immune responses following radiation therapy and whether this differs across cancers.

In this study, we investigated mechanisms that regulate why particular cancer types are either highly or poorly responsive to radiation. Using tumor models with equivalent radiosensitivity in vitro, but differing responsiveness to radiation in vivo, we demonstrate that poorly radio-immunogenic tumors fail to activate intratumoral cDC1s following treatment. Poly I:C has been shown to successfully combine with radiation therapy to improve tumor control [226]. We similarly show that by combining radiation with the exogenous adjuvant poly I:C this successfully drives cDC1 maturation resulting in tumor cures. We determine the combined efficacy of radiation and poly I:C is dependent on cDC1s, which promote the development of tumor-specific effector $CD8^+$ T cells. Finally, we establish that trafficking of $CD8^+$ T cells from LNs to the tumor is necessary for treatment efficacy. Taken together these data demonstrate that intratumoral cDC1 activation and migration following radiation is one potential mechanistic factor that limits the response to radiation therapy across different cancer pathologies.

Materials & Methods

ANIMALS & CELL LINES

Experiments utilized 6-8 week old C57BL/6 (#000664), B6.SJL (#002014) and Zbtb46^{dtr} (#019506) mice that were obtained from The Jackson Laboratories. 2C TCR transgenic mice were kindly provided by Dr. Thomas Gajewski at the University of Chicago. Survival experiments were performed with 5-8 mice per experimental group, and mechanistic experiments with 4-6 mice per group. Animal protocols were approved by the Earle A. Chiles Research Institute (EACRI) Institutional Animal Care and Use Committee (Animal Welfare Assurance No. A3913-01). The Panc02-SIY pancreatic adenocarcinoma line expressing the model antigen SIY was kindly provided by Dr. Ralph Weichselbaum at the University of Chicago. MC38 colorectal carcinoma line was obtained from Dr. Kristina Young at EACRI. Moc1 and Moc2 oral squamous cell carcinoma lines were kindly provided by Dr. Ravindra Uppaluri at the Dana Faber Cancer Institute. Panc02-SIY, Moc1 and Moc2 cell lines were grown in complete RPMI containing 10% heat inactivated fetal bovine serum (FBS), 100U/mL penicillin, 100μg/mL streptomycin. MC38 cell lines were grown in DMEM containing 10% heat inactivated FBS, 100U/mL penicillin, 100μg/mL streptomycin. Pathogen and mycoplasma contamination testing were performed on all cell lines within the past 6 months using the IMPACT II Mouse PCR Profiling from IDEXX BioAnalytics.

CLONOGENIC ASSAY

Tumor cells lines were treated with indicated dose of radiation using a cesium irradiator. After treatment 5 x 10^2 cells were seeded in a 6-well plate and allowed to grow

for 5 days. On day 5 media was removed, plates were washed with PBS and cells were fixed with methanol. The number of tumor cell colonies was counted for each well and normalized by dividing by the number of colonies in the untreated well to get the percent of surviving cells for each dose of radiation.

TUMOR TREATMENTS

Tumors were implanted subcutaneously into the right flank as follows; 2 x 10^5 MC38, 5 x 10^6 Panc02-SIY, 1 x 10^6 Moc1 and 1 x 10^5 Moc2. When tumors were approximately 5mm in average diameter, mice were randomized to receive treatment with CT-guided radiation using the Small Animal Radiation Research Platform (SARRP) from XStrahl. Dosimetry was performed using Murislice software from XStrahl. The SARRP delivered a single dose of 12Gy to an isocenter within the tumor using a 10mm x 10mm collimator and a 45° beam angle to minimize dose delivery to normal tissues. For poly I:C treatments vaccine grade reagent from InvivoGen (#vac-pic) was administered intratumorally at 50 μg/tumor in a total volume of 10ul. Control mice received 10μl of vehicle. The 1st dose of poly I:C was administered concurrently with radiation and the 2nd dose was given 5 days later. For CD8 depletion, 200 μg of α-CD8β antibodies from BioXCell (clone 53-5.8) were given intraperitoneally one day prior to radiation and again 7 days later. To block T cell egress during treatment, FTY720 from Cayman Chemical Company (#10006292) was administered at 1 mg/kg/day intraperitoneally, starting 1 day prior to radiation for a total of 7 consecutive days. For Flt3L experiments, compound was provided by Bristol Myers-Squibb and administered intraperitoneally at 30μg/mouse/day for 9 consecutive days. In all survival experiments, tumor length and width were measured 2-3 times per week using

calipers. Mice were euthanized when tumor size exceeded 12 mm in any dimension, or when body condition score declined 1 level.

TISSUE PROCESSING

Following dissection, tumors were weighed and minced into small fragments, then transferred into C tubes from Miltenyi Biotec containing enzyme digest mix with 250U/mL collagenase IV (Worthington Biochemical, #LS004188), 30U/mL DNase I (Millipore-Sigma, #4536282001), 5mM CaCl2, 5% heat inactivated FBS and HBSS. Tissue was dissociated using a GentleMACS tissue dissociator from Miltenyi Biotech. This was followed by incubation at 37°C for 30 min with agitation. For the dLNs, capsules were cut open and incubated with enzymatic mix described above at 37°C for 15 min with agitation. Enzyme mix containing dLNs was then vigorously pipet mixed and incubated at 37°C for an additional 15 min. Enzymatic reactions for both the tumor and dLN were quenched using ice cold RPMI containing 10% FBS and 2mM EDTA. Single cell suspensions were then filtered through 100μm (tumor) or 40μm (dLN) nylon cell strainers to remove macroscopic debris. Cells were washed and counted for flow cytometry.

FLOW CYTOMETRY

For staining, 2 x 10^6 cells were stained with Zombie Aqua Viability Dye from BioLegend (#423102) in PBS for 10 min on ice, then Fc receptors were blocked with α-CD16/CD32 antibodies from BD Biosciences (2.4G2) for an additional 10 min. After centrifugation, the supernatant was removed and cell were stained with a surface antibody cocktail containing in FACS buffer (PBS, 2mM EDTA, 2% FBS) and Brilliant Stain Buffer Plus from BD Biosciences (#566385) for 20 min on ice. The following antibodies were purchased from BioLegend; F4/80-PerCP/Cy5.5 (BM8), CD11c-PE/Cy7 (N418), CCR7-

PE (4B12), CD90.2-A700 (30-H12), CD19-A700 (6D5), MHC-II-BV421 (M5/114.14.2), CD11b-BV605 (M1/70), CD8α-BV650 (53-6.7), Ly-6C-BV711 (HK1.4) and IL-12 PE (C15.6). CD40-FITC (HM40-3), CD103-APC (2E9), CD24-APC e780 (M1/69) and Granzyme B eFluor450 (NGZB) were obtained from Thermo Fisher Scientific. CD80-PE CF594 (16-10A1), CD45-BV786 (30-F11) and Ki-67 FITC (B56) were purchased from BD Biosciences. PE-conjugated Kb - SIYRYYGL pentamers (#F1803-2B) were purchased from Proimmune. After surface staining, cells were washed in FACS buffer and fixed for 20 min on ice with Fixation/Permeabilization Buffer from BD Biosciences (#554722). For intracellular and intranuclear cytokine analysis, single cell suspensions from tumors were incubated in complete RPMI +/- 50μg/mL poly I:C and 10 μg/mL GolgiPlug from BD Biosciences (#555029) at 37°C for 6 hrs. Cells were then stained as described above, except fixation and permeabilization was performed using the Foxp3/Transcription Factor Staining Buffer Set from Thermo Fisher Scientific (#00-5523-00) and then cells were incubated with intracellular antibodies for 30 min on ice. All samples were resuspended in FACS buffer and acquired on a BD Fortessa flow cytometer. Data were analyzed using FlowJo software from Tree Star, v10.5. cDC1 were gated as leukocytes/single cells/Live/CD45+/CD90.2-CD19-/Ly-6C-/MHC-II+/CD24+F4-80-/CD11b-/CD103+. $CD8^+$ T cells were gated as single cells/Live/CD45+/CD90.2+ CD19-/CD8+CD4-.

BONE MARROW CHIMERAS

Bone marrow chimeras were generated using B6.SJL ($CD45.1^+$) recipient mice that were irradiated with 10Gy of radiation. Bone marrow cells were isolated from WT C57BL/6 ($CD45.2^+$) or $Zbtb46^{dtr}$ ($CD45.2^+$) donor mice femurs and tibias using a 27G needle. Cells were filtered through a 70 μm cell strainer to generate a single cell suspension and

resuspended in PBS. Recipient mice received 3-5 x 10^6 donor bone marrow cells by retro-orbital injection. Tumors were implanted 8 weeks following bone marrow reconstitution. Diphtheria toxin from Millipore-Sigma (#D0564) was administered 3 days prior to radiation at 20 ng/g intraperitoneally for initial DC depletion. This was followed by an additional 3 doses of 5 ng/g of diphtheria toxin that were given every 3 days to maintain depletion.

CYTOKINE LUMINEX ASSAY

Tumors were harvested on ice, weighed and homogenized in PBS containing 4.5 μl HALT Protease Inhibitor Cocktail from Thermo Fisher Scientific (#78440) per mg tissue. The cell debris was removed by centrifugation at 14,000g for 15 minutes at 4°C, and supernatants were stored in aliquots at -80°C until analyzed. Cytokines and chemokines were detected using 25 μl of supernatant and the Cytokine & Chemokine 26-Plex Mouse ProcartaPlex Panel 1 kit from Life Technologies (#EPX260-26088-901). Data was acquired on a Luminex 100 array reader and cytokine/chemokine concentrations for each tumor sample was calculated using standard curves for each analyte.

STATISTICS

Data were analyzed and graphed using Prism from GraphPad Software (v7.0). Individual data sets were compared using Student's T-test and analysis across multiple groups was performed using ANOVA with individual groups assessed using Tukey's comparison. Kaplan Meier survival curves were compared using a log-rank test.

Results

IN RADIO-IMMUNOGENIC TUMORS CD8+ T CELLS CONTROL THE RESPONSE TO RADIATION INDEPENDENT OF TUMOR CELL INTRINSIC RADIOSENSITIVITY

First, we set out to identify murine tumor models with equivalent radiosensitivity in vitro, but differing responsiveness to the same dose of radiation in vivo. We compared the radiosensitivity of the murine colon tumor cell line, MC38 and the pancreatic tumor cell line, Panc02-SIY. In vitro, both tumor cell lines had comparable sensitivity to a range of radiation doses (**Figure 2-1A**), These cell lines were then used to establish syngeneic flank tumors in mice and further evaluate their response to radiation in vivo. When tumors reached an average diameter of 5 mm, they were treated with CT-guided radiation to prevent indirect targeting of the tumor dLN (**Figure 2-1B i-ii**). Both tumors types showed delayed tumor growth kinetics in response to radiation, as compared to untreated controls. Despite displaying equivalent radiosensitivity to Panc02-SIY in vitro, MC38 tumors exhibited considerable tumor regression and, in some instances, tumor cures (**Figure 2-1C i**). We also tested the head and neck tumor cell lines Moc1 and Moc2, which had comparable radiosensitivity in vitro, but differing responsiveness in vivo (**Figure 2-2A-B**). Taken together these data indicate that tumor cell intrinsic radiosensitivity is not the limiting factor controlling the response to radiation in vivo in these tumor models. To determine if the improved tumor control in MC38 tumors following radiation was dependent on the adaptive immune response, we depleted CD8+ T cells prior to treatment and found that CD8+ T cell depletion significantly abrogated the enhanced survival benefit of radiation in MC38 tumors, but had no impact on Panc02-SIY (**Figure 2-1C ii, Figure 2-2B**). We observed similar results in Moc1 tumors which required CD8+ T cells for their enhanced response to radiation, whereas Moc2 tumors did not require CD8+ T cells (Fig 2-2B). Given that MC38 and Moc1 tumors exhibited a CD8+ T cell-dependent survival advantage in

response to radiotherapy, we will refer to them as “radio-immunogenic” tumors from this point forward, while Panc02-SIY and Moc2 will be referred to as a “poorly radio-immunogenic” tumors, in the context of radiation.

RADIATION INDUCES cDC1 MATURATION IN RADIO-IMMUNOGENIC TUMORS BUT NOT IN POORLY RADIO-IMMUNOGENIC TUMORS

In radio-immunogenic MC38 tumors, improved tumor control following radiation therapy required $CD8^+$ T cells, suggesting a potential failure to generate an effective anti-tumor $CD8^+$ T cell response in poorly radio-immunogenic Panc02-SIY tumors. Since cDC1s are known to play an important role in cross-priming $CD8^+$ T cell responses, this led us to evaluate whether cDC1s were being activated equivalently in both tumor models following radiation [34]. We used flow cytometry to assess changes in both the quantity and maturation state of DC subsets within the tumor after treatment with a range of radiation doses (**Figure 2-3A, Figure 2-4A**). There was a significant reduction in total DCs, particularly within the $CD103^+$ cDC1s compartment following radiation in both tumor models (**Figure 2-4B i-ii**). Interestingly, the remaining intratumoral cDC1s in MC38 tumors expressed higher levels of markers associated with DC maturation, including CCR7, which is important for migration to the dLN (**Figure 2-4C i**) and the co-stimulatory molecule CD80 (**Figure 2-4C i-ii**) [84]. Moreover, expression of these activation markers increased in a dose dependent manner with higher doses of radiation (**Figure 2-4C i-ii**). Similarly, there was a trend towards increased intratumoral cDC1 activation following 12Gy of radiation in the radio-immunogenic Moc1 tumors, but not in the poorly radio-immunogenic Moc2 tumors (**Figure 2-2C i-iii**). To determine whether increased accumulations of intratumoral cDC1s could improve the efficacy of radiation in poorly radio-immunogenic Panc02-SIY tumors, we administered the cytokine Fms-like tyrosine kinase 3 ligand (FLT3L) in

combination with radiation (**Figure 2-5A**) [156]. Treatment with FLT3L significantly increased the accumulation of intratumoral cDC1s, but DC maturation was still impaired (**Figure 2-5B i-ii**), and treatment had no impact on animal survival following radiation (**Figure 2-5C i-ii**). Thus, while radiation is clearly capable generating signals to promote cDC1 maturation in particular tumor types, these signals are either lacking or actively suppressed in poorly radio-immunogenic tumors, leading to impaired tumor control after radiation. Importantly, these results provide one potential explanation for why equivalent doses of radiation are capable of inducing varying degrees of tumor regression across different tumor types.

ADJUVANTS THAT TARGET cDC1 MATURATION OVERCOME THE FAILURE OF RADIATION TO INDUCE INTRATUMORAL cDC1 MATURATION IN POORLY RADIO-IMMUNOGENIC TUMORS, RESULTING IN TUMOR CURES

Our results thus far had suggested that radiation alone is unable to drive cDC1 activation in poorly radio-immunogenic tumors and this failure may limit the extent of tumor control following radiation. We hypothesized that externally driving DC maturation by administration of adjuvants directly to the tumor would restore T cell mediated tumor control. To identify an optimal adjuvant, we examined toll-like receptor (TLR) expression on DC and found TLR3 expression to be highly enriched on cross-presenting cDC1 (**Figure 2-6B-D**). Importantly, signaling through this innate receptor has been shown to induce cDC1 maturation [227, 228]. Previous work has demonstrated improved tumor control in murine models when radiation is combined with poly I:C, suggesting that this agent may restore cDC1 function in tumors [226, 229, 230]. We administered intratumoral poly I:C concurrently with radiation and then again 5 days later and assessed tumors for cytokine responses and DC maturation (**Figure 2-6A**). Analysis of cytokines in tumors

revealed increased levels of type I interferon (IFNα), pro-inflammatory cytokines (TNFα, IL-6, IL-1β) and chemokines known to recruit T cells (CCL5, CXCL10) in both single agent poly I:C or the combination of radiation and poly I:C treated tumors (**Figure 2-6B**). Thus, treatment with poly I:C transforms the milieu within the tumor into an environment that is more favorable for the development of anti-tumor immunity in the context of radiation therapy.

Earlier data indicated that radiation effectively induced cDC1 maturation only in radio-immunogenic tumors (MC38, Moc1) and this process did not occur in poorly radio-immunogenic tumors (Panc02-SIY, Moc2). To address whether poly I:C was able to induce cDC1 maturation in poorly radio-immunogenic Panc02-SIY tumors, we used flow cytometry to monitor changes in the quantity and activation state of cDC1s within the tumor. Our analysis revealed that all treatment groups had fewer intratumoral cDC1s as compared to untreated controls one day following treatment (**Figure 2-6C i**). However, of the cDC1s that remained in the tumor, we noted increased expression of markers associated with DC maturation and migration (CCR7, CD80) when poly I:C was given alone or in combination with radiation (**Figure 2-6C ii-iv**). Treatment with poly I:C significantly increased production of IL-12 specifically in intratumoral cDC1s (**Figure 2-6D**), a cytokine associated with enhanced DC priming [228]. Interestingly, while single agent poly I:C induced changes in cDC1 maturation and generated a favorable cytokine environment within tumors, it failed to impact tumor growth, whereas the combination of radiation and poly I:C resulted in tumor regression (**Figure 2-6E i**). Unlike earlier studies, our dosing regimen also resulted in durable tumor cures (**Figure 2-6E ii**) [226]. These data demonstrate in tumor models where cDC1 maturation is impaired either due to active suppression or a failure for radiotherapy to release sufficient signals, we can overcome

this deficit by administering exogenous adjuvants to promote cDC1 maturation following radiation therapy and this leads to durable tumor cures. Importantly, these results suggest that adjuvant signal in the form of poly I:C alone is insufficient to induce tumor cures.

cDCs ARE REQUIRED FOR COMBINED EFFICACY OF RADIATION AND POLY I:C

Since macrophages in the tumor express some TLR3 (**Figure 2-3C**), and tumor associated macrophages can impact tumor control following radiation therapy [231], we evaluated the importance of tumor macrophages to the treatment response. We found that macrophage depletion using anti-CSF1 did not significantly impact tumor control by the combination of radiation therapy and poly I:C (**Figure 2-7A i-ii**), suggesting that cDC1s may be the critical target for TLR3 ligands. Although cDC1s were successfully activated by poly I:C when combined with radiation, the question remained whether these cells were required for treatment efficacy. One widely used approach to deplete cDC1s in murine models are Batf3-/- mice; however, these mice lack DCs through all stages of tumor development, which changes the baseline tumor immune environment prior to treatment initiation [34]. To isolate the effect of treatment on DC populations, we required an approach to selectively deplete cDCs at the time of treatment. Zbtb46^{dtr} mice express the diphtheria toxin receptor selectively in cDCs and permits their depletion at any time point by administration of diphtheria toxin [232]. To deplete cDCs, we established Panc02-SIY tumors in Zbtb46^{dtr} or wild-type (WT) C57BL/6J bone marrow chimeras and treated them with diphtheria toxin three days prior to treatment with radiation and poly I:C (**Figure 2-8A i**). Treatment with diphtheria toxin resulted in a loss of cross-presenting DCs in both the tumor (**Figure 2-7B**) and in the tumor dLN (**Figure 2-7C i-ii**) of Zbtb46^{dtr} bone marrow chimeras, but not in WT control bone marrow chimeras. Depletion of cDCs immediately

prior to radiation significantly impaired tumor control and abrogated the enhanced survival benefit of radiation and poly I:C when compared to control WT bone marrow chimeras treated with diphtheria toxin (**Figure 2-8A ii-iii**). Notably, in bone marrow chimeras given the combination of poly I:C and radiation therapy without DC depletion the overall efficacy of treatment was consistently reduced compared to that observed in WT mice (**Figure 2-6E**), suggesting some general loss of immune function through development of bone marrow chimeras. While cDC1s were clearly important for the efficacy of combination therapy, the mechanism by which they promoted tumor regression remained unclear. To determine whether DC migration was important for therapy we first quantified the total number of migratory $CD103^+$ cDC1s in the tumor dLN following treatment. The data revealed more migratory $CD103^+$ cDC1s with an activated phenotype (CD80) in the dLNs of combination treated animals as compared to untreated or single agent controls (**Figure 2-8B i-ii**), suggesting increased migration following treatment. These data demonstrate that cDC1s play important role in the anti-tumor efficacy of radiation and poly I:C.

ADJUVANT COMBINED WITH RADIATION THERAPY PROMOTES THE DEVELOPMENT OF EFFECTOR $CD8^+$ T CELLS AND REQUIRES T CELL TRAFFICKING FROM THE LN

Our data thus far suggested that $CD103^+$ cDC1 migration to the dLN is increased following combination therapy. While antigen recognition serves as signal 1 for T cell priming, DCs are known to provide additional signals in the form of co-stimulation (signal 2) and cytokines (signal 3) that further promote the expansion and quality of antigen-specific T cells [233]. This led us to first evaluate whether $CD8^+$ T cells were required for the combined efficacy of radiation and poly I:C by depleting $CD8^+$ T cells (**Figure 2-9A i, Figure 2-7D**). Depletion of $CD8^+$ T cells completely abolished the efficacy of treatment,

indicating that these cells were indeed important for treatment (**Figure 2-9A ii-iii**). Next we used flow cytometry to assess the phenotype of $CD8^+$ T cells in the tumor 7 days after treatment. While radiation alone increased the number of $CD8^+$ T cells in tumors compared to all other treatment groups, this was not the case in the combination of treated animals (**Figure 2-9B i**). Instead the combination of radiation and poly I:C significantly expanded the proportion of proliferating ($Ki67^+$) $CD8^+$ T cells in the tumor with enhanced cytotoxic potential as identified by the protease granzyme B (**Figure 2-9B ii**). This pattern was also observed in antigen-specific 2C $CD8^+$ T cells which recognize the SIYRYYGL (SIY) peptide expressed by Panc02-SIY tumor cells (**Figure 5B iii**). Moreover, we observed a similar increase in $CD8^+$ $Ki67^+$ Granzyme B^+ cells following radiation alone in radio-immunogenic MC38 tumors (**Figure 2-7E**). These data suggest that following radiation and poly I:C, cDC1 prime $CD8^+$ T cells that have improved cytolytic potential as compared to controls. The question then remained whether these T cells were being activated within the tumor or were instead being primed by cDC1s within the dLN. To address this question, we used S1P receptor agonist FTY720 to sequester T cells in the LN, thereby preventing their migration to the tumor following priming in the dLN (**Figure 2-9C i, Figure 2-7D**) [234]. When T cell egress from the LNs was impaired with FTY720, the combined efficacy of radiation and poly I:C was completely abrogated (**Figure 2-9C ii-iii**). Taken together, these results demonstrate that tumor regression following treatment radiation and poly I:C is dependent on cDC1s which play an important role in generating tumor-reactive effector $CD8^+$ T cells within the tumor dLN, and these T cells must be free to migrate through the circulation to the treatment site to result in tumor cure.

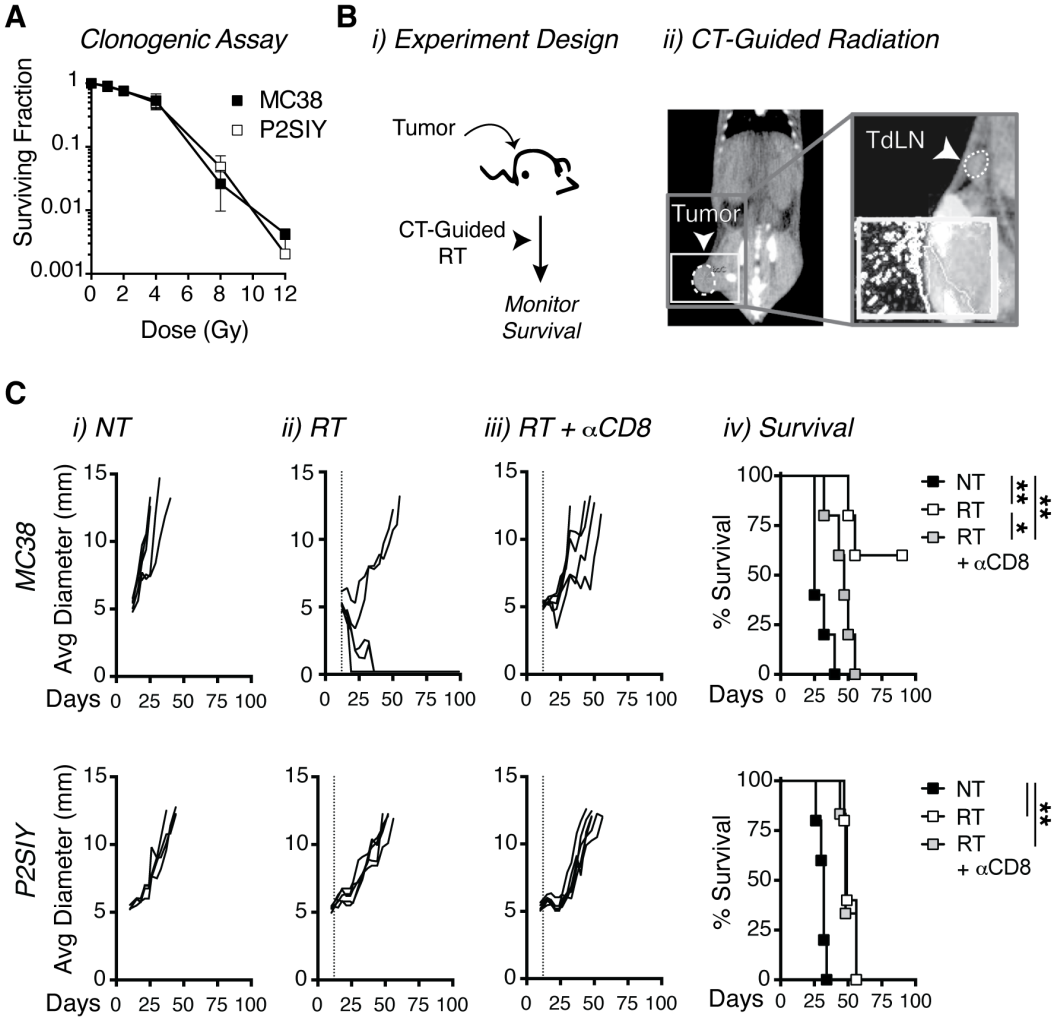


Figure 2-1: Radio-immunogenic tumors require CD8⁺ T cells for enhanced response to radiation. (A) MC38 or Panc02-SIY (P2SIY) tumors were treated *in vitro* with indicated dose of radiation, cultured for 5d and the number of surviving colonies was quantified. The colony number was then normalized to untreated control (0Gy) for each tumor type. Data represent the mean +/- SD from 3 independent experiments. **(B)** i) MC38 or P2SIY tumors were established and allowed to grow to ~5mm average diameter before being treated with 12Gy of CT-guided radiation therapy (RT). ii) Representative CT image with targeting of tumor (large dotted line) within field of radiation (solid white box) to avoid indirect targeting of the tumor dLN (TdLN) (small dotted line). **(C)** i) MC38 and P2SIY tumor growth curves for tumors that were untreated (NT), ii) treated with 12Gy focal radiation (RT), or iii) treated with αCD8β depleting antibodies one day prior 12Gy focal RT. iv) Overall survival. n = 5 animals per treatment group. Results shown are representative of two independent experiments. **p < 0.05. **p < 0.01.*

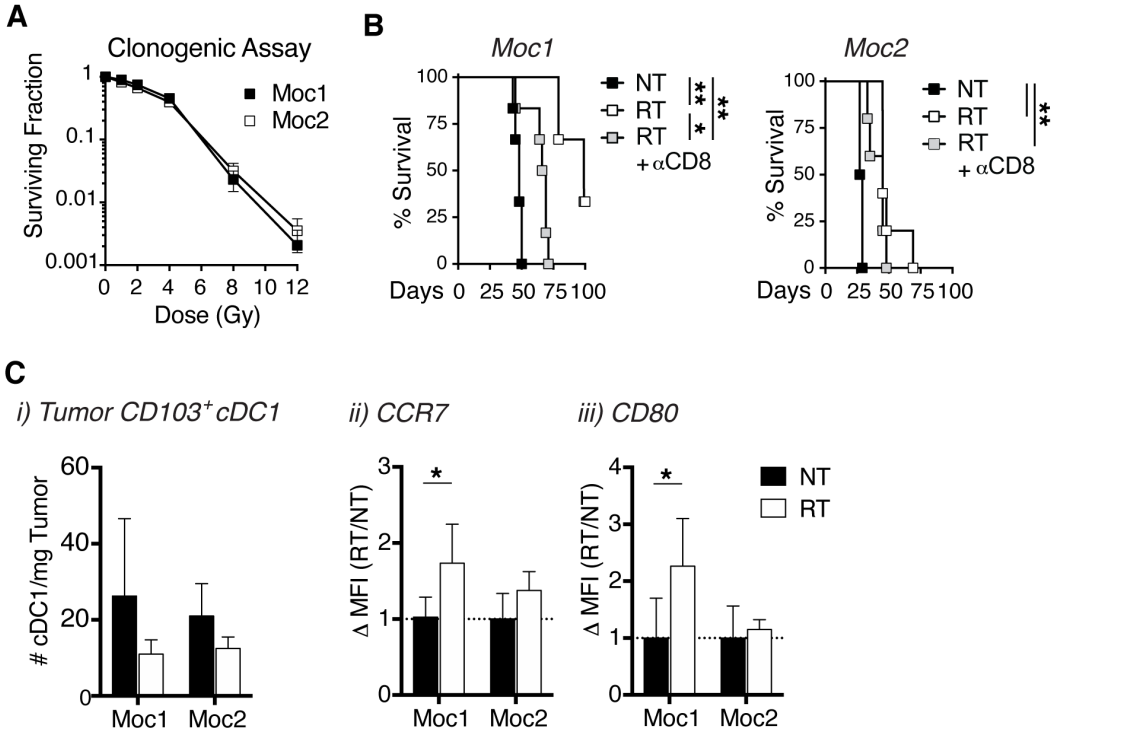


Figure 2-2: Radiation induces cDC1 maturation in radio-immunogenic Moc1 tumors. (A) Moc1 and Moc2 tumor cells lines were treated as described in fig 1A to quantify surviving fraction of cells at indicated doses of radiation *in vitro.* **(B)** Moc1 and Moc2 tumor bearing mice were treated with 12Gy of RT when tumors were ~5mm in diameter. $CD8^+$ T cells were depleted and animal survival was monitored. **(C)** i) Tumors were harvested 3 days following radiation and the number of intratumoral $CD103^+$ cDC1s per mg of tumor tissue were quantified. The MFI for ii) CCR7, and iii) CD80 was quantified on $CD103^+$ cDC1 and this value was normalized to the average of the untreated controls to determine the fold change in expression following treatment. n = 4-7 animals/group. Data represent the mean +/- SD of each group. *$p < 0.05$, **$p < 0.01$.

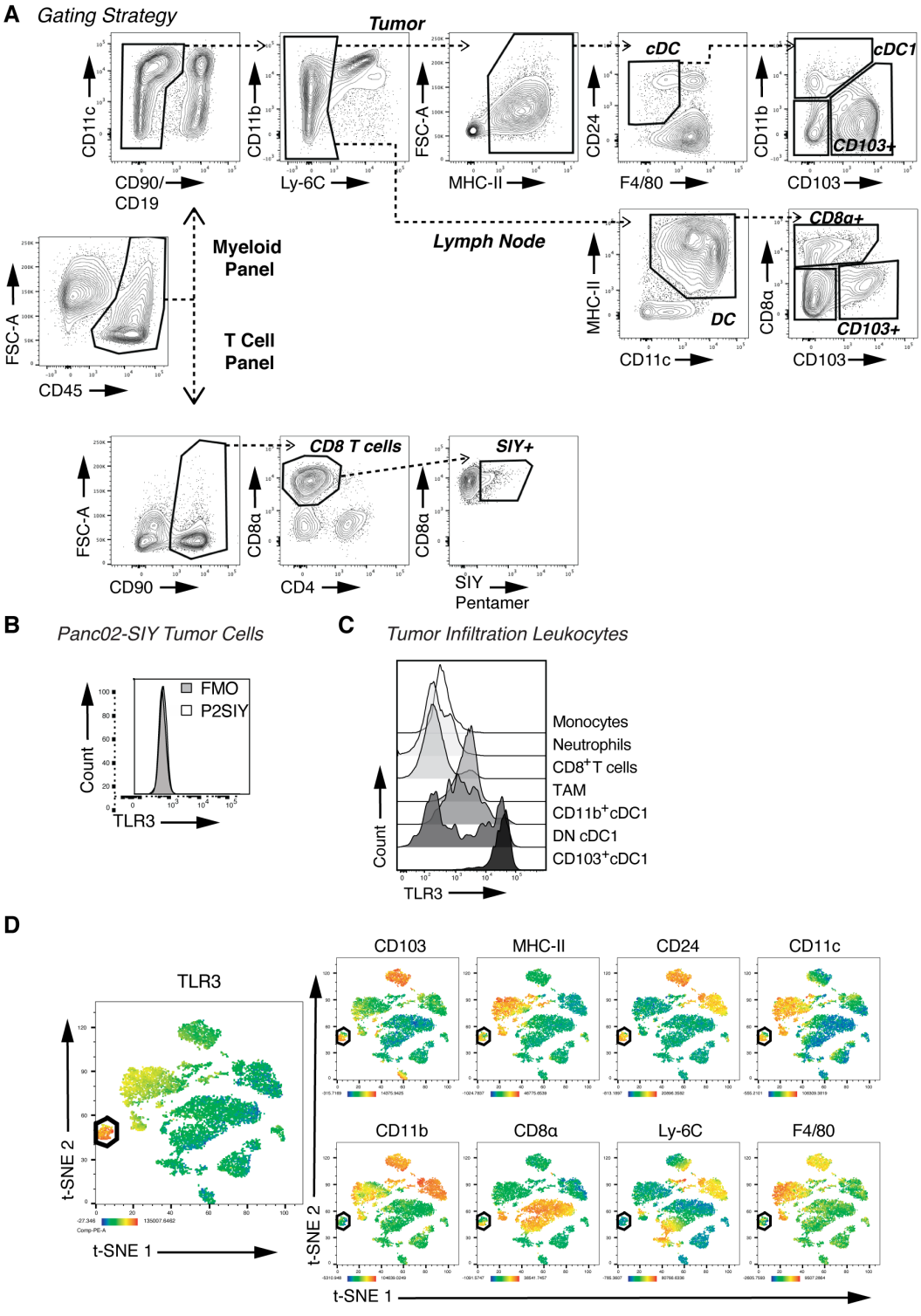


Figure 2-3: Gating strategy for flow cytometry analysis and TLR3 expression across cell types in the tumor. **(A)** Representative gating strategy from single cell suspension that begins after leukocytes were gated and doublets were removed in FlowJo. **(B)** Expression of TLR3 in Panc02-SIY (P2SIY) tumors compared to fluorescence minus one (FMO) control. **(C)** Expression of TLR3 within tumor infiltrating leukocyte populations ($CD45^+$). **(D)** Dimensionality reduction of flow cytometry data using t-SNE algorithm to identify clusters of immune cells ($CD45^+$) expressing TLR3 from P2SIY tumors with $CD103^+$ cDC1s identified in each panel by solid black hexagon.

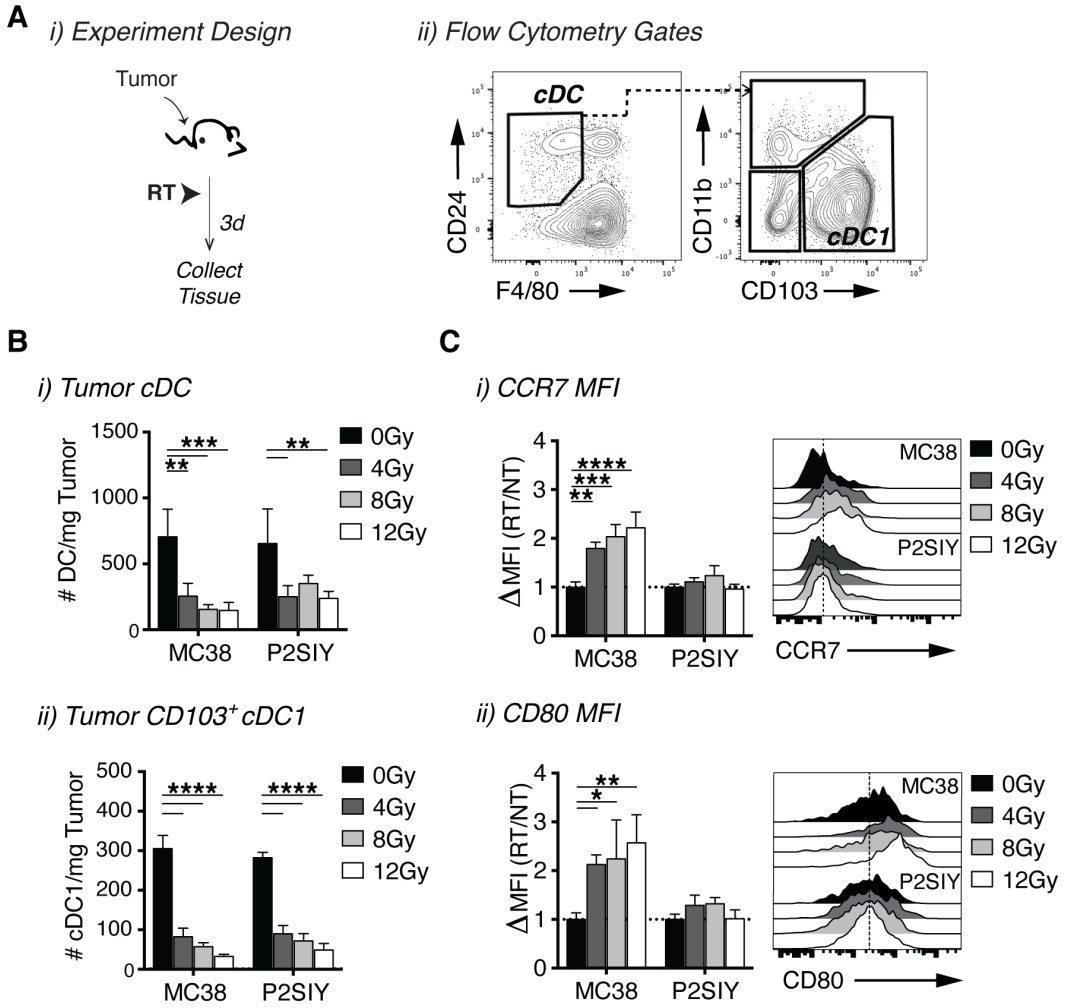


Figure 2-4: Radio-immunogenic tumors successfully activate intratumoral cDC1s following radiation. (A) i) Experiment setup for B-C and ii) flow cytometry gating strategy for cDCs and cDC1s from Live CD45$^+$ CD90.2$^-$ CD19$^-$ Ly-6C$^-$ MHC-II$^+$. When tumors reached an average diameter of 5mm they were treated +/- RT and tumor infiltrating immune cells were phenotyped three days following treatment. (B) i) The number of cDCs and ii) CD103$^+$ cDC1s per mg of tumor tissue in MC38 and Panc02-SIY (P2SIY) tumors treated with 0Gy, 4Gy, 8Gy or 12Gy of radiation. (C) The average expression (MFI) of i) CCR7 and ii) CD80 on intratumoral CD103$^+$ cDC1s for each radiation dose was divided by the average MFI for 0Gy samples in each tumor type to calculate the fold increase in expression following treatment with radiation. n = 5 animals/group. Data represent the mean +/- SD of each group. Results shown are representative of two independent experiments. *$p < 0.05$. **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

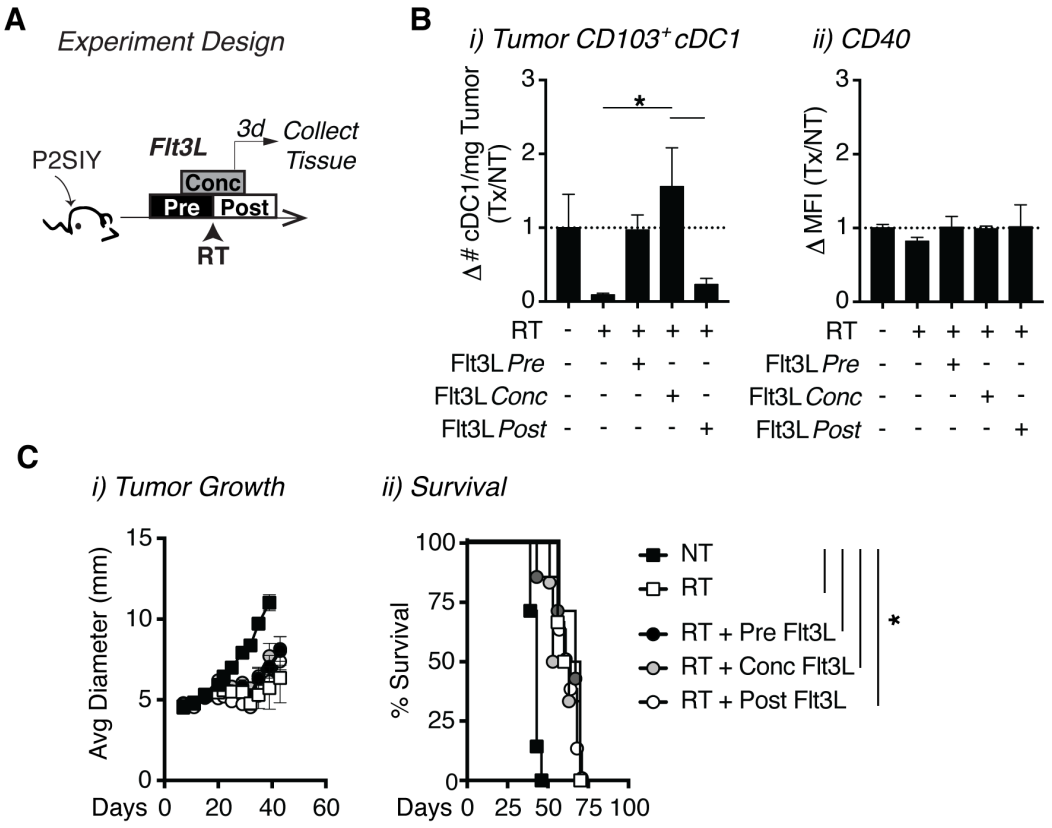


Figure 2-5: Increased accumulations of intratumoral cDC1s fails to improve the efficacy of radiation. (**A)** Experiment setup for B-C. Pacn02-SIY (P2SIY) tumor bearing mice were treated with 9 consecutive, daily doses of Flt3L starting either prior to RT (Pre), concurrently with RT (Conc) or after treatment with RT (Post). Tissue was collected 3 days following treatment with 12Gy RT for phenotypic analysis. **(B)** The number of i) CD103+ cDC1 per mg of tumor tissue and ii) CD40 MFI on CD103+ cDC1s was quantified and normalized to the average value for untreated controls to calculate fold change following treatment. **(C)** i) Tumor growth curves and ii) animal survival after treatment with radiation and Flt3L. n = 5-8 animals/group. Data represent the mean +/- SD of each group. Results shown are representative of two independent experiments. *$p < 0.05$.

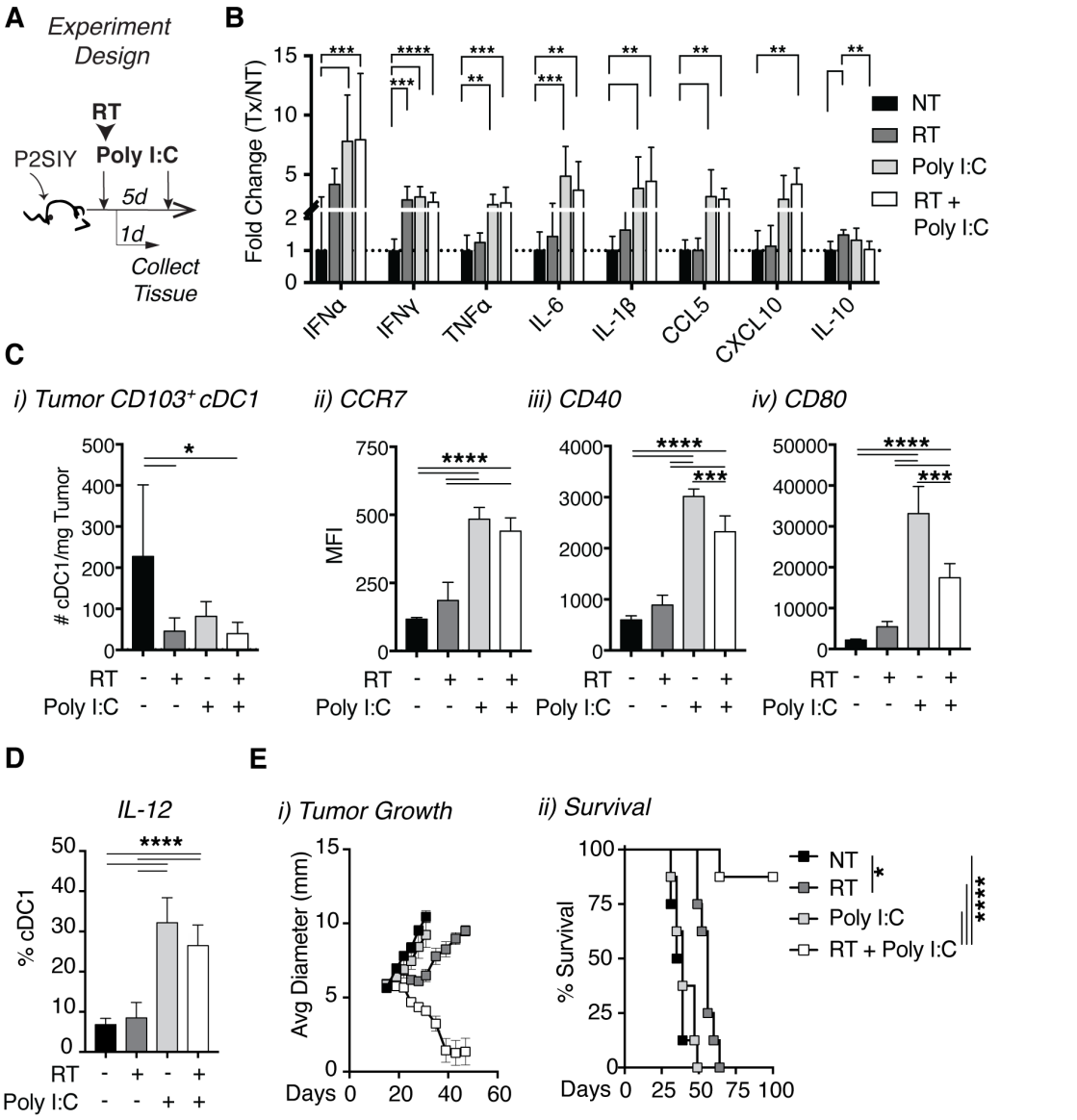


Figure 2-6: The adjuvant poly I:C induces intratumoral cDC1 activation resulting in tumor cures when combined with radiation. (A) Experiment setup for B-D. Panc02-SIY (P2SIY) tumor bearing animals were treated with 12Gy of RT and 50ug of intratumoral poly I:C on day 15, followed by a second dose of intratumoral poly I:C on day 20. Tumors were harvested and analyzed on day 16. (B) Tumors were homogenized, and cytokines were quantified using a multiplex Luminex assay. (C) i) The number of $CD103^+$ cDC1s per mg of tumor tissue was quantified. Intratumoral $CD103^+$ cDC1 expression of ii) CCR7 MFI, iii) CD40 MFI and iv) CD80 MFI. (D) Treated tumors were harvested one day following treatment in vivo, processed into a single cell suspension and cultured with brefeldin A +/- poly I:C in vitro for 6 hours before intracellular cytokine staining. The percentage of $CD103^+$ cDC1 expressing IL-12 was quantified using FACS. (E) i) Tumor growth curves and ii) animal survival following treatment with radiation and poly I:C. n = 5-8 animals/group. Data represent the mean +/- SD of each group. Data are representative of 2-3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

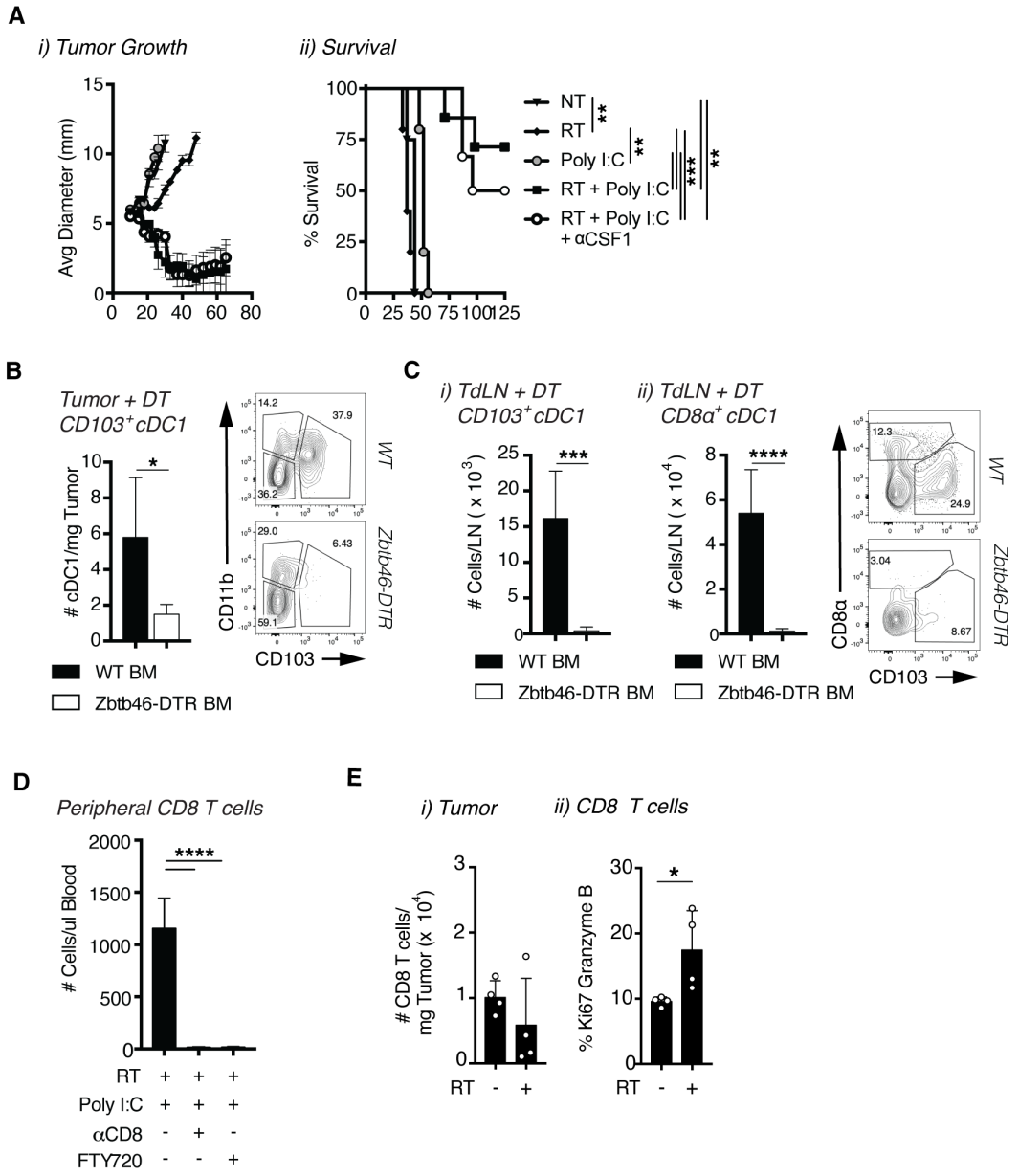


Figure 2-7: The role of individual immune cell subsets in the efficacy of radiation and poly I:C. (**A)** Panc02-SIY (P2SIY) tumor bearing mice were treated with anti-CSF1 antibodies during treatment with 12Gy radiation and poly I:C. i) Tumor growth curves and ii) animal survival after treatment. **(B)** P2SIY tumors were harvested from wild-type (WT) C57BL/6 or Zbtb46^{dtr} bone marrow (BM) chimeras with no treatment or treatment with diphtheria toxin (DT) in addition to radiation and poly I:C. The number of intratumoral CD103$^+$ cDCs/mg of tissue was quantified. **(C)** i) The number of migratory CD103$^+$ cDC1s/dLN and ii) resident CD8a$^+$ cDC1s/dLN was quantified. **(D)** Blood was collected 1 day following radiation and poly I:C in P2SIY tumors after administering FTY720 or CD8 depleting antibodies. The number of CD8$^+$ T cells/µL of blood was quantified. **(E)** MC38 tumors were harvested 7 days following treatment with radiation and poly I:C. The number of i) CD8$^+$ T cells/mg of tumor and ii) the percentage of CD8$^+$ T cells co-expressing Ki67$^+$ and Granzyme B$^+$ was quantified. n = 4-7 animals/group. Data represent the mean +/- SD of each group. **p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

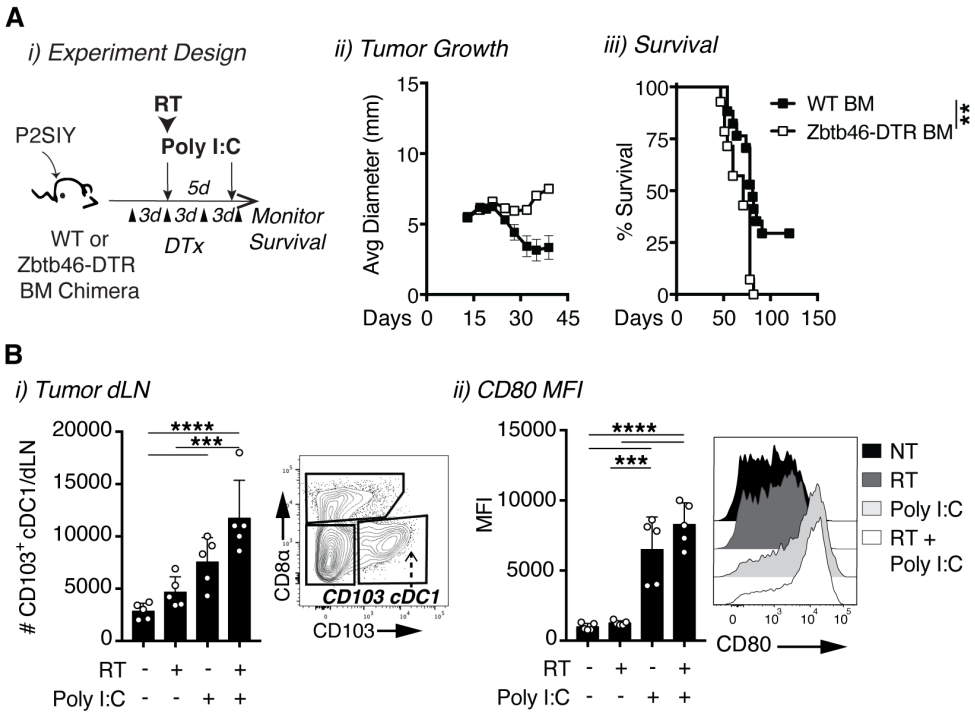


Figure 2-8: The efficacy of radiation and poly I:C is dependent on cDCs. (A) i) Bone marrow (BM) chimeras were generated by transferring wild-type (WT) C57BL/6 or Zbtb46-DTR donor bone marrow into lethally irradiated B6.SJL hosts. Panc02-SIY (P2SIY) tumors were established ~8 weeks following bone marrow reconstitution. Each chimeric group was treated with diphtheria toxin (DTx) starting 3 days prior to 12Gy radiation and poly I:C. n = 12-16 animals/group ii) Tumor growth and iii) animal survival following cDC depletion with diphtheria toxin. (B) i) The tumor dLN was harvested one day following radiation and poly I:C to quantify the number of migratory $CD103^+$ cDC1s and ii) CD80 MFI on migratory $CD103^+$ cDC1s. n = 5-8 animals/group. Data represent the mean +/- SD of each group. Data is representative of two independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

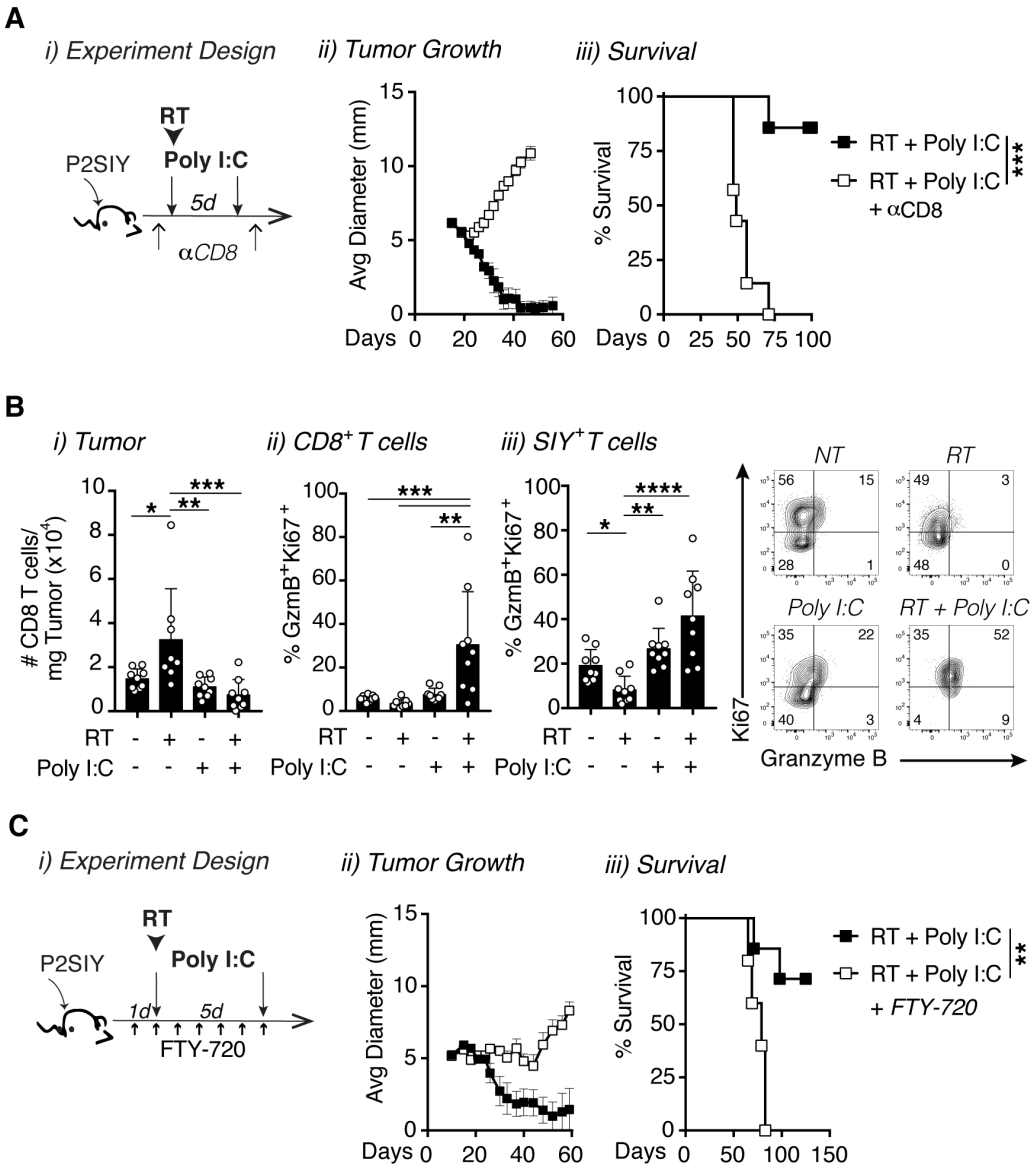


Figure 2-9: Combination therapy increases the recruitment of effector CD8⁺ T cells to the tumor. (A) i) Panc02-SIY (P2SIY) tumor bearing mice were treated with CD8 depleting antibodies one day prior to treatment with 12Gy radiation and intratumoral poly I:C. ii) Tumor growth and iii) animal survival were monitored following treatment. **(B)** i) Tumors were harvested 7 days following treatment with radiation plus poly I:C and CD8⁺ T cells were gated as Live CD45⁺ CD19⁻ CD90.2⁺ CD4⁻ CD8⁺. The number of intratumoral CD8⁺ T cells per mg of tumor tissue were quantified. ii) The expression of Ki67 and Granzyme B was assessed on all intratumoral CD8⁺ T cells and iii) within tumor antigen SIY⁺ CD8 T cells within P2SIY tumors. **(C)** i) P2SIY tumor bearing mice were treated daily with intraperitoneal FTY720 injections starting one day before treatment with radiation and poly I:C. ii) Tumor growth and iii) animal survival following radiation and poly I:C with FTY720 treatment. n = 4-7 animals/group. Data represent the mean +/- SD of each group. Data represent two independent experiments. **p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

Discussion

The treatment response to radiation is highly variable across different cancer pathologies. While radiation is capable of directly killing tumor cells, this is not the sole mechanism responsible for tumor shrinkage following treatment [211]. Our studies confirm that tumor cell intrinsic radiosensitivity in vitro is a poor predictor for the overall response to radiation in vivo and instead implicates other mechanisms. Given that radiation has been shown to elicit tumor-specific adaptive immune responses, we investigated immune-related mechanisms that might explain this variable response across cancer pathologies [212, 235]. Our findings demonstrate that when a range of tumor types were treated with equivalent doses of radiation in vivo, improved treatment responses were dependent on the presence of $CD8^+$ T cells only in radio-immunogenic tumors (MC38, Moc1), and independent of tumor cell intrinsic radiosensitivity. These data highlight the importance of generating a productive tumor-specific adaptive immune response following radiation and provide useful insight into the potential immune-related mechanisms that explain the differential response to radiation across different cancers.

cDC1s are a critical cross-presenting cell type capable of linking the innate and adaptive immune system [34]. We discovered that intratumoral cDC1 activation following radiation is not uniform across different tumor types. Instead, radiation induces cDC1 maturation only in particular tumor types (MC38, Moc1) that corresponds with the tumor types reliant on $CD8^+$ T cells for an improved response to radiation. These data suggest that cDC1 maturation fails to occur in poorly radio-immunogenic tumors either due to active suppression or the absence of adequate signals following radiation therapy. Ultimately, this failure results in impaired generation of tumor-specific $CD8^+$ T cell responses and limits the extent of tumor control following radiation. While we did see a

modest increase in the DC-suppressive cytokine IL-10 following radiation in the poorly radio-immunogenic Panc02-SIY tumors [139], each tumor type may have its own unique pathways or cell types potentially responsible for DC suppression following radiation. These could include other cytokines or metabolites such as PGE_2 or IDO that are increased following radiation and function to suppress intratumoral cDC1 activation [142]. Additional studies are needed to identify the specific factors and signaling pathways within various tumors that prevent cDC1 maturation after treatment in order to improve responses to radiation.

Previous studies have demonstrated that bone marrow-derived DCs injected into irradiated tumors can take up antigens and cross-present in the draining lymph nodes, but have a limited ability to recruit activated T cells back to the irradiated site [236]. Similarly, Jahns et al demonstrated that radiation of monocyte-derived DCs *in vitro* did not directly cause DC maturation, but also did not prevent their maturation following exposure to appropriate stimuli [237]. One approach to overcome the failure of radiation to induce intratumoral cDC1 activation is to provide exogenous adjuvants that drive DC maturation. In this study we used the adjuvant poly I:C to target the innate receptor, TLR3, which is highly expressed by cDC1s [228]. Yoshida et al. previously demonstrated that poly I:C in combination with radiation improved tumor control, resulting in DC activation in the tumor-draining lymph node [226]. We similarly demonstrate that concurrent administration of poly I:C and radiation with a second dose of poly I:C given 5 days later successfully drives intratumoral cDC1 maturation in poorly radio-immunogenic Panc02-SIY tumors. Importantly this treatment combination leads to durable tumor cures that are dependent on cDCs. The prior reports have suggested that when poly I:C is given one day prior to radiation can temporarily delay tumor growth, but treatment ultimately fails to cure tumors

[226]. Timing adjuvant delivery with radiation-mediated tumor cell death is likely critical in coordinating the release of tumor associated antigens with the adjuvant signals that function to promote DC maturation.

Our data suggest that while radiation alone is capable of generating signals that promote cDC1 maturation in radio-immunogenic tumors, these signals are either absent or suppressed in poorly radio-immunogenic tumors. We have previously demonstrated that macrophages suppress T cell control of tumors following radiation therapy [215, 238], and others have shown they can secrete factors such as IL-10 that suppress DC maturation in tumors [139]. In addition, other cell populations present in the tumor environment can alter patterns of DC maturation following radiation therapy [239], suggesting that the immune milieu may regulate the ability of DCs to mature. In poorly radio-immunogenic tumors a bolus of innate adjuvant was sufficient to provide the missing signal or overcome suppressive mechanisms. In our studies in poorly radio-immunogenic tumors we provided this signal in the form of poly I:C which was selected based on the enriched expression of its receptor TLR3 in cDC1s, but other innate adjuvants that activate DC maturation have also shown synergy with radiation therapy [240-242]. While we see no evidence of other cells contributing to cDC1 maturation following TLR3 ligation, this possibility has not been excluded. While TLR3 is expressed by cDC1s and necessary for their activation by poly I:C, cDC1 maturation to full antigen presenting and processing capacity following TLR3 ligation is dependent on their production and response to type I IFN [77, 227, 228]. Thus, TLR3 ligation likely causes additional positive pro-inflammatory effects in the tumor environment secondary to TLR3 ligation in DCs. Together, these data indicate that the presence of immunological adjuvant in the tumor and the capability of

DCs to respond to these released adjuvants are critical determinants for the success of radiation therapy.

A long-standing question within the field of radiation therapy is whether treatment can lead to the development of new tumor-reactive $CD8^+$ T cell responses and essentially function as an endogenous cancer vaccine. Here we provide evidence that radiation fails to drive intratumoral cDC1s maturation in poorly radio-immunogenic tumors, one of the first steps in developing a productive anti-tumor $CD8^+$ T cell response. However, by combining radiation with poly I:C, we overcome this barrier and demonstrate that when T cells have been sequestered in the LNs during treatment tumors fail to cure. DC maturation through signals such as TLR3 ligation results in a decreased phagocytosis and a shift to a migratory and antigen presentation phenotype via expression of markers such as CCR7 and CD80, respectively [28, 243]. Our data suggests that in poorly radio-immunogenic tumors DCs are actively phagocytosing material from irradiated cancer cells, but fail to receive the signals that allow them to mature. In radio-immunogenic tumors, or in poorly radio-immunogenic tumors given adjuvants, these cells complete their cycle and travel to the dLN to prime T cells [28, 243]. These data suggest that in these circumstances that combination therapy is generating new $CD8^+$ T cells responses within the dLN and indicate that under optimal conditions radiation therapy can function as an endogenous cancer vaccine. Importantly, this work also demonstrates the importance of selecting diverse tumor models to evaluate treatments. The non-responsive tumors may provide the greatest source of information to understand how treatments succeed, and critically guide novel interventions to help patient populations who currently do not respond to treatment.

In patients, $CD8^+$ T cell infiltration within tumors tends to correlate with improved outcomes across a range of malignancies [244-246]. Even in the absence of radiation,

recent studies have demonstrated that the presence of DCs within tumors is highly impactful to the success of other therapies [225, 247]. We propose that patients with a poor immune environment are similar to our poorly responsive murine models, whereby radiation therapy fails to drive DC maturation either due to absence of adjuvant signals or by active suppression within the tumor microenvironment. In these patients, radiation would be unable to generate high quality tumor-reactive T cell responses despite the release of tumor antigens that have the potential to be recognized by the immune system. Thus, these unresponsive patients may benefit from the addition of adjuvants that enable radiation therapy to fully function as an endogenous cancer vaccine by driving cDC1 maturation and effective cross-presentation of tumor antigens to $CD8^+$ T cells. We believe that by combining radiation therapy with adjuvants that target these deficiencies, we can restart the cycle of immunity and convert otherwise dismal radiation responses into more favorable outcomes.

Chapter 3:

Dendritic Cell Migration is Required for the Immunological Efficacy of Radiation Therapy

Blair TC, Bambina S, Kramer GF, Alice AF, Baird JR, Crittenden MR, Gough MJ.

Introduction

Radiation therapy is regularly used to treat cancer patients but the mechanisms underlying its effectiveness as a cancer therapeutic remain to be fully determined. Early studies suggested that tumor control and regression following treatment were the result of lethal DNA damage sustained by the tumor cells that resulted in widespread tumor cell death [248]. However, recent evidence has demonstrated that this extensive tumor cell death is capable of interacting with the immune system to promote tumor-specific immunity [183, 199]. The local tumor microenvironment is critical in determining whether therapy drives immune activation or suppression, as both types of responses have been reported following treatment [209, 210, 249]. It has been proposed that radiation therapy can function as an in situ vaccine against tumor by promoting the release of tumor-associated antigens and adjuvant signals from dying tumor cells capable of activating dendritic cells (DCs). For radiation to generate T cells capable of killing tumor cells this likely requires tumor antigen uptake by DCs in the tumor, followed by their subsequent maturation and migration to the tumor draining lymph node (TdLN).

Priming of tumor-specific $CD8^+$ T cells requires specialized DCs capable of cross-presenting tumor associated antigens. Conventional type 1 DCs (cDC1s) have been shown to excel at cross-presenting antigens to $CD8^+$ T cells [34, 221, 222]. In murine models cDC1s are defined by their expression of the transcription factors ID2, IRF8, ZBTB46 and BATF3 [37]. cDC1s can be further divided into those with the capacity to migrate from the tissue to lymphoid organs (migratory $CD103^+$ cDC1s) and those that remain resident to lymphoid organs (resident $CD8\alpha^+$ cDC1s) [222, 223]. Migratory $CD103^+$ cDC1s are one of the primary cell types capable of trafficking intact tumor associated from the tumor to the TdLN to initiate T cell priming and cDC1 migration to LN is thought to occur via a CCR7 dependent mechanism [224]. In preclinical models cDC1s have been shown to support anti-tumor immunity as $Batf3^{-/-}$ mice which lack cross-presenting cDC1s fail to reject highly immunogenic tumors and many immunotherapies depend on the presence of cDC1s [34, 144, 159, 225].

Radiation therapy has been reported to drive the release of adjuvant like compounds capable of inducing DC maturation [199, 200]. However, the specific contribution of cross-presenting cDC1s to radiation mediated tumor regression is poorly understood. We have previously demonstrated that radiation drives intratumoral cDC1 maturation in radio-immunogenic tumors that depend on the adaptive immune system for their enhanced response to radiation, and this process fails to occur in tumors that are poorly radio-immunogenic [248]. Other groups have reported that the efficacy of radiation therapy is significantly diminished in $Batf3^{-/-}$ mice that lack cross-presenting cDC1s [205, 206]. However, cDC1s were depleted in the animals throughout tumor development in these studies making it difficult to determine whether cDC1s contribute to the initial priming of tumor-reactive T cell at tumor implantation or whether they function to prime new T cells

following radiation therapy. cDC1 migration to the tumor draining LN is likely required to initiate priming of tumor-reactive T cells, however, to date it remains unclear whether DC migration from the tumor is required for the immunological efficacy of radiation therapy.

In this section we aimed to understand the role cDC1 migration plays in the response to radiation therapy. By using a radio-immunogenic tumor model that is known to depend on the immune system for improved tumor control following radiation therapy, we demonstrate that cDC migration is required for tumor control and regression following treatment. We demonstrate in radio-immunogenic tumors that radiation does not change the number of cDC1s migrating from the tumor to the TdLN. Instead, treatment with radiation increases the expression of activation markers on tumor migratory cDC1s. Finally, we demonstrate that in poorly radio-immunogenic tumors that fail to induce cDC1 maturation in the tumor, treatment with radiation therapy decreases the number of tumor migratory cDC1s in the TdLN. Taken together these data demonstrate DC migration from the tumor to the TdLN is critical to generating a productive immune response directed towards tumors following radiation therapy.

Materials and Methods

ANIMALS & CELL LINES

Experiments utilized 4-8 week old C57BL/6 (#000664), B6.SJL (#002014), CCR7$^{-/-}$ (#006621) and Zbtb46^{dtr} (#019506) mice that were obtained from The Jackson Laboratories. Kaede transgenic mice were kindly provided by Amanda Lund at Oregon Health and Science University [250]. Survival experiments were performed with 8-14 mice per experimental group, and mechanistic experiments with 4-6 mice per group. Animal protocols were approved by the Earle A. Chiles Research Institute (EACRI) Institutional Animal Care and Use Committee (Animal Welfare Assurance No. A3913-01). The Panc02-SIY pancreatic adenocarcinoma line expressing the model antigen SIY was kindly provided by Dr. Ralph Weichselbaum at the University of Chicago. MC38 colorectal carcinoma line was obtained from Dr. Kristina Young at EACRI. The Panc02-SIY cell line was grown in complete RPMI containing 10% heat inactivated fetal bovine serum (FBS), 100U/mL penicillin, 100μg/mL streptomycin. The MC38 cell line was grown in DMEM containing 10% heat inactivated FBS, 100U/mL penicillin, 100μg/mL streptomycin. Pathogen and mycoplasma contamination testing were performed on all cell lines within the past 6 months using the IMPACT Mouse PCR Profiling from IDEXX BioAnalytics.

TUMOR TREATMENTS

Tumors were implanted subcutaneously into the right flank as follows; 2 x 10^5 MC38 and 5 x 10^6 Panc02-SIY. When tumors were approximately 5mm in average diameter, mice were randomized to receive treatment with CT-guided radiation using the Small Animal Radiation Research Platform (SARRP) from XStrahl. Dosimetry was performed using Murislice software from XStrahl. The SARRP delivered a single dose of 12Gy to an

isocenter within the tumor using a 10mm x 10mm collimator and a 45° beam angle to minimize dose delivery to normal tissues. For poly I:C treatments vaccine grade reagent from InvivoGen (#vac-pic) was administered intratumorally at 50 μg/tumor in a total volume of 10ul. Control mice received 10μl of vehicle. The 1st dose of poly I:C was administered concurrently with radiation and the 2nd dose was given 5 days later. For photoconversion experiments using the Kaede mice, tumors were converted as described by Steele et al [251]. Briefly, animals were completely covered in aluminum foil except for tumors which were exposed to 405nm LED light source using a collimator for 5 minutes (Prizmatix). In all survival experiments, tumor length and width were measured 2-3 times per week using calipers. Mice were euthanized when tumor size exceeded 12 mm in any dimension, or when body condition score declined 1 level.

TISSUE PROCESSING

Following dissection, tumors were weighed and minced into small fragments, then transferred into C tubes from Miltenyi Biotec containing enzyme digest mix with 250U/mL collagenase IV (Worthington Biochemical, #LS004188), 30U/mL DNase I (Millipore-Sigma, #4536282001), 5mM CaCl2, 5% heat inactivated FBS and HBSS. Tissue was dissociated using a GentleMACS tissue dissociator from Miltenyi Biotech. This was followed by incubation at 37°C for 30 min with agitation. For the dLNs, capsules were cut open and incubated with enzymatic mix described above at 37°C for 15 min with agitation. Enzyme mix containing dLNs was then vigorously pipet mixed and incubated at 37°C for an additional 15 min. Enzymatic reactions for both the tumor and dLN were quenched using ice cold RPMI containing 10% FBS and 2mM EDTA. Single cell suspensions were

then filtered through 100μm (tumor) or 40μm (dLN) nylon cell strainers to remove macroscopic debris. Cells were washed and counted for flow cytometry.

FLOW CYTOMETRY

For staining, 2 x 10^6 cells were stained with Zombie Aqua Viability Dye from BioLegend (#423102) in PBS for 10 min on ice, then Fc receptors were blocked with α-CD16/CD32 antibodies from BD Biosciences (2.4G2) for an additional 10 min. After centrifugation, the supernatant was removed and cell were stained with a surface antibody cocktail containing in FACS buffer (PBS, 2mM EDTA, 2% FBS) and Brilliant Stain Buffer Plus from BD Biosciences (#566385) for 20 min on ice. The following antibodies were purchased from BioLegend; F4/80-PerCP/Cy5.5 (BM8), CD11c-PE/Cy7 (N418), CCR7-PE (4B12), CD90.2-A700 (30-H12), CD19-A700 (6D5), MHC-II-BV421 (M5/114.14.2), CD11b-BV605 (M1/70), CD8α-BV650 (53-6.7), and Ly-6C-BV711 (HK1.4). CD40-FITC (HM40-3), CD103-APC (2E9) and CD24-APC e780 (M1/69) were obtained from Thermo Fisher Scientific. CD80-PE CF594 (16-10A1) and CD45-BV786 (30-F11) were purchased from BD Biosciences. After surface staining, cells were washed in FACS buffer and fixed for 20 min on ice with Fixation/Permeabilization Buffer from BD Biosciences (#554722). All samples were resuspended in FACS buffer and acquired on a BD Fortessa flow cytometer. Data were analyzed using FlowJo software from Tree Star, v10.7. cDC1 in the tumor were gated as leukocytes/ single cells/ Live/ $CD45^+$ /$CD90.2^-CD19^-$ /$Ly\text{-}6C^-$ /$MHC\text{-}II^+$ /$CD24^+F4\text{-}80^-$ /$CD11b^-$ /$CD103^+$. In the TdLN migratory $CD103^+$ cDC1 were gated as leukocytes/ single cells/ Live/ $CD45^+$ /$CD90.2^-CD19^-$ /$Ly\text{-}6C^-$ /$MHC\text{-}II^+$ $CD11c^+$ / $CD8\alpha^-$ /$CD103^+$ and resident $CD8\alpha^+$ cDC1 were gated as leukocytes/ single cells/ Live/ $CD45^+$ /$CD90.2^-CD19^-$ /$Ly\text{-}6C^-$ /$MHC\text{-}II^+$ $CD11c^+$ /$CD103^-$ / $CD8\alpha^+$. cDC2 in the tumor were gated

as leukocytes/ single cells/ Live/ $CD45^+$ /$CD90.2^-CD19^-$ /$Ly\text{-}6C^-$ /$MHC\text{-}II^+$ /$CD24^+F4\text{-}80^-$ /$CD103^-$ /$CD11b^+$. In the TdLN migratory $CD11b^+$ cDC2 were gated as leukocytes/ single cells/ Live/ $CD45^+$ /$CD90.2^-CD19^-$ /$Ly\text{-}6C^-$ /$MHC\text{-}II^{high}$ $CD11c^+$ / $CD8\alpha^-$ /$CD103^-$/ $CD11b^+$ and resident $CD11b^+$ cDC2 were gated as leukocytes/ single cells/ Live/ $CD45^+$ /$CD90.2^-CD19^-$ /$Ly\text{-}6C^-$ /$MHC\text{-}II^{int}$ $CD11c^+$ / $CD8\alpha^-$ /$CD103^-$/ $CD11b^+$.

BONE MARROW CHIMERAS

Bone marrow chimeras were generated using B6.SJL ($CD45.1^+$) recipient mice that were irradiated with 10Gy of radiation. Bone marrow cells were isolated from WT C57BL/6 ($CD45.2^+$), CCR7 KO ($CD45.2^+$), or $Zbtb46^{dtr}$ ($CD45.2^+$) donor mice femurs and tibias using a 27G needle. Cells were filtered through a 70 μm cell strainer to generate a single cell suspension and resuspended in PBS. Recipient mice received 1.5-2.5 x 10^6 of each specified donor bone marrow cells for a total of 3-5 x 10^6 cells/recipient animal that were transferred by retro-orbital injection. Tumors were implanted 8-10 weeks following bone marrow reconstitution. Diphtheria toxin from Millipore-Sigma (#D0564) was administered 3 days prior to radiation at 20 ng/g intraperitoneally for initial DC depletion. This was followed by an additional 3 doses of 5 ng/g of diphtheria toxin that were given every 3 days to maintain depletion.

STATISTICS

Data were analyzed and graphed using Prism from GraphPad Software (v9.0). Individual data sets were compared using Student's T-test and analysis across multiple groups was performed using ANOVA with individual groups assessed using Tukey's comparison. Kaplan Meier survival curves were compared using a log-rank test.

Results

DC MIGRATION IS REQUIRED FOR THE IMMUNOLGICAL EFFICACY OF RADIATION THERAPY IN RADIO-IMMUNOGENIC MC38 TUMORS

First, we set out to determine whether cDC1 migration was required for the efficacy of radiation therapy. The chemokine receptor CCR7 is upregulated upon DC maturation and this receptor has been shown to play an important role in guiding cDC1 migration from the tissue through the lymphatics to the dLN [84, 89]. Animals that completely lack CCR7 have impaired T cell and DC migration, resulting in disrupted architecture within the LN [89]. To overcome this issue and formally examine the role of CCR7 mediated migration, we use a mixed bone marrow (BM) chimera approach to deplete only cDCs expressing CCR7 [224]. Animals were given 50% $CCR7^{-/-}$ BM and 50% BM from mice where the human diphtheria toxin receptor (dtr) expression is driven by the cDC specific transcription factor Zbtb46 (**Figure 3-1A i**). Thus, $Zbtb46^{dtr}$ BM ensured that cDCs expressing CCR7 are present during animal and tumor development but enabled us to deplete these $CCR7^{+/+}$ cDCs by administering diphtheria toxin, leaving behind only $CCR7^{-/-}$ deficient cDCs. This ensured normal tumor and LN biology prior to and during experiments. As controls we gave another group of mice 50% WT (C57BL/6) BM and 50% $CCR7^{-/-}$ BM (**Figure 3-1A i**). BM was allowed to reconstitute for 8-10 weeks and then tumors were implanted (**Figure 3-1A i**). We first aimed to block cDC migration in the radio-immunogenic MC38 colorectal carcinoma tumor model which we have previously published is very responsive to radiation therapy and successfully induces intratumoral cDC1 maturation [248]. When tumors reach ~5mm average diameter animals were given diphtheria toxin to deplete cDCs expressing CCR7, leaving behind only $CCR7^{-/-}$ cDCs and then tumors were subsequently treated with 12Gy of CT-guided radiation (**Figure 3-1A i**). The TdLN was

harvested 1 day following treatment for analysis by flow cytometry (**Figure 3-1A i**). As expected, the number of migratory CD103^{+} cDC1s in the TdLN were significantly reduced in the animals where cDC migration was impaired (**Figure 3-1A ii**). We also saw a reduction in the number of migratory CD11b^{+} cDC2s in the TdLN following treatment, though not to same degree as migratory CD103^{+} cDC1s (**Figure 3-1A iii**). This is to be expected because not all cDC2s are depleted in Zbtb46$^{-/-}$ mice, and as a result would not be depleted with diphtheria toxin in our model and could still migrate normally because they express CCR7 [232]. These data confirm that our mixed BM chimera approach impairs migratory cDC migration to the TdLN. Next, we setup experiments to monitor tumor growth and animal survival when cDC migration was impaired during treatment with radiation (**Figure 3-1B i**). When control animals (WT:CCR7$^{-/-}$) were treated with radiation there was a significant survival advantage in these animals and half of the tumors were cured as compared to untreated control animals (**Figure 3-1B ii-iii**). However, in animals where cDC migration was impaired (Zbtb46^{dtr}:CCR7$^{-/-}$), this survival advantage disappeared following radiation therapy and tumor cures were no longer observed (**Figure 3-1B ii,iv**). These data provide convincing evidence that cDC migration is required for the efficacy of radiation therapy in radio-immunogenic MC38 tumors.

THE EFFICACY OF RADIATION COMBINED WITH ADJUVANT IN POORLY RADIO-IMMUNOGENIC PANC02-SIY TUMORS DOES NOT REQUIRE cDC MIGRATION

Our next question was whether cDC migration was required for the efficacy of therapy in poorly radio-immunogenic tumors that have been treated with adjuvant to drive cDC1 maturation. We previously demonstrated that the pancreatic ductal adenocarcinoma tumor model, Panc02-SIY is poorly responsive to radiation and fails to successfully drive

cDC1 maturation unless adjuvant (poly I:C) is provided at the time of treatment to mature cDC1s [248]. Using the same approach described above, control (WT:CCR7$^{-/-}$) and migration impaired (Zbtb46^{dtr}:CCR7$^{-/-}$) BM chimeras were established (**Figure 3-2A i**). cDCs expressing CCR7 were depleted and tumors were treated with the combination of 12Gy radiation therapy in combination with 50 μg of intratumoral poly I:C and the TdLN was harvested 1 day after treatment (**Figure 3-2A i**). Analysis of DC numbers in the TdLN 1 day following treatment revealed that the number of both migratory CD103^{+} cDC1s and migratory CD11b^{+} cDC2s in the TdLN was significantly reduced following treatment with diphtheria toxin when CCR7 expressing cDCs were depleted (**Figure 3-2A ii,iii**). We then setup a survival experiment to determine whether cDC migration was required for the combined efficacy of radiation and poly I:C (**Figure 3-2B i**). Radiation combined with poly I:C resulted in a significant survival advantage and tumor cures in WT control BM chimeras (WT:CCR7$^{-/-}$) when compared to radiation alone or untreated animals (**Figure 3-2B ii-iii**). However, when cDC migration was impaired, this surprisingly had no impact on animal survival or tumor growth (**Figure 3-2B ii,iv**). We have previously demonstrated that in the Panc02-SIY tumors treated with the combination of radiation therapy and poly I:C, that cDCs are required for the efficacy of treatment [248]. While cDCs are clearly important for the efficacy of radiation and poly I:C in the poorly radio-immunogenic Panc02-SIY tumor model, it appears that cDC migration to the TdLN is not required for the efficacy of treatment. These data suggest in poorly radio-immunogenic Panc02-SIY tumors treated with radiation in combination with adjuvant that cDCs either function locally within the tumor or adjuvant might enable antigen delivery and/or cross-presentation by a non-CCR7- and/or Zbtb46-dependent population.

MIGRATORY DC SUBSETS IN THE TDLN ARE ENRICHED FOR DC THAT WERE IN THE TUMOR AT THE TIME OF TREATMENT

Given that cDC migration was required for the efficacy of radiation therapy in radio-immunogenic MC38 tumors, we next aimed to understand how radiation influences the kinetics and phenotype of cDCs migrating directly from the tumor. In our models, the inguinal LN drains the tumor along with other surrounding tissues, and as a result makes it difficult to assess changes in only tumor migratory DC populations following treatment [252, 253]. To overcome this issue, we utilized the Kaede photoconvertible mice, which express the Kaede-green fluorescent protein that can be converted into the Kaede-red fluorescent protein upon exposure to violet light [250]. Radio-immunogenic MC38 tumors were implanted into Kaede mice and when tumors reached an average diameter of 5-6mm the animals were covered in aluminum foil except for the tumor, which was exposed to a 405nm LED light source for 5 minutes, followed by treatment with 12Gy radiation (**Figure 3-3A**). The TdLN was harvested at 1, 2 and 3 days post photoconversion for analysis by flow cytometry (**Figure 3-3A**). The majority of tumor migratory converted (Kaede-red$^+$) cells were found in T cell (data not shown) and DC populations as has been previously published (**Figure 3-3B**) [251, 254]. Strikingly, we noted that within DC populations in the TdLN, the majority of converted Kaede-red$^+$ cells were found in migratory CD103$^+$ and CD11b$^+$ DC subsets, validating that these were indeed migratory populations (**Figure 3-3B-C**). Further confirming the specificity of this model, we did not find any converted DCs in the contralateral inguinal LN (**Figure 3-3B**). These data revealed that converted Kaede-red$^+$ cells represented less than half of the migratory CD103$^+$ and CD11b$^+$ DC subsets 1 day after photoconversion (**Figure 3-3C i-ii**). The frequency of converted cells in LN-resident CD8α^+ and CD11b$^+$ DC populations was

significantly lower (**Figure 3-3C iii-iv**). Over time the frequency of converted tumor migratory DCs slowly declined in migratory CD103$^+$ and CD11b$^+$ DC populations (**Figure 3-3C i-ii**). Treatment with radiation did not change the proportion of converted DC subsets in the dLN (**Figure 3-3C i-iv**). Taken together these data suggest that the Kaede mice are a useful model to directly study tumor migratory DC populations following treatment with radiation therapy.

IN RADIO-IMMMUNOGENIC TUMORS, THE FREQUENCY OF ACTIVATED TUMOR MIGRATORY DCs ARE INCREASED IN THE TDLN AFTER RADIATION THERAPY

There are two potential ways that radiation could be influencing cDC migration; 1) by increasing the number of migratory cDCs following treatment or 2) changing the phenotype of these cells after therapy. The data from the Kaede mice demonstrated that migratory cDCs encompassed the highest frequency of converted cells. Using the same experiment setup as above for radio-immunogenic MC38 tumors (**Figure 3-3A**), we decided to focus in specifically on these tumor migratory populations to assess changes in their numbers and phenotype following radiation. Radiation did not change the total number of migratory or converted CD103$^+$ cDC1s when compared to untreated controls (**Figure 3-4A i-ii**). While the number of total migratory CD103$^+$ cDC1s remained relatively constant over time in both groups, the number of converted CD103$^+$ cDC1s slowly declined in both groups (**Figure 3-4A i-ii**). We noted a similar trend in both the total number and converted number of migratory CD11b$^+$ cDC2s (**Figure 3-4B i-ii**). Thus, radiation does not appear to be change the kinetics of DC migration from the tumor to the TdLN. The next question was whether treatment impacted the phenotype of DC populations migrating from the tumor. We first looked at the expression of the co-

stimulatory molecules CD40 and CD80 which are upregulated during DC maturation. Starting 2 days following treatment there was a significant increase in the proportion of converted tumor migratory $CD103^+$ cDC1s co-expressing CD40 and CD80 in the radiation treated group (**Figure 3-4C i**). Moreover, in the radiation treated group we noted that the MFI of CD80 was significantly higher in converted $CD103^+$ cDC1s 3 days post treatment (**Figure 3-4C ii**). While there was some evidence that converted migratory $CD11b^+$ DCs from the radiation treated group had an increased proportion of DC co-expressing CD40 and CD80, this effect was significantly reduced in this DC subset (**Figure 3-4 i-ii**). These data suggest that while radiation therapy fails to increase the number of $CD103^+$ DCs migrating from the tumor, treatment does increase the proportion of tumor migratory $CD103^+$ DCs expressing CD40 and CD80 in the TdLN.

RADIATION DECREASES THE NUMBER OF TUMOR MIGRATORY DCs IN THE TDLN AFTER TREATMENT IN POORLY RADIO-IMMUNOGENIC PANC02-SIY TUMORS

Our data thus far has indicated that cDC migration is required for the efficacy of radiation therapy in radio-immunogenic tumors and that treatment in this model increases the proportion of tumor migratory cDCs in the TdLN with an activated phenotype. Moreover, our previous work had demonstrated that radiation failed to induce cDC maturation in poorly radio-immunogenic Panc02-SIY tumors. This raised the question of how radiation impacted cDC migration in the Panc02-SIY model. Given that the timepoint 3 days post treatment seemed to yield the most significant differences in the radio-immunogenic MC38 tumor model we opted to test this timepoint in Panc02-SIY tumors. Panc02-SIY tumor were implanted into Kaede mice and when they reached 5-6mm average diameter, tumors were photoconverted followed by treatment 12Gy radiation

(**Figure 3-5A**). In the Panc02-SIY tumor model we again noticed that within DC subsets in the TdLN, it was predominantly the migratory $CD103^+$ and $CD11b^+$ populations that contained the highest frequency of converted tumor migratory DCs (**Figure 3-5B**). Interestingly, when we compared untreated and radiation treated animals, we also noticed that radiation decreased the frequency of converted cells within both migratory $CD103^+$ and $CD11b^+$ DC populations (**Figure 3-5B**). Next, we evaluated the total number in addition to the number of converted migratory $CD103^+$ and $CD11b^+$ DCs in the TdLN (**Figure 3-5C-D**). Similarly, we noted significantly fewer converted $CD103^+$ and $CD11b^+$ DCs in the TdLN after treatment with radiation (**Figure 3-5C i-ii, Figure 3-5D i-ii**). These data suggested that radiation impairs the migration of cDCs from the tumor to the TdLN in the poorly radio-immunogenic Panc02-SIY tumor model. Finally, we analyzed the expression of the co-stimulatory molecules CD40 and CD80 which are upregulated upon DC maturation. In the radiation treated group, the converted tumor migratory $CD103^+$ DCs had an increased proportion of cells co-expressing CD40 and CD80 (**Figure 3-5 i**). However, we did not detect any differences in the expression of these markers in converted tumor migratory $CD11b^+$ DCs between untreated and radiation treated groups (**Figure 3-5 ii**). Thus, treatment with radiation reduces the number of tumor migratory cDCs that are found in the TdLN and this may explain why the Panc02-SIY tumor model is poorly responsive to radiation therapy.

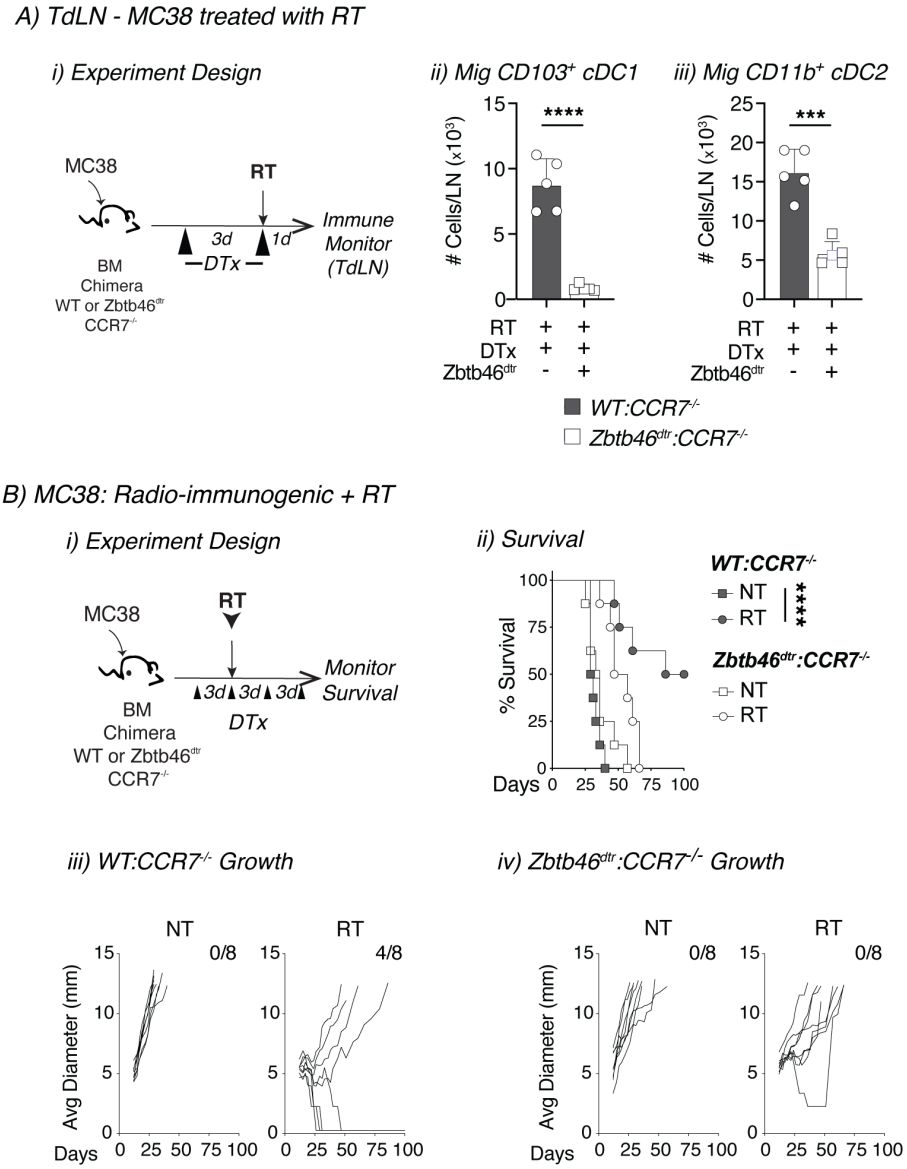


Figure 3-1: Radio-immunogenic MC38 tumors require cDC migration for the immunological efficacy of radiation therapy. (A) i) Experiment layout for Aii-Aiii. Mixed bone chimeras (BM) (WT:CCR7$^{-/-}$ or Zbtb46^{dtr}:CCR7$^{-/-}$) were established for 8-10 wks prior to MC38 tumor implantation. Animals were given diphtheria toxin (DTx) to deplete cDC expressing CCR7 as indicated and treated with 12Gy radiation. The tumor draining LN (TdLN) was harvested 1 day following treatment (n=4-5 animals/group) for immune monitoring. ii) The number of migratory CD103^{+} cDC1s and iii) migratory CD11b^{+} cDC2s in the TdLN in WT:CCR7$^{-/-}$ or Zbtb46^{dtr}:CCR7$^{-/-}$ mixed BM chimeras. **(B)** i) Experiment layout for Bii-Biv. Mixed BM chimeras (WT:CCR7$^{-/-}$ or Zbtb46^{dtr}:CCR7$^{-/-}$) were established for 8-10 wks prior to MC38 tumor implantation. Animals were given diphtheria toxin (DTx) to deplete cDCs expressing CCR7 as indicated and treated with 12Gy radiation. Animal survival was monitored following cDC depletion and radiation therapy. ii) Animal survival following treatment as detailed in Bi (n=8 animals/group). iii) Individual growth curves for control animals (WT:CCR7$^{-/-}$ mixed BM chimeras) and iv) animals where CCR7 expressing cDCs were depleted prior to treatment with radiation (Zbtb46^{dtr}:CCR7$^{-/-}$ mixed BM chimeras). ***p < 0.001, ****p < 0.0001.

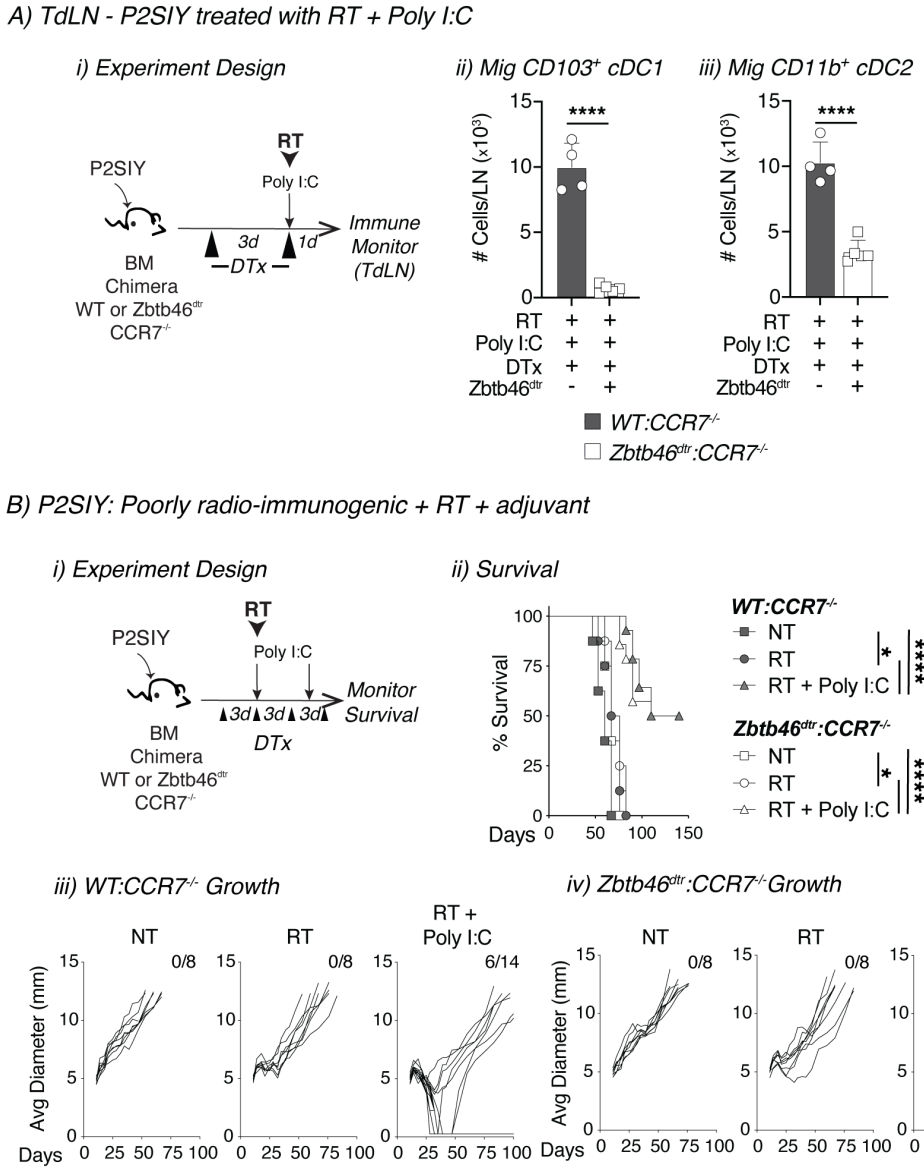


Figure 3-2: cDC migration is not required for the combined efficacy of radiation and adjuvant in poorly radio-immunogenic Panc02-SIY tumors. (A) i) Experiment layout for Aii-Aiii. Mixed bone chimeras (BM) (WT:CCR7$^{-/-}$ or Zbtb46^{dtr}:CCR7$^{-/-}$) were established for 8-10 wks prior to Panc02-SIY (P2SIY) tumor implantation. Animals were given diphtheria toxin (DTx) to deplete cDCs expressing CCR7 as indicated and treated with 12Gy radiation and 50ug of intra-tumoral poly I:C. The TdLN was harvested 1 day following treatment (n=4-5 animals/group) for immune monitoring. ii) The number of migratory CD103^{+} cDC1s and iii) migratory CD11b^{+} cDC2s in the TdLN in WT:CCR7$^{-/-}$ or Zbtb46^{dtr}:CCR7$^{-/-}$ mixed BM chimeras. **(B)** i) Experiment layout for Bii-Biv. Mixed BM chimeras (WT:CCR7$^{-/-}$ or Zbtb46^{dtr}:CCR7$^{-/-}$) were established for 8-10 wks prior to P2SIY tumor implantation. Animals were given diphtheria toxin (DTx) to deplete cDCs expressing CCR7 as indicated and treated with 12Gy radiation + 50ug of intra-tumoral poly I:C. 5 days later animals were given a second dose of 50ug poly I:C intratumorally. Animal survival was monitored following cDC depletion and radiation therapy. ii) Animal survival following treatment as detailed in Bi (n=8-14 animals/group). iii) Individual growth curves for control animals (WT:CCR7$^{-/-}$ mixed BM chimeras) and iv) animals where CCR7 expressing cDCs were depleted prior to treatment with radiation and poly I:C (Zbtb46^{dtr}:CCR7$^{-/-}$ mixed BM chimeras). Results shown are representative of two independent experiments. *$p < 0.05$, ****$p < 0.0001$.

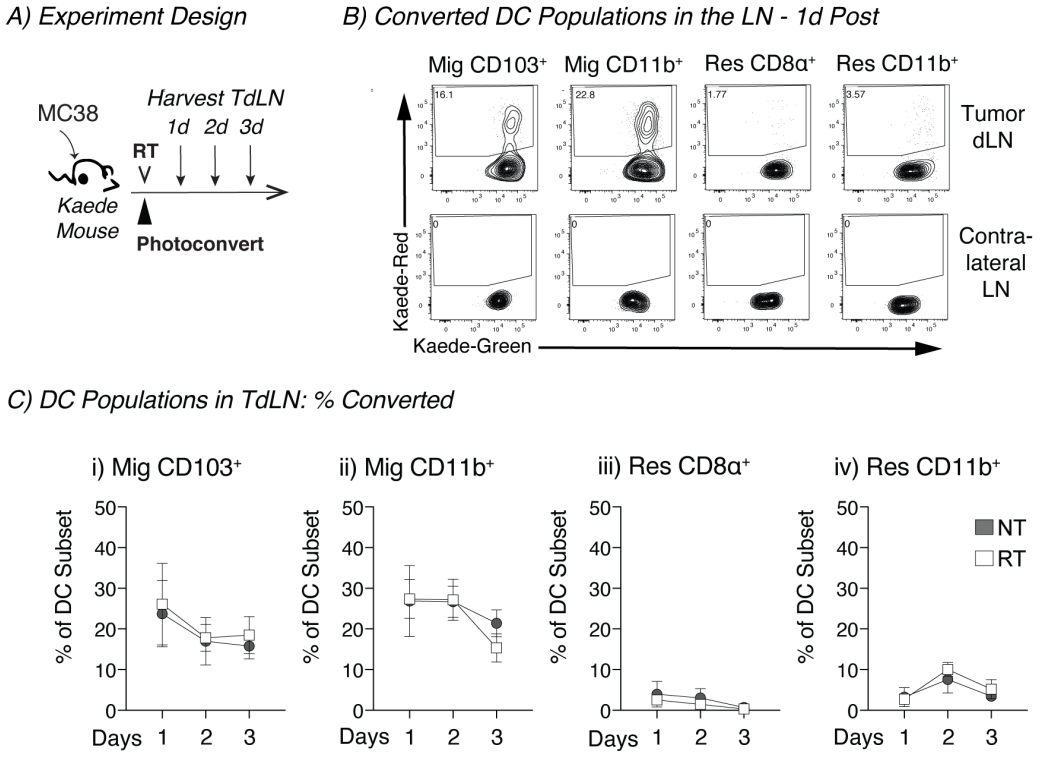


Figure 3-3: Tumor migratory DC populations in the TdLN. (A) Experiment design for B-C. MC38 tumors were implanted into Kaede mice and treated with 12Gy of RT when tumors reached ~5-6mm average diameter. All tumors were photoconverted at the time of radiation for 5min with a 405nm LED light sources while covering the rest of the animal with foil. The TdLN was harvested 1d, 2d, and 3d following treatment. **(B)** Representative flow plots from the TdLN and contralateral non-draining LN 1d post photoconversion in an untreated (NT) animal. Kaede-Red positive were gated in DC populations within the LN. **(C)** Comparing the frequency of converted (Kaede-Red$^+$) cells in each DC subset; i) Mig CD103$^+$, ii) Mig CD11b$^+$, iii) Res CD8! $^+$ and iv) Res CD11b$^+$ in the TdLN at 1d, 2d, and 3d post photoconversion between untreated (NT, grey) and radiation treated (RT, white). n = 3 animals/group.

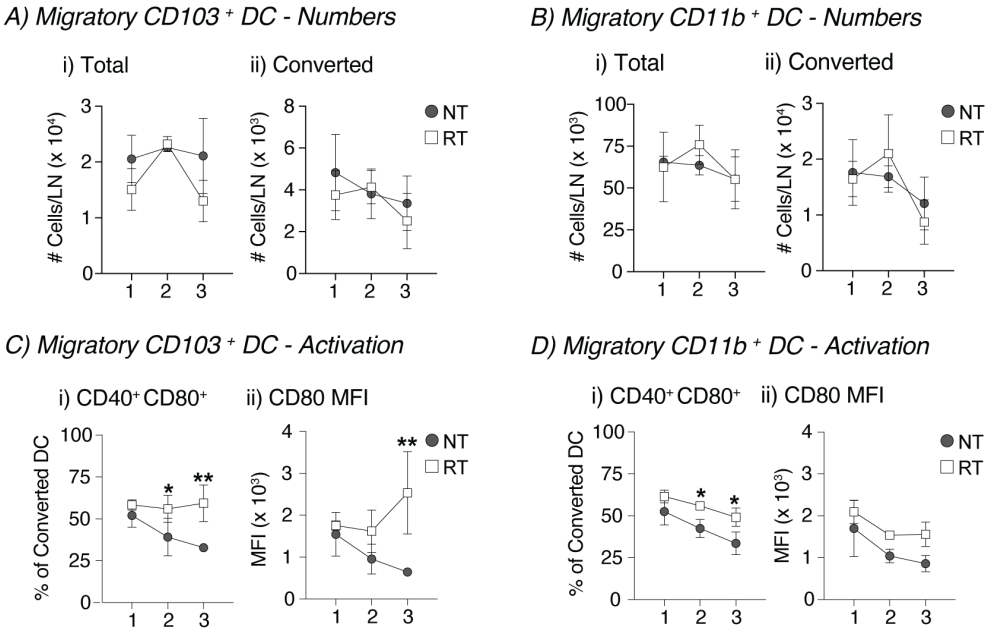


Figure 3-4: Radiation increases the frequency of activated tumor migratory DCs in the TdLN. MC38 tumors were established and treated as described in 3-3A. **(A)** The total number of i) migratory CD103$^+$ DCs and ii) converted (Kaede-Red$^+$) migratory CD103$^+$ DCs cells were quantified in the TdLN of untreated (NT, grey) and treated with 12Gy radiation (RT, white) over time. **(B)** The total number of i) migratory CD11b$^+$ DCs and ii) converted (Kaede-Red+) migratory CD11b$^+$ DCs cells were quantified in the TdLN of NT and RT treated animals. **(C)** Expression activation markers on converted tumor migratory CD103$^+$ DCs compared between NT and RT. Comparing the i) frequency of CD40$^+$ CD80$^+$ and ii) CD80 MFI. **(D)** Expression activation markers on converted tumor migratory CD11b$^+$ DCs compared between NT and RT. Comparing the i) frequency of CD40$^+$ CD80$^+$ and ii) CD80 MFI. *$p < 0.05$, **$p < 0.01$. n = 4-5 animals/group.

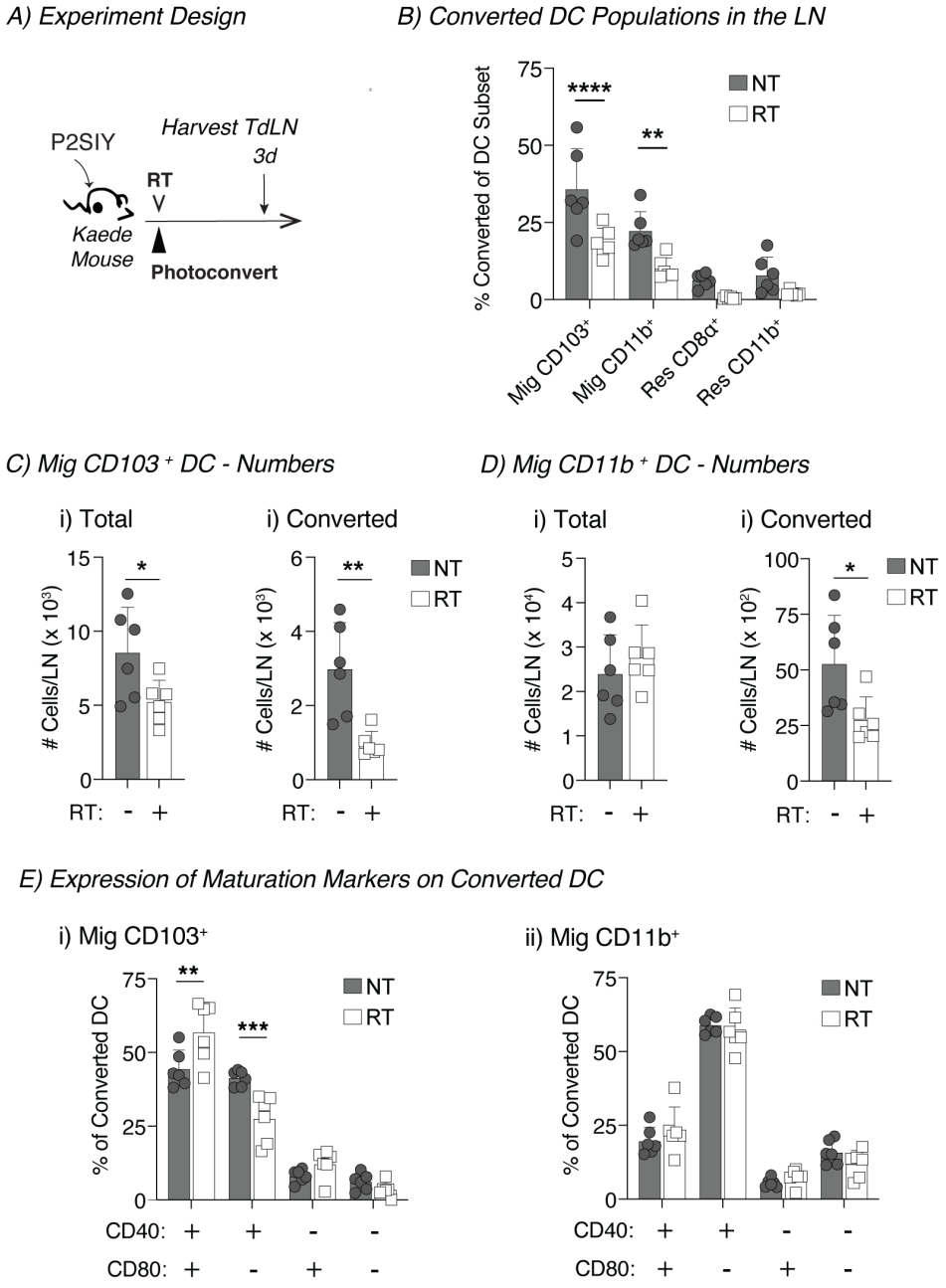


Figure 3-5: Radiation decreases the number of tumor migratory DCs in the TdLN of poorly radio-immunogenic Panc02-SIY tumors. (A) Experiment design for B-E. Panc02-SIY (P2SIY) tumors were established and photoconverted for 5min with 405nm LED light prior to being treated with 12Gy radiation (RT). The TdLN was harvested 3d post treatment and analyzed by flow cytometry **(B)** The percentage converted (Kaede-Red$^+$) cells within each DC subset was quantified in the TdLN for untreated (NT) and RT groups. **(C)** The number of i) total mig CD103$^+$ DCs and ii) converted mig CD103$^+$ DCs in the TdLN were quantified for NT and RT groups. **(D)** The number of i) total mig CD11b$^+$ DCs and ii) converted mig CD11b$^+$ DCs in the TdLN were quantified for NT and RT groups. **(E)** Expression of activation markers (CD40 & CD80) on converted i) mig CD103$^+$ DCs and ii) mig CD11b$^+$ DCs. Each symbol represents an individual animal . *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. n = 6 animals/group.

Discussion

A long-standing question within the field of radiation oncology is whether radiation therapy can function as an in situ vaccine capable of priming new $CD8^+$ T cells responses directed against tumors. To function in this capacity, treatment with radiation therapy would likely require mature cDC1s to carry tumor-associated antigens from the tumor to the TdLN for cross-presentation to T cells, and to date there has been limited evidence supporting the role of DC migration in radiation. Here we demonstrate that cDC migration from the tumor to the TdLN is necessary for the immunological efficacy of radiation in the radio-immunogenic MC38 tumor models. This is one of the first steps in generating tumor-specific immunity and these data suggest that radiation has the capacity to initiate the process of priming new $CD8^+$ T cell responses. Once in the TdLN these tumor migratory cDCs may either function to directly cross-present antigens to naïve tumor antigen-specific $CD8^+$ T cells or they may instead hand off antigen to other cDC1 subsets in the LN for cross-presentation [224, 255]. Our previous work indicated that radiation can drive intratumoral cDC1 maturation in radio-immunogenic tumors [248]. These new studies build on this work and provide convincing evidence that radiation is capable of inducing both cDC maturation and migration to the TdLN following treatment and these events are critical to maximizing the benefits or radiation therapy.

Previous studies have demonstrated that by combining radiation with adjuvant in poorly radio-immunogenic tumors this could overcome the failure of radiation alone to induce intratumoral cDC1 maturation, resulting in improved tumor control following treatment that required the presence of cDCs [248]. Interestingly, our data suggested that cDC migration was not required for combined efficacy of radiation and poly I:C in the poorly radio-immunogenic Panc02-SIY tumor model. Given that treatment requires the presence

but not necessarily the migration of cDCs, these data suggest that cDCs may exert their function locally in this tumor model. For example, Spranger et al have reported that Batf3^{+} cross-presenting DCs reside in tumors and function to secrete the cytokines that recruit tumor-specific T cells into the tumor for re-priming [225]. Alternatively, treatment with the adjuvant poly I:C may enable expression of other migratory receptors to compensate for the loss of CCR7 on cDCs. The chemokine receptor CXCR4 has been shown to be involved in cDC migration from the skin to dLN and this receptor could allow DCs to still migrate from the tumor to the TdLN in the absence of CCR7 [96]. Another possibility is that tumor-associated antigens are being delivered to LN-resident cDC1s for cross-presentation independent of the migratory cDC populations that are being targeted for depletion in our BM chimera models. Future studies utilizing the Kaede photoconvertible mice would enable us to address whether a CCR7$^{-/-}$ or Zbtb46$^{-/-}$ DC population within the tumor is still capable of trafficking to the TdLN following treatment with radiation and poly I:C.

In our tumor models the inguinal LN drains the tumor in addition to other surrounding tissues which makes it difficult to determine whether migratory DC populations in the TdLN came from the tumor or other surrounding tissues [251, 254]. By using the Kaede photoconvertible mice we were able to specifically identify tumor migratory DC populations within the TdLN. Similar to previous reports, DCs were one of the main cell types migrating from the tumor to the TdLN and converted cells were predominantly found in populations that we identified as being migratory DCs using flow cytometry markers [251, 254]. These data confirm that our flow cytometry panel accurately identifies migratory DC populations. Given that less than half of migratory DCs were converted cells, these data also highlight why it has been difficult in the past determine how different treatments specifically impact

DC migration from the tumor since these cells make up a relatively small proportion of total migratory DC subsets in the TdLN. Thus, the Kaede mice are a powerful tool to specifically track and characterize changes in DC populations migrating from the tumor to the TdLN.

Unsurprisingly, our data also suggest that radiation has differential impacts on DC migration depending on the tumor model being studied. We found that radiation does not change the number of tumor migratory DCs in the TdLN in the radio-immunogenic MC38 tumor model. By contrast, treatment with radiation impaired DC migration to the TdLN in poorly radio-immunogenic Panc02-SIY tumors. It remains possible that the kinetics of migration in the poorly radio-immunogenic model is different than in radio-immunogenic tumors. However, in radio-immunogenic tumors, radiation had no impact on the migration across several time points, indicating that the impairment in DC migration might be a sustained phenomenon in the Panc02-SIY tumor model. CD40 and CD80 are widely used to define mature DC, as they are consistently upregulated adjuvant signals [58, 78, 79]. While it would be expected the majority of DCs found in the LN have a “mature” phenotype since they were able to successfully migrate from the tissue, it’s likely that migrating DCs exist on a spectrum of maturity. This maturity level likely determines their ability to successfully cross-present antigens and activate adaptive immune responses. For instance, It’s been reported that semi-mature DCs are capable of migrating from the tissue to the dLN, however, these DCs are poor stimulators of T cell immunity [84]. Future studies are needed to perform a comprehensive analysis to identify additional migratory DCs maturation markers such that we can determine whether more subtle difference exist between theses DCs.

Our data suggest that in certain tumor models, radiation is capable of functioning similarly to an endogenous cancer vaccine. However, in tumors that have cDC1s, this raises the question of why radiation activates the immune system successfully in one model, but then fails to do so in another tumors model. As has been previously reported there are many potential mechanisms that could be responsible for the suppression of DC maturation following treatment, whether coming from directly from the tumor or intratumoral immune populations such as macrophages [139]. For instance, it has been reported that oxidized cholesterol ligands secreted from tumors can suppress the expression of CCR7 in DCs and this impairs the migration from the tumor to TdLN [166]. Taken together, these data suggest that individual tumor microenvironments determine whether DCs are activated or suppressed following treatment with radiation, and this in turn determines whether DCs are able to migrate to the TdLN to cross-present antigens. Additional work is needed unravel the potential mechanisms that prevent DCs from initiating the priming of new tumor-reactive $CD8^+$ T cells. In the following chapter we directly address this question by using single cell RNA sequencing to compare DC gene expression patterns in radio-immunogenic versus poorly radio-immunogenic tumors. In this way we can explore the direct effect of treatment on DCs within the tumor that might explain their differing response to radiation therapy.

Chapter 4:

Radiation Induced Transcriptional Changes in Tumor Dendritic Cells

Blair TC, Dowdell A, Bambina S, Kramer GF, Alice AF, Piening B, Crittenden MR, Gough MJ.

Introduction

Radiation therapy has the capacity to promote the maturation of intratumoral dendritic cells (DCs) through the release of endogenous adjuvants from dying tumor cells [199, 200]. DC maturation and migration to the tumor draining lymph node (TdLN) is likely critical to the development of tumor-specific immunity following treatment with radiation therapy. Within the tumor conventional DCs (cDCs) are the primary professional antigen presenting cell (APC) type with the capacity to respond to these inflammatory signals to initiate the development of tumor-specific T cell responses. There are two main types of cDCs; 1) conventional DCs type 1 (cDC1s) which are superior at cross-presenting antigens to $CD8^+$ T cells; and 2) conventional DC type 2 (cDC2s) which excel at presenting antigens to $CD4^+$ T cells [25]. Previous work has demonstrated that cDC1s are critical for the development of anti-tumor immunity and given the role of $CD4^+$ T cell help in developing a comprehensive $CD8^+$ T cells responses, it is likely that cDC2s play an important role as well [34]. Radiation therapy has been reported to drive the maturation of cDC1s in tumors

that require the development of an adaptive immune response for an enhanced response to treatment, termed radio-immunogenic tumors, and this process fails to occur in poorly radio-immunogenic tumors [248]. The mechanisms that determine why some tumors successfully drive DC maturation while others fail to are poorly understood.

DCs express multiple types of pattern recognition receptors (PRRs) that enable them to sense different signals within their environment to determine whether they promote or suppress the generation of an adaptive immune response against foreign pathogens [68]. Radiation therapy has been shown to promote the release of signals from dying tumor cells such as nucleic acids, or extracellular ATP that have the potential to trigger signaling through PRRs expressed by DCs to drive their maturation [200, 201]. In preclinical models, sensing of cytosolic DNA through the stimulator of interferon genes (STING) pathway in DC is one such mechanism that has been reported to promote anti-tumor immune responses [201]. The individual tumor microenvironment is likely critical to shaping the response to radiation as treatment has also been reported to drive immune suppression [214, 215]. Tumors are known to secrete compounds such as oxidized cholesterol ligands or prostaglandin E_2 (PGE_2) that are capable of suppressing DC maturation [166, 169]. Thus, the cumulative integration of these activating and suppressive signals is likely critical to determining whether a productive tumor-specific T cell response is generated following radiation therapy.

In this section we aimed to understand which transcriptional pathways are activated or suppressed in DCs following radiation therapy. We have previously reported that radiation drives cDC maturation in some tumors, but not in other following treatment with radiation [248]. In this chapter we compare the transcriptional signatures between cDCs from radio-immunogenic tumor that successfully induce cDC maturation following

radiation, to the signatures in cDCs from poorly radio-immunogenic tumors that fail to induce cDC maturation. Using these transcriptional signatures, we identify cDCs that were likely in the tumor at the time of treatment with radiation. Furthermore, we demonstrate that radiation drives the upregulation of genes that are associated with nucleic acid sensing pathways in radio-immunogenic tumors, but not in poorly radio-immunogenic tumors.

Materials and Methods

ANIMALS & CELL LINES

Experiments utilized 4-8 week old C57BL/6 (#000664) mice that were obtained from The Jackson Laboratories 3 mice per group. Animal protocols were approved by the Earle A. Chiles Research Institute (EACRI) Institutional Animal Care and Use Committee (Animal Welfare Assurance No. A3913-01). The Panc02-SIY pancreatic adenocarcinoma line expressing the model antigen SIY was kindly provided by Dr. Ralph Weichselbaum at the University of Chicago. MC38 colorectal carcinoma line was obtained from Dr. Kristina Young at EACRI. The Panc02-SIY cell line was grown in complete RPMI containing 10% heat inactivated fetal bovine serum (FBS), 100U/mL penicillin, 100μg/mL streptomycin. The MC38 cell line was grown in DMEM containing 10% heat inactivated FBS, 100U/mL penicillin, 100μg/mL streptomycin. Pathogen and mycoplasma contamination testing were performed on all cell lines within the past 6 months using the IMPACT Mouse PCR Profiling from IDEXX BioAnalytics.

TUMOR TREATMENTS

Tumors were implanted subcutaneously into the right flank as follows; 2 x 10^5 MC38 and 5 x 10^6 Panc02-SIY. When tumors were approximately 5mm in average diameter, mice were randomized to receive treatment with CT-guided radiation using the Small Animal Radiation Research Platform (SARRP) from XStrahl. Dosimetry was performed using Murislice software from XStrahl. The SARRP delivered a single dose of 12Gy to an isocenter within the tumor using a 10mm x 10mm collimator and a 45° beam angle to minimize dose delivery to normal tissues.

TISSUE PROCESSING

Following dissection, tumors were weighed and minced into small fragments, then transferred into C tubes from Miltenyi Biotec containing enzyme digest mix with 250U/mL collagenase IV (Worthington Biochemical, #LS004188), 30U/mL DNase I (Millipore-Sigma, #4536282001), 5mM CaCl2, 5% heat inactivated FBS and HBSS. Tissue was dissociated using a GentleMACS tissue dissociator from Miltenyi Biotech. This was followed by incubation at 37°C for 30 min with agitation. Enzymatic reactions for the tumor were quenched using ice cold RPMI containing 10% FBS and 2mM EDTA. Single cell suspensions were then filtered through 100μm nylon cell strainers to remove macroscopic debris. Cells were washed and counted.

SINGLE CELL RNA SEQUENCING

Panc02-SIY or MC38 tumors were treated +/- 12Gy radiation as described above. Tumors were harvested 24 hours post treatment (n=4 animals/group), processed into a single cell suspension as described above and magnetically enriched using CD45 (TIL) MicroBeads (Miltenyi Biotec). Enriched cells were labeled with viability dye and CD45-APC. Live CD45$^+$ cells were sorted using a 100uM nozzle on a BD Biosciences Aria cell sorter and cells were processed according the manufacturers protocol for the Chromium Single Cell 3' Reagent kit (v3.0) from 10X Genomics. Libraries were sequenced using an Illumina NovaSeq 6000 using a NovaSeq 6000 S2 reagent kit (v1.0). Data were processed using the Cell Ranger pipeline (v3.1) and subsequently analyzed with the Loupe Browser from 10X Genomics (v5.0). Using the Loupe Browser differentially expressed genes between groups were considered significant if the log2 fold change of gene expression was > 0.58 and the Benjamini-Hochberg adjusted p value was < 0.1. Volcano plots were

generated using the EnhancedVolcano package (v1.7.16) and heatmaps were generated using pheatmap package (v1.0.12) in R (v4.0.2). Gene pathway and gene ontology analysis was performed using the STRING database (v11.0). Additional upstream molecule pathway analysis was performed with Ingenuity Pathway Analysis (IPA) software from Qiagen (v01-19-00) using default settings for Core Analysis.

Results

IDENTIFICATION OF cDCs FROM MC38 AND PANC02-SIY TUMORS USING TRANSCRIPTIONAL SIGNATURES

Our previous data demonstrated that radiation induced cDC maturation in radio-immunogenic MC38 tumors, and this process failed to occur in poorly radio-immunogenic Panc02-SIY tumors [248]. To identify which pathways might be differentially regulated between cDCs from radio-immunogenic MC38 tumors and poorly radio-immunogenic Panc02-SIY tumors we opted to use a transcriptomics-based approach. MC38 or Panc02-SIY tumors were established in mice and when tumor reached ~5-6mm average diameter they were treated with 12Gy of CT-guided radiation (**Figure 4-1A**). Tumors were harvested 24 hours after treatment with radiation, processed, and live $CD45^+$ cells were run through the 3' Gene Expression single cell RNA sequencing platform from 10X Genomics (**Figure 4-1A**). Data were processed using the Cell Ranger Pipeline from 10X Genomics, then all samples were aggregated into a single cloupe file and subsequently analyzed in the Loupe Browser. We first set out to use an unbiased approach to identify cDCs within tumor using a graph-based clustering algorithm within the Loupe Browser to identify clusters of cells that expressed cDC genes. The graph-based clustering algorithm in the Loupe Browser builds a sparse nearest-neighbor graph, followed by Louvain Modularity Optimization to find modules within the graph that are highly connected and then adds an additional cluster-merging step using hierarchical clustering. This graph-based clustering approach identified 18 unique clusters (**Figure 4-1B**). Gene expression analysis across each cluster demonstrated that cluster 16 expressed many genes associated with cDCs, including Flt3, Zbtb46 and CD24a (**Figure 4-1C**). However, when we plotted the $\log_2$ expression of the cDC-specific transcription factor Zbtb46 across

treatment groups, we noted that not all of the cells expressed the Zbtb46 transcription factor (**Figure 4-1D**). This led us to examine the expression of cDC-specific transcription factors to identify DCs within the tumor. We first used the cDC1 transcription factor Batf3, but noted its widespread expression (**Figure 4-1E**). The cDC-specific transcription factor Zbtb46 was more specific, though there was still expression in other $CD45^+$ cells (**Figure 4-1E**). We next used the combination of the transcription factors Zbtb46 and Batf3 to identify cDCs, and found that dual expressing cells were more tightly associated with graph-based cluster 16 in Fig 4-1B (**Figure 4-1E**). Since we used the Zbtb46 transcription factor to deplete cDCs in our previous studies, we opted to use a combination of the graph-based clustering and targeted gene expression to identify cDCs. Using this approach, we identified cDCs as cells within cluster 16 from the graph-based clustering that expressed 1 or more transcripts of Zbtb46 (**Figure 4-1F i**). We confirmed that all cDCs in our analysis expressed Zbtb46 by comparing expression across all cells from each treatment group (**Figure 4-1F ii**). Thus, moving forward we will use the combination of graph-based clustering and the transcription factor Zbtb46 to define cDCs for downstream analysis.

DIFFERENTIAL GENE EXPRESSION BETWEEN cDCs FROM MC38 AND PANC02-SIY TUMORS

Using the approach described above we identified cDCs within each tumor and treatment type. We identified 12 cDCs in untreated MC38 tumors, and 12 cDCs in radiation treated MC38 tumors (**Figure 4-2A i**). A total of 62 cells were identified as cDC in untreated Panc02-SIY tumors and 71 cells in radiation treated Panc02-SIY tumors (**Figure 4-2A ii**). We then compared these cDCs using differential gene expression (DGE) analysis to identify genes that either up or down regulated in each treatment group. The following comparisons were assessed; 1) MC38: untreated versus radiation treated; 2) Panc02-SIY:

untreated versus radiation treated; 3) Untreated: Panc02-SIY versus MC38; and 4) Radiation treated: Panc02-SIY versus MC38. Each comparison was plotted using a volcano plot with significantly upregulated genes colored red (BH-adjusted p-value > 0.1, Log_2 fold change > 0.58) and significantly downregulated genes colored as blue (BH-adjusted p-value > 0.1, Log_2 fold change < -0.58) (**Figure 4-2B i**). The total number of differentially expressed genes, including the number of up versus down regulated genes were displayed for each comparison (**Figure 4-2B ii**). When comparing untreated to radiation treated cDCs we identified 179 (MC38) and 180 (Panc02-SIY) genes that were differentially expressed after treatment. Comparing genes in Panc02-SIY versus MC38 in the untreated setting we identified 395 significant genes, and in the radiation treated setting there were 370 differentially expressed genes (**Figure 4-2B ii**). We then set out to determine whether there was shared overlap in genes that were upregulated across groups (**Figure 4-2B iii**). This analysis revealed that between individual comparisons there was shared overlap between genes that were significantly increased (**Figure 4-2B iii**).

INTRATUMORAL cDCs IN THE RADIATION TREATED GROUPS WERE IN THE TUMOR AT THE TIME OF TREATMENT

One of the first questions we had when analyzing these shared genes was whether we could identify a radiation induced transcriptional signature that would be consistent with cDCs being in the tumor at the time of radiation treatment. In Fig 4-B iii there were a total of 15 genes that were upregulated following radiation in cDCs from both MC38 and Panc02-SIY tumors (**Figure 4-2C i**). We identified Cdkn1 as being one of the genes with the most significant increase following radiation and this is a gene that has previously been reported to be upregulated by treatment with radiation (**Fig 4-2B i**) [256, 257]. Pathway and gene ontology analysis of these shared 15 genes showed that gene ontology terms

for cellular response to radiation (GO:0071478), regulation of IFNγ production (GO:0032649) and calcium-dependent protein binding (GO:0048306) were increased in cDC following radiation (**Figure 4-2C ii**). These data suggest that the cDCs included in our analysis from radiation treated groups express genes that are consistent with them being in the tumor at the time of treatment.

RADIATION INCREASES THE EXPRESSION OF GENES ASSOCIATED WITH PRR ACTIVATION AND SIGNALING IN cDC FROM RADIO-IMMUNOGENIC TUMORS

Next, we set out to obtain a more global overview of how the genes upregulated in MC38 cDCs might be connected. The 211 genes in Fig 4-2B ii that were identified as being significantly upregulated (BH adjusted p-value of < 0.1 and $\log_2$ fold change expression of 0.58) in cDCs from radiation treated MC38 tumors as compared to Panc02-SIY tumors were input into pathway and gene ontology enrichment analysis programs. This analysis revealed that genes associated with antigen processing and presentation (GO:0048002) were significantly increased in MC38 cDCs (**Figure 4-3A**). These data indicate that MC38 cDCs treated with radiation are processing and presenting the antigens they have already taken up, suggesting they are more mature than Panc02-SIY cDCs. Response to both interferon-alpha (GO:0035455) and interferon-beta (GO:0035456) were also predicted to upregulated in cDCs from MC38 tumors relative to Panc02-SIY tumors (**Figure 4-3A**). Type I IFN expression is increased following PRR stimulation and has been reported to signal back on DCs to further promote their maturation [77]. Finally, other pathways associated with innate immune activation were also predicted to be increased, including positive regulation of IL-6 secretion (GO:2000778), as well as response to IL-1 (GO:0070555) and TNF (GO:0070555), which has been shown to be capable of driving

DC maturation (**Figure 4-3A**) [76]. Activation of these pathways is consistent with cDCs receiving maturation signals following radiation therapy in MC38 tumors.

To gain more insight into the transcriptional pathways that were upregulated in cDCs from radiation treated MC38 tumors as compared to Panc02-SIY tumors we took the 470 genes that were identified as being differentially expressed and input them in Qiagen's Ingenuity Pathway Analysis (IPA) software. IPA predicted that within DCs from MC38 tumors the transcription factors IRF3, IRF7 and STAT1 were likely to be active given the pattern of increased gene expression in these cells (**Figure 4-3B**). IRF3 and IRF7 are known to be activated during innate immune responses and signal downstream of many PRRs within immune cells [258, 259]. The transcription factor MXD1 was also predicted to be activated and previous work has demonstrated that MXD1 expression is associated with mature DC (**Figure 4-3B**) [260]. Meanwhile the transcription factor E2F1 was predicted to be down regulated in MC38 cDCs, and its expression has been shown to be associated with the suppression of DC maturation (**Figure 4-3B**) [261]. These data suggest that innate PRRs are likely activated in cDCs from MC38 tumors treated with radiation therapy.

Our next question was which PRRs might be activated in radiation treated cDCs from MC38 tumors and responsible for triggering DC maturation following treatment. We used the IPA upstream regulator analysis to predict which PRRs may be activated in MC38 cDCs relative to Panc02-SIY cDCs based the pattern of differentially expressed genes. This analysis indicated that a number of PRRs that are capable of sensing and responding to different forms of nucleic acids within the cell might be activated in MC38 cDCs (**Figure 4-3C-D**). These included RNA sensors that are located in the cytosol (DDX58, IFIH1) and endosomes (TLR3, TLR7) (**Figure 4-3C-D**). In addition, the PRRs involved in sensing

different forms of DNA in the cytosol (STING) and endosomal (TLR9) were also predicted to be activated (**Figure 4-3C-D**). These data led us to explore which of these PRRs were expressed in the cDCs from each tumor and treatment type (**Figure 4-3E**). The average expression of each gene was plotted using a heatmap and the data revealed the genes for many of these nucleic acid sensing proteins were expressed by cDCs (**Figure 4-3E**). We did not detect any significant differences between groups in the expression for any of these sensors (**Figure 4-3E**). These data indicate that cDCs in Panc02-SIY express transcripts for receptors that would be necessary to respond to nucleic acids but may either lack sufficient signaling through these receptors because the ligands are either absent in Panc02-SIY tumors or signaling through these receptors is actively suppressed. Taken together these data suggest that pathways involved in both DNA and RNA sensing are increased in cDCs in radiation responsive tumors, which may make these cells more responsive to endogenous adjuvants and explain the differential response. At the same time, negative regulation of nucleic acid sensing may be occurring in unresponsive tumors, which may limit their ability to mature. Thus, both positive and negative regulation may control responses to treatment in different tumors.

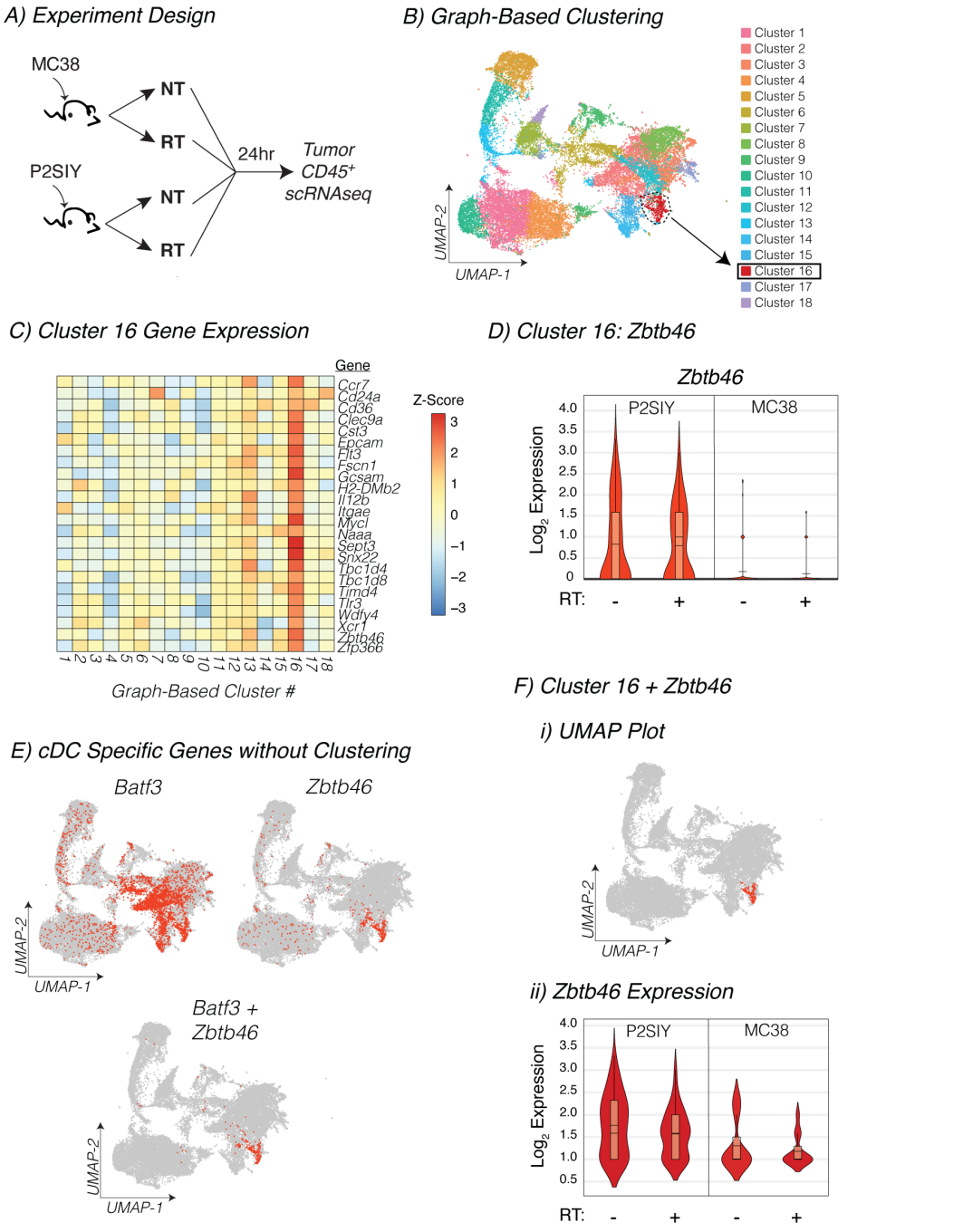
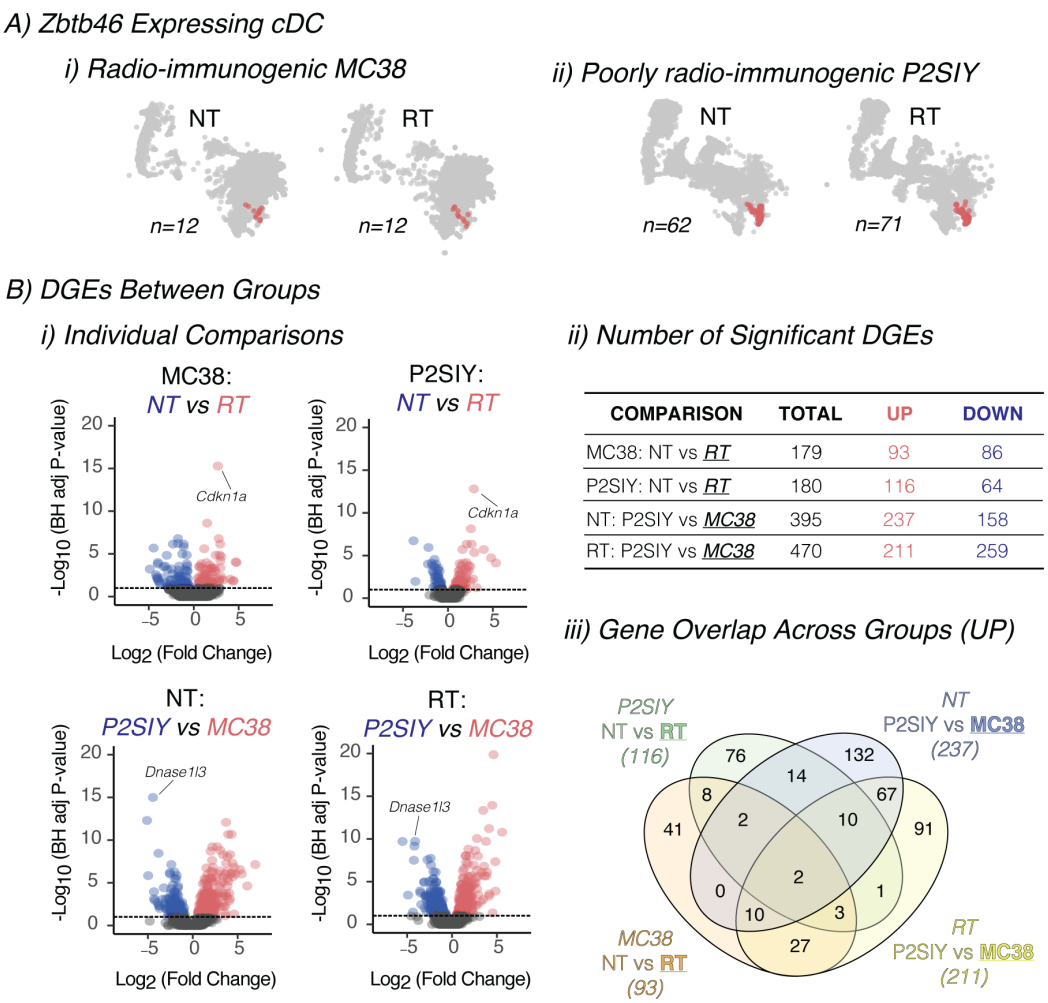


Figure 4-1: Identification of cDCs in tumors following treatment with radiation. (A) Experiment design: MC38 or Panc02-SIY (P2SIY) tumors were implanted into mice and were either untreated (NT) or treated with 12Gy radiation (RT) when they reached 5-6mm average diameter with n=3 animals/group. 24 hours post treatment tumors were harvested and sorted CD45$^+$ cells processed for single cell RNA sequencing (scRNAseq). **(B)** In the Loupe Cell Browser cell were subjected to graph-based clustering (UMAP plot) with cluster 16 (red dots surrounded by dotted outline) representing DCs. Each dot represents an individual cell. **(C)** Heatmap showing the expression of cDC specific genes across each cluster represented in B. **(D)** Log_2 expression of Zbtb46 within graph-based cluster 16 from C across cells from P2SIY and MC38 tumors treated +/- radiation. **(E)** Expression of cDC genes Batf3, Zbtb46 or Batf3 + Zbtb46 across sequenced cells from each tumor and treatment group. **(F)** UMAP plot showing all cells within cluster 16 that express Zbtb46 transcripts (red dots) and expression of Zbtb46 in each tumor type and treatment group. Violin plots show log_2 expression of each individual cell for each treatment group. The dark colored box inside violin plot represents quartile 1 (q1) through quartile 3 (q3). The mean is the solid line inside the box and the median is dashed line.

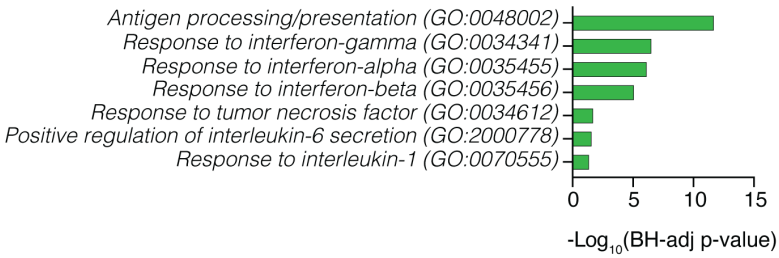


COMPARISON	TOTAL	UP	DOWN
MC38: NT vs *RT*	179	93	86
P2SIY: NT vs *RT*	180	116	64
NT: P2SIY vs *MC38*	395	237	158
RT: P2SIY vs *MC38*	470	211	259

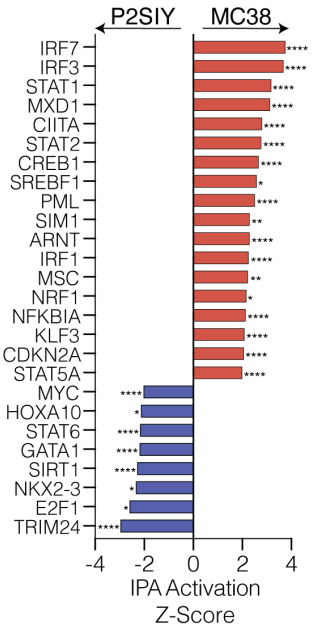
Term	Description	Genes in Term
GO:0071478	Cellular response to radiation	*Cdkn1a, Ccnd2, Ptgs2*
GO:0032649	Regulation of IFNγ production	*Cd274, Il1b, Isg15*
GO:0048306	Calcium-dependent protein binding	*S100a4, S100a6*

Figure 4-2: Differential gene expression between cDCs from MC38 and Panc02-SIY tumors following treatment with radiation. (A) Within cluster 16 from Fig 4-1, Zbtb46 expressing cDCs were selected for analysis in i) radio-immunogenic MC38 tumors with 12 cells selected for analysis from untreated (NT) and radiation (RT) groups and ii) in poorly radio-immunogenic Panc02-SIY (P2SIY) tumors there were 62 cells selected for analysis from the NT group and 71 cells selected from the RT group. **(B)** Differential gene expression (DGE) was calculated between cDCs from different tumor and treatment types. Genes with a BH-adjusted p-value of <0.1 and $\log_2$ fold change >0.58 were considered significant. Each dot represents an individual gene with significantly increased genes for each comparison were colored red, significantly decreased genes were colored blue and genes that weren't significant were colored grey. ii) Table with the number of significantly increased or decreased number of DGE for each listed comparison calculated in Bi. ii) Venn diagram illustrating the numbered of shared genes that were upregulated for each DGE comparison listed in the table in Bii. **(C)** i) The expression of 15 shared genes that were identified in Biii were represented on heat map for each tumor type +/- RT. Data represent Z-score of the average expression for each gene scaled across each column. ii) Gene ontology and pathway analysis of 15 shared genes that were upregulated following RT in both MC38 and P2SIY cDCs.

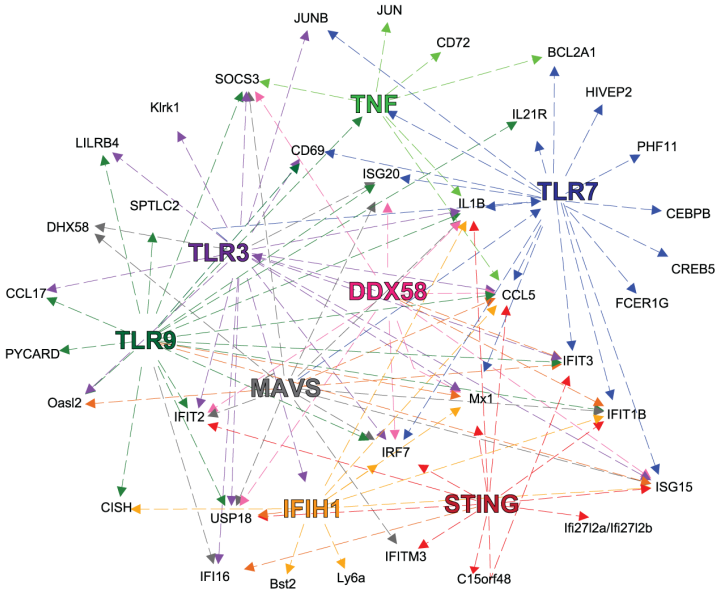
A) Pathway analysis: UP in RT MC38 cDCs



B) Predicted transcription factor activation in cDCs



C) Predicted interactions between genes upregulated in RT MC38 cDC versus P2SIY cDC



D) Activated upstream regulators

Upstream Regulator	Activation Z-Score	P-value
DDX58	2.549	3.45E-10
IFIH1	2.699	1.61E-09
STING	3.116	1.27E-12
TLR3	2.641	6.78E-13
TLR7	3.014	3.11E-09
TLR9	3.457	1.63E-10
TNF	2.607	3.11E-02

E) Expression of PRRs in cDCs

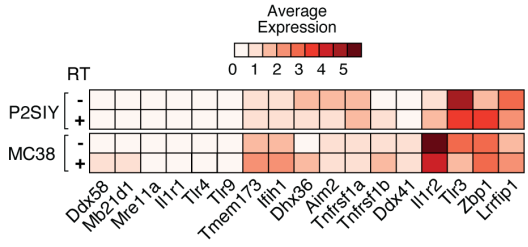


Figure 4-3: Increased expression of genes associated with PRR activation in radiation treated cDCs from MC38 tumors compared to cDCs from Panc02-SIY tumors. (A) The 211 genes that were significantly upregulated in the differential gene expression analysis between radiation (RT) treated cDCs from MC38 compared cDCs from Panc02-SIY (P2SIY) tumors were input into gene ontology and pathway analysis. Select gene ontology pathways with BH-adjusted p-value >0.05 were listed in table. **(B)** Following DGE analysis in 4-2B, all 470 differentially expressed genes were input into Qiagen IPA to predict which transcription factors are active based on changes in gene expression between radiation treated cDCs from MC38 versus P2SIY tumors. Predicted transcription factors with p-value of >0.05 and IPA activation Z-score with > 2 (red, MC38) or < -2 (blue, P2SIY) were considered significant. *p>0.05, **p>0.01, ***p>0.001, ****p>0.0001. **(C)** Using the genes upregulated in RT cDCs from MC38 tumors IPA was used to predict upstream molecule activation and connections between signaling molecules. **(D)** Table listing potential upstream regulators that might be responsible for inducing gene expression pattern observed in RT treated cDCs from MC38 tumors as compared to cDCs from P2SIY tumors. **(E)** The average expression of PRR sensors for each tumor and treatment group.

Discussion

Using a transcriptional-based approach we identified cDCs that were present in both radio-immunogenic MC38 and poorly radio-immunogenic Panc02-SIY tumors. There are multiple ways to define DC subsets within transcriptional datasets, either through the use of computational-based approaches or gene set specific approaches. In our studies we opted to first use an unbiased clustering algorithm to identify a population or populations resembling cDCs, and once a cluster was identified we selected only the cells that expressed the cDC-specific transcription factor Zbtb46. One of the concerns with using a set of genes to identify cross-presenting cDC1s, is that the expression of any of these genes many change across different tumor types with different treatments. For example, it has been reported that the cDC1-specific chemokine receptor, XCR1 is down regulated both at the transcript and protein level in cDC1 found in tumors expressing PGE_2 [142]. Thus, if we choose to use this particular gene to identify cDC1s within tumors it is possible that we might have missed some of these cells in our analysis. While we could have used the combination of multiple genes that are often expressed by DCs to identify specific subsets, we wanted our analysis to be broad. Instead, after using a graph-based clustering algorithm, we choose to use only the cDC-specific transcription factor Zbtb46 as our previous studies utilized this transcription factor to deplete cDCs [248]. For future studies it would be interesting to use a range of DC-specific genes to define individual DC subsets to determine how this changes the results following data analysis.

In our studies we identified a transcriptional signature in cDCs that was consistent with these cells being in the tumor at the time of treatment with radiation therapy. One of the genes that was most significantly upregulated in cDCs following treatment was Cdkn1a (p21). Within tumors we identified both DCs and macrophages (data not shown) as the

primary cell types that upregulate the expression of this gene following treatment. Cdkn1a has been shown to increased following radiation and plays an important role in responding to DNA damage by promoting cell survival after radiation [256, 262]. It is likely that increased expression of Cdkn1a enables these cell types to survive treatment with radiation. While our data suggests that Cdkn1a is potentially a useful transcriptional biomarker for DCs that were in the tumor at the time of radiation, this finding need to be further validated. Additional studies assessing the kinetics of Cdkn1a expression following treatment using flow cytometry would be useful. Moreover, single cell transcriptional analysis from DCs in other tumors types would further validate the utility of this gene as a biomarker of radiation treatment. Pending the outcome of these results, it might be possible to use Cdkn1a expression to identify DCs that migrate to the TdLN from the tumor after treatment with radiation. This would be particularly important when studying the effects of radiation on DCs in patient tumors where we are unable to use tools such as photoconvertible Kaede mice.

Why does radiation therapy successfully drive intratumoral DC maturation in some tumors but not in others? While multiple mechanisms are likely responsible, our data suggest that nucleic acid sensing through PRRs may be one potential pathway that determines whether radiation successfully induces DC maturation. These data are consistent with previous reports suggesting that DNA and RNA sensors are required for the response to radiation therapy [201, 263]. This is also consistent with our previous results demonstrating that in poorly radio-immunogenic Panc02-SIY tumors which fail to drive intratumoral DC maturation, administration of adjuvants that trigger recognition through nucleic sensing PRRs are sufficient to drive cDC maturation leading to improved tumor control [248]. While additional studies are clearly needed to further validate our

results, the data we have collected thus far suggests that nucleic acid sensing in DCs is one of the critical pathways that determines whether a successful immune response is generated following radiation therapy.

Chapter 5:

Discussion

Summary of Key Findings

Our work establishes a critical role for DC maturation and migration in radiation mediated tumor regression. We first characterized the maturation status of intratumoral cross-presenting DCs across tumor types and the data revealed that radiation induces DC maturation only in radio-immunogenic tumors that required $CD8^+$ T cells for their enhanced response to treatment. This analysis revealed that cross-presenting DCs failed to upregulate maturation markers in tumors that were poorly responsive to radiation alone and co-administration of exogenous adjuvant with radiation overcame this maturation failure, resulting in tumor cures. Depletion studies demonstrated that cDCs were necessary for the combined treatment efficacy of radiation and adjuvant in poorly radio-immunogenic Panc02-SIY tumors. Taken together, these data suggest that radiation is capable of driving intratumoral DC maturation, and this process is not uniform across different tumor models.

What signaling pathways are responsible for driving DC maturation and migration in radio-immunogenic tumors? Using single cell RNA sequencing we compared transcriptional signatures between DCs from radio-immunogenic and poorly radio-

immunogenic tumors. This analysis revealed genes associated with PRR signaling through nucleic acid sensing pathways were significantly upregulated in cDCs from radio-immunogenic tumors following radiation therapy. While these predictions need to be further validated, they suggest that nucleic acids may be released and available to bind and signal through PRRs on DCs following treatment with radiation. These data also suggest that this process is impaired in poorly responsive tumors, either through degradation of these signals or active suppression of these signaling pathways in DCs.

One of the unanswered questions within the radiation field is whether radiation therapy can function similar to a vaccine by initiating the priming of new tumor-reactive T cell responses following treatment. For this to occur, radiation likely needs to promote the migration of mature DCs from the tumor to TdLN. Using a mixed bone marrow chimera approach, we demonstrate that DC migration is required for the efficacy of radiation therapy in radio-immunogenic MC38 tumors. We showed that radiation does not change the kinetics of tumor DC migration in radio-immunogenic tumors but rather treatment increases the frequency of tumor migratory DCs with an activated phenotype that appear in the TdLN. On the contrary, we discovered that radiation does impair DC migration from the tumor to the TdLN in poorly radio-immunogenic Panc02-SIY tumor. These data are consistent DCs having a more immature phenotype in these tumors after treatment with radiation and demonstrate the importance of DC migration in the response to radiation. Given that DC maturation and migration to the LN are requisites for the generation of new T cell responses and that radiation appears to successfully initiate the early steps that are required for T cell priming in radio-immunogenic MC38 tumors, our data suggest that radiation may be capable of priming new tumor-reactive T cell responses.

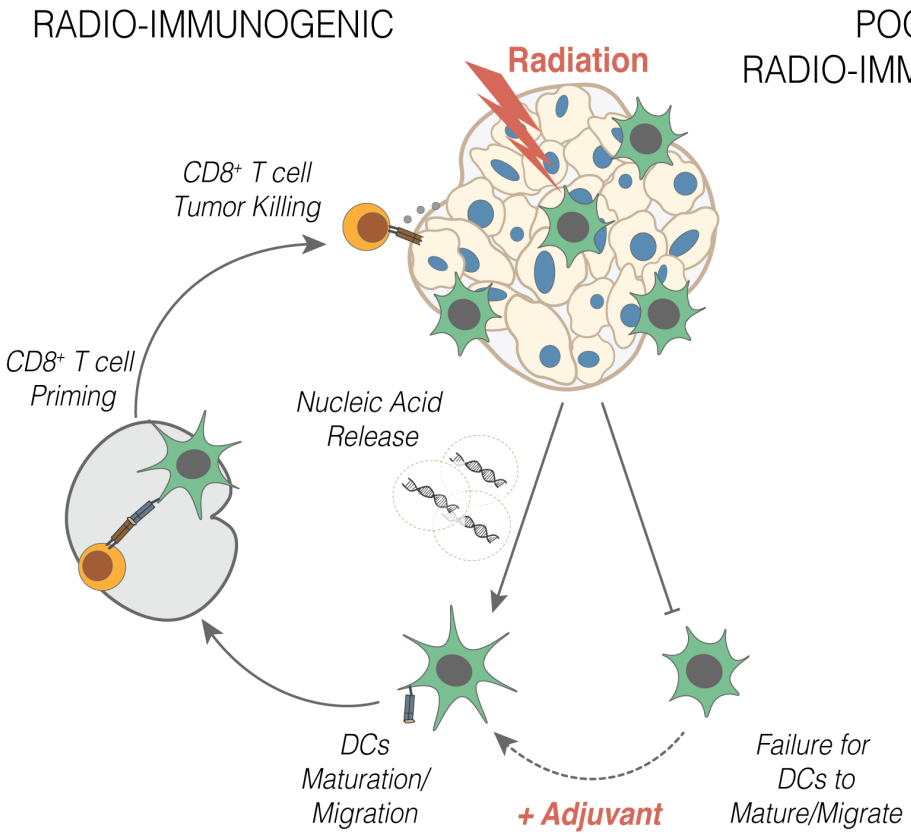


Figure 5-1: Model illustrating the role of DCs in radiation mediated tumor regression. In radio-immunogenic MC38 tumors radiation results in the release of nucleic acids or other PRR signals from dying tumors cells that drives intratumoral DC maturation. Mature DCs then migrate to the TdLN where they prime tumor antigen specific CD8⁺ T cells. These CD8⁺ T cells then migrate to the tumor to directly kill target tumor cells. In poorly radio-immunogenic Panc02-SIY tumors these nucleic acids or other PRR signals either are either degraded or fail to adequately stimulate cDCs following treatment, resulting in a failure to promote intratumoral DC maturation. Since DCs fail to mature this results in a failure to migrate to the TdLN to prime tumor-reactive CD8⁺ T cells leading to a diminished response following treatment.

Taken together, our studies demonstrate that DCs are key players in the response radiation therapy. The work presented here supports a model whereby radiation drives the release of immunostimulatory nucleic acids or PRR signals from dying tumors cells, which are then sensed by intratumoral DCs in radio-immunogenic tumors, and this drives DC maturation and subsequent migration to the TdLN (**Figure 5-1**). This process fails to occur

in poorly radio-immunogenic tumors, as DCs fail to mature and migrate to the TdLN after treatment (**Figure 5-1**). Finally, we can overcome this failure by administering exogenous adjuvants that function as ligands to induce DC maturation following radiation. We propose that in tumors where DCs fail to mature that DC maturation signals may either be lacking or there is active suppression these signals in DCs.

Future Directions

Our work demonstrates that radiation therapy promotes intratumoral DC maturation in radio-immunogenic (MC38, Moc1) tumors, while this process fails to occur in poorly radio-immunogenic tumors (Panc02-SIY, Moc2). Prior to our studies it was unclear whether radiation was capable of driving intratumoral cross-presenting DC maturation and whether this varied across tumor types. Reports had suggested that radiation promoted DC maturation, as determined by increased expression of surface of co-stimulatory markers on bulk $CD11c^+$ populations in tumors, and through indirect evidence of increased tumor-specific $CD8^+$ T cell proliferation in the TdLN [183, 203, 204]. However, non-DC subsets within the tumor microenvironment, including macrophages, express high levels of the integrin CD11c, making this a poor marker to assess phenotypical changes exclusively on DCs [25]. To address this issue, we used a comprehensive flow cytometry panel to identify and characterize the phenotype of DC subsets across multiple tumor models, including cross-presenting DCs [141]. We revealed that radiation drives intratumoral cross-presenting DC maturation in radio-immunogenic tumors, but not in poorly radio-immunogenic tumors. Future experiments that characterize DCs in additional tumor models, including spontaneous models, will enable us to understand determinants of radiation's capacity to drive DC maturation.

Radiation therapy alone failed to promote DC maturation in poorly radio-immunogenic Panc02-SIY tumors, but co-administration of exogenous adjuvant was able to drive DC maturation and improve tumor responses to radiation. In our studies, we utilized the TLR3 agonist poly I:C, as it is known for its ability to promote cross-presenting DC maturation [73, 228]. Recent work in preclinical lymphoma models has demonstrated that the addition of a TLR3 agonist in combination with radiation therapy and FLT3L to increase DC numbers also leads to improved tumor responses as compared to radiation alone, and clinical trials are currently underway investigating this combination in T and B cell lymphomas (NCT01976585, NCT03789097) [230]. While we demonstrate that radiation is capable of inducing DC maturation in preclinical models, it remains to be determined if this process also occurs in patient samples. A deeper understanding for the mechanisms that dictate why certain tumor are poorly radio-immunogenic has important implications for our ability differentiate which patients might benefit from the addition of adjuvant to radiation therapy. Our data suggest that patients with tumors where DCs fail to mature following radiation therapy would benefit the most from this type of combination therapy. Given how rare DCs are in patient tumor biopsies it has been technically challenging to assess DC maturation in patients using conventional flow cytometry. However, transcriptional-based approaches may allow us to overcome these obstacles. We have RNA sequencing data that were generated from a clinical trial where tumor biopsies were collected pre- and post-radiation in the same patient (NCT03247712). By using deconvolution algorithms and computational approaches, we can use this dataset to validate whether radiation is capable of driving DC maturation in patient samples, and this will assist us in identifying features or signatures that predict whether DC maturation will occur in response to

radiation [264-266]. This information will be valuable in determining which patients are most likely to benefit from radiation combined with adjuvants that drive DC activation.

Our work demonstrates that DC migration plays a critical role in the immunological efficacy of radiation therapy. Prior to our research it was unknown whether DCs were required to migrate from the tumor to the TdLN to prime tumor-reactive T cell responses following radiation. Reports had indicated that Batf3$^{+/+}$ cross-presenting DCs were required for the efficacy radiation therapy, which suggested that cross-presentation of antigens by DCs was important for therapeutic efficacy [205, 206]. However, mice were lacking cross-presenting DCs throughout animal and tumor development in these studies, making it difficult to isolate whether DCs were required to cross-present tumor antigens at tumor implantation or following radiation treatment. Importantly, it was unknown whether cross-presentation by DCs occurred within the tumor itself or in the TdLN. Studies monitoring the proliferation of antigen-specific T cells in the TdLN following radiation therapy had suggested that immunological efficacy of radiation required DC migration to the TdLN [203, 204]. To directly address whether DC migration was important for radiation, we utilized a mixed bone marrow chimera approach whereby tumors could develop with CCR7 expressing cDCs and then following the administration of diphtheria toxin just prior to radiation we could block CCR7-dependent cDC migration only during treatment [224]. From these experiments, we now understand that DC migration is essential for tumor cures following radiation therapy in radio-immunogenic MC38 tumors. We had previously demonstrated that cDCs were required for the combined efficacy of radiation and poly I:C in poorly radio-immunogenic Panc02-SIY tumors. However, cDC migration did not appear to be required for the combined treatment efficacy in Panc02-SIY tumors since depletion of CCR7 expressing cDCs had no effect on treatment efficacy. One possibility is that other

subsets in the tumor, which are not depleted by diphtheria toxin are capable of carrying antigen to the TdLN and delivering it to LN-resident cDC populations for cross-presentation. Alternatively, the strong adjuvant poly I:C may allow cDCs to migrate independently of CCR7 to the TdLN. Future studies that utilize the Kaede photoconvertible mice to specifically track tumor migratory DC populations will enable us to address whether a CCR7$^{-/-}$ or Zbtb46$^{-/-}$ population within the tumor is still capable of trafficking to the TdLN following treatment with radiation and poly I:C.

The work presented here suggest that radiation therapy is capable of modulating both the kinetics and phenotype of tumor migratory DC populations. Prior to our research there were no studies that examined how radiation impacted cDC migration from the tumor to the TdLN. Photoconvertible mice are one tool that have been used to specifically track tumor migratory immune populations and these studies have reported that it's primarily T cells and DCs that migrate from the tumor to the TdLN [251, 254]. Using Kaede mice we showed that radiation does not change the kinetics of DC migration in radio-immunogenic MC38 tumors, but treatment does increase the proportion of migratory DCs in the TdLN with a mature phenotype. However, in the poorly radio-immunogenic Panc02-SIY tumor model we observed that radiation therapy does impair the migration of DCs to the TdLN. Prior to using the Kaede photoconvertible system, we were only able to identify migratory DC populations in the TdLN based on the expression of surface markers that had been shown to correlate with migratory DCs [25]. We had previously been unable to detect any differences in DC populations within the TdLN following treatment in either tumor model using this approach to identify tumor migratory DC populations. Our results demonstrate that there is a significant proportion of non-tumor migratory DCs that end up in the TdLN and these populations had masked our ability to detect the differences between tumor

migratory DC populations following treatment. Moreover, these data suggest that tumor migratory DCs are a rare population in the TdLN and underscore the utility of the Kaede photoconvertible system in being able to carefully identify and assess populations that were specifically in the tumor at the time of treatment. It remains to be seen how radiation impacts the kinetics and phenotype of DC migration to the TdLN in other tumor models. Additional experiments using the Kaede mice to monitor and track tumor migratory DC populations across a range of tumor models will provide useful insight into the changes that occur in DC migration following radiation.

Our studies demonstrate that cancer cells dictate the local immune environment within tumors, which in turn determines whether radiation successfully drives DC maturation. How do individual tumors control whether DCs are successfully matured following treatment? One possibility is that cancer cells themselves secrete molecules to directly suppress DC function. As discussed in earlier chapters, tumors are known to secrete metabolites capable of suppressing DC maturation, including PGE_2, oxidized cholesterol ligands, adenosine, and kynurenine derivatives [166, 169, 267, 268]. We assessed changes in the quantity of different metabolomic mediators in poorly radio-immunogenic Panc02-SIY tumors following treatment with radiation. Of the 155 metabolites assayed, we found that the metabolite kynurenine was significantly increased following treatment with radiation (**Figure 5-2**). DCs express the enzyme indoleamine 2,3-dioxygenase (IDO) which functions to break down tryptophan into kynurenine which can signal through the aryl hydrocarbon receptor (AhR), resulting the suppression of DC function [269]. Recent reports have demonstrated improved tumor control when radiation is combined with IDO inhibitors in preclinical models [268, 270, 271]. Thus, kynurenine products may be produced as part of the breakdown of tryptophan in IDO pathway, and

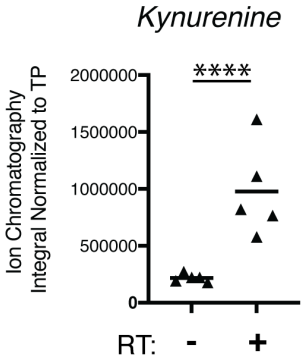


Figure 5-2: Radiation increases the level of suppressive kynurenine in poorly radio-immunogenic Panc02-SIY tumors. Panc02-SIY (P2SIY) tumors were implanted into mice and treated with 12Gy of radiation. Untreated and radiation tumors were harvested 3 days following treatment and kynurenine levels were quantified using conventional liquid chromatography mass spectrometry. ****p > 0.0001 using students t-test. Each symbol represents an individual tumor from one animal. n = 5 animals/group. TP = total protein.

this might provide one potential mechanism as to how DCs are suppressed in the poorly radio-immunogenic Panc02-SIY tumor model following radiation. Using our transcriptional dataset could also further validate this finding by determining whether components of the AhR signaling pathway are increased in radiation treated cDCs from Panc02-SIY tumors. These data would suggest that the addition of an IDO inhibitor to radiation in Panc02-SIY tumors might relieve DC suppression and improve tumor control following radiation therapy.

Comparative transcriptional analysis between cDCs from radio-immunogenic MC38 and poorly radio-immunogenic Panc02-SIY tumors suggested that DCs receive immunostimulatory signals either from nucleic acids or other PRR ligands following treatment in MC38 tumors, but not in Panc02-SIY tumors. Both IRF3 and IRF7 signaling pathways were predicted to be activated in cDCs from MC38 tumors following radiation. While these data need to be further validated by assessing phosphorylation of these transcription factors in MC38 cDC following treatment, these data suggest PRR signaling pathways are being activated in MC38 cDCs by radiation. What are these signals? How are they transferred to DCs and why don't they trigger signaling in cDCs from Panc02-SIY

tumors? Tumor derived exosomes have been shown to contain nucleic acids such as double stranded DNA and previous work has demonstrated that radiation is capable of altering the contents of exosomes such that they promote DC maturation in a cGAS/STING dependent manner [272, 273]. It is possible that certain tumor cells express enzymes that degrade these immunostimulatory signals before they are loaded into exosomes. For example, radiation has been shown to increase the expression of the exonuclease TREX1 which functions to degrade immunostimulatory nucleic acids in dying tumor cells and as a result reduces the immunostimulatory capacity of tumor derived exosomes [206]. ENPP1 is another enzyme capable of degrading cGAMP, an intermediate signaling molecule in the cGAS/STING signaling pathway [274]. Alternatively, tumor cells may also drive the expression enzymes in DCs themselves that are capable of degrading the immunostimulatory signals they receive from dying cancer cells. From our transcriptional analyses, we identified one gene, Dnase1l3, that was significantly increased in cDCs from Panc02-SIY tumors as compared to cDCs from MC38 tumors following radiation (**Figure 5-3A**). Dnase1l3 is secreted from DCs and it functions to prevent inflammatory responses to self-DNA by degrading DNA found in microparticles released from dying cells [275]. Patients and mice with loss of function mutations in Dnase1l3 fail to clear immunostimulatory DNA, resulting in autoimmunity and the development of systemic lupus erythematosus [275, 276]. Thus, in the context of tumors, it is possible that Dnase1l3 secreted from DCs in Panc02-SIY tumors, and it functions to degrade stimulatory DNA signals in microparticles before they have the ability to trigger DC maturation (**Figure 5-3B**). Dnase1l3 expression has been shown to be increased in DCs following treatment with IL-4 [277]. It remains to be determined whether other factors are capable of driving the expression of Dnase1l3, but it is possible that Panc02-SIY

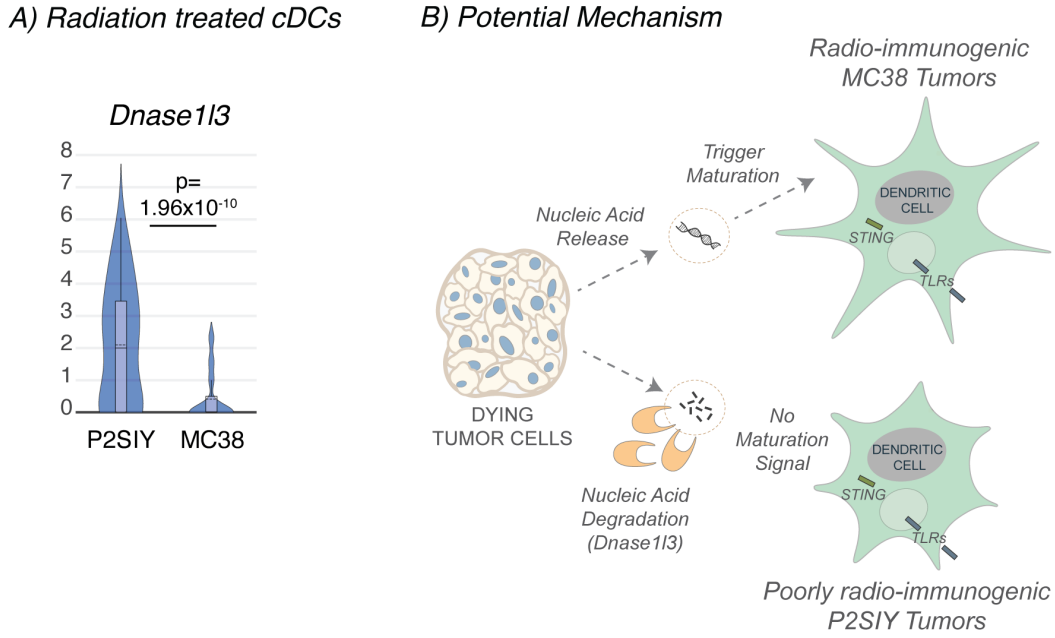


Figure 5-3: Potential mechanism involving Dnase1l3 mediated suppression of DC maturation in Panc02-SIY tumors. A) Average expression of Dnase1l3 in cDCs from MC38 versus Panc02-SIY (P2SIY) tumors. The light-colored box inside violin plot represent quartile 1 (q1) through quartile 3 (q3). The mean is the solid line inside the box and the median is dashed line. **(B)** Potential model for how Dnase1l3 may degrade immuno-stimulatory nucleic acid signals released by tumor cell thereby preventing the activation of cDCs in P2SIY tumors.

tumors secrete one of these factors. We plan to address whether increased Dnase1l3 expression is responsible for DC suppression by implanting Panc02-SIY tumors into mice with conditional deletion of Dnase1l3 in DCs. These experiments will enable us to determine whether degradation of nucleic acid signals following radiation is responsible for the failure of DCs to mature following treatment with radiation in Panc02-SIY tumors.

We used a comparative approach to understand how DCs in the tumor are modulated by radiation therapy. By comparing radiation responsive tumors to poorly responsive tumors we determined that treatment is capable of driving DC maturation; however, this process is not uniform across tumor models. It was through these direct comparisons that we identified that the tumors which were most responsive to radiation were the tumors that successfully drove DC maturation following treatment. In our studies we termed these

tumors as being “radio-immunogenic” given their ability to utilize the adaptive immune system to enhance their baseline cytotoxic response to radiation. Moving forward we believe the term “radio-immunogenic” will be a useful tool to discriminate those tumors that may be responsive and treatable by the addition of radiation therapy. Furthermore, future studies comparing radio-immunogenic and poorly radio-immunogenic tumors will be valuable in identifying features within individual tumors that dictate responsiveness to radiation therapy.

Finally, previous data had suggested that radiation primarily functions to boost the function of pre-existing tumor-reactive T cells [278]. Our work suggests that radiation may be capable of initiating the priming of new tumor-reactive T cell responses. While additional studies are needed to better understand the mechanisms and circumstances that enable radiation to function in this capacity, the data presented here have important implications for patients. Multiple reports have demonstrated that increased $CD8^+$ T cell infiltration in tumors tends to correlate with improved patient survival across a range of cancer pathologies [244-247]. Thus, patients lacking tumor-reactive T cells or patients with few poor-quality T cells would be predicted to benefit from therapies that have the ability to promote the development of new tumor-reactive T cells capable of destroying their tumors. We propose that radiation therapy has capacity to overcome this deficit by restarting the cancer-immunity cycle and initiating the priming of new T cells in these patients.

Materials & Methods

METABOLOMIC ANALYSIS

Panc02-SIY tumors were implanted subcutaneously into the right flank using 5 x 10^6 Panc02-SIY. When tumors were approximately 5mm in average diameter, mice were randomized to receive treatment with CT-guided radiation using the Small Animal Radiation Research Platform (SARRP) from XStrahl. Dosimetry was performed using Murislice software from XStrahl. The SARRP delivered a single dose of 12Gy to an isocenter within the tumor using a 10mm x 10mm collimator and a 45° beam angle to minimize dose delivery to normal tissues. Tumors were harvested from untreated (NT) or radiation treated (RT) animals 3 days post radiation. There were 5 animals in each treatment group. Harvested tumors were weighed, placed into a cryovial and subsequently submerged in liquid nitrogen. Tumor were then homogenized in buffer that proportional to tumor weight at 10uL/mL. Homogenates were centrifuged and total protein was quantified. Following quantification, supernatants were treated with 3X volume of methanol with 0.1% formic acid. Samples were centrifuged, then the supernatant was dried and reconstituted for LC-MS analysis of 155 individual metabolites. Extracted ion chromatography integrals were normalized by dividing the unnormalized integrals by the total protein content.

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