Elucidating the functional states of tumor-resident dendritic cells that drive productive anti-tumor immunity

by

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Elucidating the functional states of tumor-resident dendritic cells that drive productive anti-tumor immunity

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Submitted to the Department of Biology on November 15, 2021 in Partial Fulfillment of the Requirements for Degree of Doctor of Philosophy in Biology

ABSTRACT

While checkpoint blockade immunotherapy (CBT) has demonstrated remarkable clinical efficacy, its success is tempered by an inability to induce responses in the majority of cancer patients. Efforts to understand mechanisms of CBT resistance have unveiled a requirement for a subset of dendritic cells (DC) called Batf3-driven conventional DC1, given their superior ability to initiate tumor-reactive cytotoxic $CD8^+$ T cell responses. The exclusion or functional suppression of DC1 in tumors impedes CBT efficacy and enables tumor immune evasion and outgrowth. While the role of DC1 to anti-tumor immunity has been well-established, much less is known about the contribution of other DC subsets which can be found infiltrating tumors. Furthermore, under inflammation, DC subsets can exist in distinct functional states with differential impacts on their function. In this work, we sought to study DC states associated with productive or dysfunctional anti-tumor immune responses and dissect the signals that drive them.

To study DC states, we compared the DC infiltrate of a spontaneously regressing tumor (MC57-SIY; productive anti-tumor immunity) with a progressing tumor (MC38-SIY; dysfunctional anti-tumor immunity). We identified a novel activation state of $CD11b^+$ conventional DC expressing an interferon-stimulated gene signature (ISG^+ DC) that was enriched in regressor tumors. Like DC1, ISG^+ DC was capable of driving anti-tumor $CD8^+$ T cell immunity. However, unlike cross-presenting DC1, ISG^+ DC activated T cells by cross-dressing with pre-formed tumor-derived peptide-MHC complexes. We determined that constitutive tumor cell-derived type-I-interferon (IFN-I) production in regressor tumors was driving the ISG^+ DC state. Ablation of tumor cell-derived IFN-I in regressor tumors led to complete loss of anti-tumor T-cell responses in mice lacking DC1. Conversely, addition of IFNβ to progressor tumors induced ISG^+ DC and rescued anti-tumor T-cell responses in the absence of DC1.

Our study highlights the untapped stimulatory potential of the DC compartment that can be harnessed to drive anti-tumor $CD8^+$ T cell responses. In ongoing work, we are dissecting the mechanistic signals driving dysfunctional DC in progressor tumors over time. Engaging the functional states of DC or rewiring dysfunctional DC towards these functional states has the potential to strengthen anti-tumor immunity and may improve CBT responses.

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EDUCATION AND ACADEMIC HONORS

Massachusetts Institute of Technology, Cambridge, MA
Candidate for Ph.D. in Biology, Dr. Stefani Spranger Lab, 2016-present
- Research Focus: Investigating the contribution of tumor-resident dendritic cells to anti-tumor immunity
- Relevant Coursework: Principles of Biochemical Analysis, Graduate Genetics, Hallmarks of Cancer, Quantitative Biology, Methods and Logic in Molecular Biology
- SITC Young Investigator Award Recipient, 2018, 2019, 2020, and 2021
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Brown University, Providence, RI, 2007-2011
Sc.B. Chemistry (Honors), May 2011. GPA: 3.80/4.00
- Leallyn B. Clapp Prize for Outstanding Undergraduate Thesis in Chemistry: *"Exploring the Relationship Between the Sigma-1 Receptor and Lipid-Enriched Microdomains,"* 2011
- Elected to Sigma Xi, The Scientific Research Society, 2011
- U.S. Department of State Critical Language Scholarship (Beijing, China), 2011
- Doris M. Deming '30 Fund Undergraduate Teaching Research Award, 2010
- Sidney E. Frank Endowed Scholarship, 2007-2011

SCIENTIFIC RESEARCH AND WORK EXPERIENCE

Koch Institute for Integrative Cancer Research at MIT, Cambridge MA
Graduate Student, Dr. Stefani Spranger Lab, 2017-present
- Investigated the contributions of dendritic cell subsets to anti-tumor immunity using single-cell RNA-sequencing and *ex vivo* functional assays
- Identified a stimulatory dendritic cell subset (ISG^+ DC) in spontaneously regressing tumors that can activate T cells by cross-dressing with tumor-derived peptide-MHC complexes
- Established and optimized *ex vivo* coculture assays of dendritic cells and T cells
- Presented research findings at Koch Institute meetings and conferences (SITC)
- Co-authored a review on tissue-specific myeloid antigen-presenting cells

Pfizer, Cambridge, MA
Graduate Student Intern, Internal Medicine Research Unit: Metabolic Diseases, Fall 2019
- Investigated the effect of lysophosphatidic acid (LPA) on human monocyte and hepatic stellate cell motility, gene expression, and cytokine/chemokine secretion
- Designed, optimized, and performed transwell migration assays and scratch assays

- Attended and presented data at scientific meetings

Jounce Therapeutics, Cambridge, MA
Associate Scientist, Tumor Immunology, 2015-2016
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- Performed functional characterization of monoclonal antibodies against immune checkpoint receptors as an anti-tumor immunotherapeutic in both *in vitro* and *in vivo* experiments
- Performed *ex vivo* immunophenotyping studies on tissues from therapy-treated tumor-bearing mice to elucidate drug mechanism
- Investigated the effect of different Fc isotypes on the *in vitro* agonistic activity of an antibody
- Designed, optimized, and performed *in vitro* pre-clinical assays for Investigational New Drug filing
- Documented protocols and experiments clearly in up-to-date electronic lab notebook
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Research Technician II, Dr. Sylvie Le Gall Lab, 2012-2014
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- Maintained an organized digital record of experiments and protocols
- Presented data and research papers in weekly lab meetings and attended weekly immunology seminars
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Brown University Department of Chemistry, Providence, RI
Undergraduate Researcher, Dr. Carthene Bazemore-Walker Lab, 2009-2011
- Investigated the relationship between the sigma-1 receptor and lipid rafts in the promotion of survival in human cancer cell lines
- Performed proteomic characterization of detergent-resistant membranes using mass spectrometry
- Analyzed experimental data using various research databases (NCBI, UniProtKB) and interpreted results against a literature survey of current knowledge and understanding
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Teaching Assistant, Immunology (Course 7.23/7.63/20.23), Spring 2020;
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Landmark Medical Hospital, Woonsocket, RI
Volunteer, 2005-2009
- Catalogued new issues of scientific journals and assisted hospital staff with retrieving research articles
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- Assisted the in-patient pharmacy staff in various tasks including drug inventory
- Collaborated in the editing and revision process of weekly medical newsletters

Rhode Island College Upward Bound Program, Providence, RI
Residential Tutor Counselor, Summer 2008
- Tutored 40 inner-city high school students in chemistry, biology, and trigonometry and saw improvement for all by at least one letter grade at the end of the program
- Monitored students' overall academic, social, and personal progress
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I would like to conclude by thanking my friends and family for their unwavering support and encouragement. Special thanks to my sister, Karen, who requested this shoutout for sitting through every single one of my practice talks and reading through multiple drafts of my manuscript and dissertation. Last but not least, my heartfelt thanks go to my turtle, Thaddeus, and my puppies, Iorek and Hana, for being a constant source of joy and distraction while I was writing this dissertation.

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INTRODUCTION

Cancer immunotherapy has revolutionized the treatment of cancer. In particular, the clinical success of a form of immunotherapy called checkpoint blockade therapy (CBT) has quelled any skepticism about the role of the immune system in regulating tumor growth. While CBT is able to drive highly durable anti-tumor immunity across a broad spectrum of tumor types, its success is greatly tempered by the inability to induce clinical responses in the vast majority of cancer patients. Thus, there is an imminent need to understand why CBT might fail and to identify strategies that will sensitize resistant tumors to this therapy.

The presence of a T cell infiltrate in tumors has been associated with sensitivity to CBT and is often used as a biomarker for response. However, T cell presence alone is insufficient to dictate therapeutic response, as exemplified by cases wherein T cell-rich tumors fail to respond to CBT. One possibility is that these tumors are infiltrated by non-tumor-reactive T cells that do not functionally contribute to anti-tumor immunity and are thus irrelevant 'bystander' cells. Increasing focus is also being placed on the functional quality of tumor-reactive T cell responses rather than just their numbers in the tumor. While many factors can influence T cell quality, it is conceivable that the initial signals delivered to T cells during their activation play a dominant role.

The generation or priming of anti-tumor T cell responses is a critical function of antigen-presenting cells (APC) called dendritic cells (DC). While the role of DC in T cell priming is well-accepted dogma, the contributions of DC to the orchestration of anti-tumor

immunity have only been uncovered from studies in the past decade or so. These studies have spotlighted a subset of DC called Batf3-driven DC1 as being particularly adept at activating tumor-reactive $CD8^+$ T cell responses. The exclusion of DC1 or their functional impairment in tumors has been shown to blunt the efficacy of CBT and other immunotherapies, thereby enabling tumor immune evasion. Importantly, the presence of the DC1 signature in patient tumors has been associated with improved responses to immunotherapy and better clinical outcomes.

However, the DC compartment is heterogeneous and consists of several distinct subsets whose function can be impacted by different inflammatory contexts. Recent advances in genomic and transcriptomic technologies have enabled for an unparalleled view of the complexity of the DC compartment in cancer, yet much remains unknown about the specific contributions of different DC subsets and activation states to anti-tumor immunity. Depending on their activation states, DC can instruct T cells to mount an immunogenic or tolerogenic immune response against a given antigen, which has profound implications for the immune response against cancer and other diseases. Thus, the overall focus of my doctoral work has been to elucidate the specific DC states that are associated with productive or dysfunctional anti-tumor immunity and to identify the signals that drive them. A more nuanced understanding of the range of intratumoral DC subsets and activation states can facilitate the development of new strategies to strengthen anti-tumor T cell responses and improve immunotherapy responses.

In this introductory chapter, I will briefly review the origins of the concept of cancer immunosurveillance and provide an overview of immunotherapy types with a specific emphasis on the success and limitations of T cell-targeted immunotherapies. I will then

discuss our current understanding of the requirements for productive anti-tumor immunity. Following this overview, I will review DC biology, their contributions to anti-tumor T cell immunity, and how they can be impacted by the tumor microenvironment (TME). Finally, I will close the chapter by discussing the similarities and difference between murine and human DC subsets, which is critical for establishing translational relevance.

1. Cancer Immunotherapy: The Fourth Pillar of Cancer Treatment

1.1. A Brief History of Cancer Immunotherapy

Cancer immunotherapy is now considered the fourth pillar of cancer treatment after surgery, chemotherapy, and radiation therapy. While it may seem like a recent development, the idea of harnessing the immune system to fight cancer has had a long and rather turbulent history, dating back over 130 years (Oiseth & Aziz, 2017). During that time, the notion of whether an effective immune response could be generated against tumors was hotly contested and controversial (Parish, 2003).

The earliest evidence of immune control of tumors dates back to the eighteenth century and derives from a number of anecdotal reports on spontaneously regressing tumors. These remissions were rather rare, with a frequency of 1 in every 60,000 to 100,000 cases (Oiseth & Aziz, 2017). Interestingly, they were more frequently documented in cancer patients that also suffered from feverish infections. Two German physicians, Friedrich Fehleisen and Wilhelm Busch, each independently documented spontaneous tumor regression in cancer patients who suffered from erysipelas, a bacterial skin infection (Oiseth & Aziz, 2017). While they were unable to reliably replicate

these observations, they succeeded in identifying *Streptococcus pyogenes* as the pathogenic bacterial strain causing erysipelas (Oiseth & Aziz, 2017).

Years later, William Coley, a surgeon at Memorial Hospital in New York, also made the intriguing observation of spontaneous regression of a sarcoma in a patient infected with erysipelas. After reviewing medical records and medical literature, he uncovered at least 47 similar case reports, which encouraged him to further investigate the link between acute bacterial infection and cancer regression (McCarthy, 2006). In 1891, Coley began injecting patients' tumors with a mixture of live and inactivated *Streptococcus pyogenes* and *Streptococcus marescens* (McCarthy, 2006). His treatment, which became known as Coley's toxins, garnered much excitement and attention for its ability to induce tumor regression or even cures in a large number of patients (McCarthy, 2006). In spite of its success, however, Coley's toxins ultimately fell into obscurity for a number of reasons. Coley was rather inconsistent with his formulation and delivery of his toxins, and patients were at a high risk of contracting bacterial infections. Furthermore, the mechanism of Coley's toxins was entirely unknown. Thus, with the development of radiation therapy and chemotherapy, oncologists gradually discontinued the use of Coley's toxins, favoring more reliable modalities of cancer treatment instead. Despite this outcome, Coley is recognized as the Father of Immunotherapy and is accredited as developing the first cancer immunotherapy. Unbeknownst to him, the principle of using bacteria to treat cancer would eventually re-emerge. A 1959 murine study demonstrated the anti-cancer effects of Bacillus Calmette-Guérin, a live attenuated strain of *Mycobacterium bovis* (Old, Clarke, & Benacerraf, 1959). This work later supported its evaluation in a clinical trial,

where BCG was shown to be effective in the treatment of bladder cancer (Morales, Eidinger, & Bruce, 1976).

Tumor transplantation studies in the early 1900s provided further support for immune control of tumors. The German physician Paul Ehrlich observed regression of transferred tumor tissue from animals of the same species (Ehrlich, 1909; Ribatti, 2017). He also found that animals that had already rejected a tumor transplant could reject a secondary challenge with faster kinetics (Ehrlich, 1909; Ribatti, 2017). These findings led him to propose that the immune system played a significant role in controlling tumor growth. Ehrlich's hypothesis, however, did not gain much traction with his contemporary scientific community. Because of his use of outbred mice, it is now believed that many of his observations could be driven by allograft rejection. Nonetheless, subsequent work using inbred mice would support much of Ehrlich's original findings (Gross, 1943).

In 1957, Frank Macfarlane Burnet and Lewis Thomas expanded upon Ehrlich's work by proposing the immunosurveillance theory, which argues that a major function of immune system is to detect and eliminate cancerous cells (F. M. Burnet, 1970). The revival of this notion was in large part due to advances in understanding the mechanistic underpinnings of immune recognition of cancer cells. From work using carcinogen or virally induced tumors, it was increasingly understood that transformed or neoplastic cells could be distinguished from normal or non-transformed cells through the expression of unique proteins. Thus, the immune system could specifically recognize and destroy cancer cells through detecting these so-called tumor-associated antigens.

However, this favorable viewpoint towards immune control of tumors would be rather short-lived. In the 1970s, work by Argentinian physician Osias Stutman at Memorial

Sloan-Kettering directly challenged Burnet and Lewis' immunosurveillance theory. Stutman used the carcinogen methylcholanthrene (MCA) to induce tumors in wild-type or T cell-deficient athymic nude mice and showed that the incidence of tumor development and burden was comparable between the two experimental groups (Stutman, 1974, 1975, 1979a, 1979b). These findings suggested that the immunosurveillance theory might only apply to chemically or virally induced tumors and not to spontaneously arising tumors, which is more common in humans, thus casting doubt on its clinical relevance. This skepticism was further reinforced by a study showing that unlike chemically or virally induced tumors, spontaneous tumor cell lines were not recognized by the immune system and unable to generate anti-tumor immunity (Hewitt, Blake, & Walder, 1976).

Despite these discouraging setbacks, interest in cancer immunology would soon resurface in the mid-1980s and 1990s. It is now known that there were critical flaws in Stutman's experimental design and interpretations. Athymic nude mice are not completely immunodeficient; they still have natural killer cells and basal levels of T cells that increase with age, which could contribute to immune control of tumors (Budzynski & Radzikowski, 1994; Giovanella & Fogh, 1985; Ikehara, Pahwa, Fernandes, Hansen, & Good, 1984). A study performed 20 years after Stutman's work demonstrated that low dose MCA actually led to higher incidence of tumor formation in athymic nude mice compared to wild-type mice (Engel et al., 1996). Eventually, studies using more specific mouse strains that ablated various mediators of T cell function, such as interferon-γ receptor knockout mice and perforin-deficient mice, would provide sufficient evidence for the immunosurveillance of cancer as they showed increased susceptibility to and higher tumor burden as a result of MCA treatment (Kaplan et al., 1998; van den Broek et al., 1996).

Significant advancements in knowledge of basic immunology and cancer biology in the second half of the twentieth century also contributed to the revival of the cancer immunosurveillance theory. The discovery of interferons in 1957, T cells in 1967, dendritic cells in 1973, MHC restriction of T cells in 1974, and the T cell receptor (TCR) in 1982 set the foundation for our modern understanding of how immune responses are initiated and provided a framework with which to study the interaction of immune cells and tumor cells (Allison, McIntyre, & Bloch, 1982; Doherty & Zinkernagel, 1975; Haskins et al., 1983; Isaacs & Lindenmann, 1957; Isaacs, Lindenmann, & Valentine, 1957; Meuer et al., 1983; Miller & Mitchell, 1967; R. M. Steinman & Cohn, 1973; Zinkernagel & Doherty, 1974). These breakthroughs coupled with the demonstration that tumor cells are genetically unstable and thus can express genomic alternations quelled the fear that spontaneously arising tumors would be invisible to the immune system (Fenton & Longo, 1995; Stoler et al., 1999). This notion was reinforced by the identification of the first human T cell antigen, MAGE-1, in 1991, and by the characterization of numerous other murine and human tumor-associated antigens shortly thereafter (Boon, Cerottini, Van den Eynde, van der Bruggen, & Van Pel, 1994).

It is now understood that the relationship between the immune system and cancer is dynamic. In 2001, Robert Schreiber's group at Washington University published a seminal study wherein he compared the transplantation kinetics of MCA-induced sarcomas derived from immunocompetent WT mice or immunodeficient $Rag2^{-/-}$ mice, which lack T cells and B cells (Shankaran et al., 2001). He demonstrated that transplantation of tumors from $Rag2^{-/-}$ mice into syngeneic WT mice were more frequently rejected compared to transplantations of tumors derived from WT mice (Shankaran et al.,

2001). This observation suggested that tumor immune escape in immunocompetent mice is likely attributable to T cell-mediated destruction of cancer cells expressing immunogenic antigens, thus enabling cancer cells that did not express immunogenic antigens to grow out. This phenomenon later became known as “immunoediting” and was the first example of active immune evasion by the tumor. Since then, other mechanisms of immune evasion have been described, such as tumor cell-intrinsic downregulation of various components of the antigen processing machinery or even complete loss of MHC-I antigen presentation, which renders tumors invisible to cytotoxic attack by $CD8^+$ T cells (Dhatchinamoorthy, Colbert, & Rock, 2021; Vinay et al., 2015). Tumors can also actively suppress the immune response by fostering an immunosuppressive microenvironment, which is achieved through upregulated expression of immunoinhibitory molecules (i.e. programmed death-ligand 1, PD-L1) that signals to dampen T cell function, the secretion of immunosuppressive cytokines (i.e. IL-10, IL-4, TGF-β) to induce T_H2 responses, and the recruitment of immunoregulatory and immature cell types (i.e. regulatory T cells, myeloid-derived suppressor cells, tumor-associated macrophages) (Drake, Jaffee, & Pardoll, 2006; Vinay et al., 2015).

As we continue to build on our understanding of the complex interplay between cancer and the immune system, it will be important to identify ways to apply this knowledge to the design and development of novel therapies to treat cancer. The following section will provide a high-level overview of different types of immunotherapies that have been clinically developed with a particular emphasis on T cell-targeted immunotherapies as they have recently garnered the most attention and excitement.

1.2. Types of Immunotherapy: Spotlight on T Cell-Targeted Therapies

Over the last half century, there have been several innovative developments in cancer immunotherapy. The earliest forms of immunotherapy entailed the use of cytokines, which are small secreted proteins involved in cell signaling and communication. Interferon alpha (IFNα), a subtype of type-I-IFN (IFN-I), was both the first cytokine to be discovered in 1957 (Isaacs & Lindenmann, 1957; Isaacs et al., 1957) as well as the first cancer immunotherapy approved by the U.S. Food and Drug Administration (FDA) in 1986 as a treatment for hairy-cell leukemia (Golomb et al., 1986). Because IFNα has direct anti-proliferative and anti-angiogenic effects, its efficacy as an anti-cancer agent was thought to derive from its direct effects on tumor cells (Ferrantini, Capone, & Belardelli, 2007). It was not until later that the effects of IFNα (and other IFN-I) on immune cells was recognized (Belardelli & Gresser, 1996; Ferrantini et al., 2007). IFN-I and its impact on the anti-tumor immune response will be discussed in greater detail in **Section 2.4.2a**. Another cytokine, interleukin-2 (IL-2), was discovered in 1976 and was found to be effective against metastatic cancers, leading to its FDA approval for the treatment of metastatic renal cell carcinoma in 1992 and metastatic melanoma in 1998 (Tao Jiang, Zhou, & Ren, 2016). High-dose IL-2 was believed to be efficacious against cancer through its ability to expand T cells as a “T cell growth factor” (Tao Jiang et al., 2016). However, the toxicities associated with IFNα and IL-2 treatment have greatly tempered enthusiasm for these cytokine therapies, though there are ongoing efforts to engineer safer versions of these drugs (Berraondo et al., 2019; Overwijk, Tagliaferri, & Zalevsky, 2021; Xue, Hsu, Fu, & Peng, 2021).

Another strategy to treat cancer has been to target various immunosuppressive components of the TME, which refers to the ecosystem around the tumor and comprises the extracellular matrix, blood vessels, fibroblasts and other stromal cells, as well as immune cells (T. Tang et al., 2021). Inhibitors against indoleamine 2,3-dioxygenase 1 (IDO1) are currently being developed and tested in clinical trials (Tang, Wu, Song, & Yu, 2021). IDO1 is an enzyme that degrades tryptophan, which is a key nutrient needed for T cell proliferation and survival (Platten, Wick, & Van den Eynde, 2012). Thus, overexpression of IDO1 in tumors is associated with poor prognosis and comprises a major mechanism of immunosuppression by the tumor (Platten et al., 2012) (K. Tang et al., 2021). Other TME-related immunosuppressive targets for immunotherapy include vascular endothelial growth factor (VEGF), which promotes angiogenesis, and the immunoregulatory cytokines transforming growth factor beta (TGF-β) and interleukin-10 (IL-10). Depleting immunosuppressive cell types in the TME such as regulatory T cells (T_{reg}), myeloid-derived suppressor cells (MDSC), and tumor-associated macrophages (TAM) is another strategy to overcome the immunosuppressive TME (Dobosz & Dzieciątkowski, 2019; T. Tang et al., 2021).

Cancer vaccines comprise another significant development in the immunotherapy of cancer and are categorized as prophylactic or therapeutic vaccines (Crews, Dombroski, & King, 2021; Saxena, van der Burg, Melief, & Bhardwaj, 2021). Prophylactic vaccines are used to train the immune system to recognize cancer-causing viruses, such as the hepatitis B virus (HBV) and human papillomavirus (HPV), in order to prevent viral infection and subsequent development of HBV and HPV-related cancers (Crews et al., 2021). In contrast, therapeutic vaccines are used to treat an existing cancer and include both

autologous and allogeneic cancer vaccines (Saxena et al., 2021). Autologous cancer vaccines are personalized vaccines where a patient's cells are isolated, manipulated *ex vivo*, and subsequently reintroduced back into the patient. The first autologous cancer vaccine, Sipuleucel-T, was approved by the FDA in 2010 for the treatment of castration-resistant prostate cancer (Handy & Antonarakis, 2018). It consisted of an infusion of patient-derived dendritic cells that were *ex vivo* activated and loaded with prostate cancer-associated antigens (Handy & Antonarakis, 2018). While Sipuleucel-T prolonged overall survival in clinical trials, it failed to impact the time to disease progression (Kantoff et al., 2010) and thus is rarely used today. In contrast to autologous cancer vaccines, allogeneic vaccines entail infusion of lab-grown cells that are not derived from the patient. While there is yet to be an FDA-approved allogeneic vaccine, efforts are ongoing to develop them (Avigan et al., 2007; Jou, Harrington, Zocca, Ehrnrooth, & Cohen, 2021; Rousseau, Hirschmann-Jax, Takahashi, & Brenner, 2001). Another promising vaccination strategy under development involves the administration of tumor neoantigens to elicit anti-tumor T cell responses (Castle, Uduman, Pabla, Stein, & Buell, 2019; T. Jiang et al., 2019).

The most successful immunotherapies to date have largely targeted $CD8^+$ T cells, which are believed to be the predominant drivers of clinical response given their ability to recognize and kill tumor cells (Durgeau, Virk, Corgnac, & Mami-Chouaib, 2018). One major strategy has been to increase the numbers of tumor-reactive T cells via adoptive cell therapy (ACT), a strategy initially pioneered by Steven Rosenberg at the National Cancer Institute in the 1980s (Rosenberg & Dudley, 2009). In the traditional form of ACT, tumor-reactive T cells from a patient are isolated and expanded *in vitro* before being reinjected into the patient's blood circulation. In more recent forms of ACT, however, the

isolated T cells are often modified prior to expansion and reinfusion into patients. The most notable example has been the incorporation of chimeric antigen receptors (CARs) into T cells (Sadelain, Brentjens, & Rivière, 2013). CARs are recombinant receptors that comprise an external antigen-binding domain (antibody fragment) and a cytoplasmic T cell activation domain (Larson & Maus, 2021; Sadelain et al., 2013). Their utility as a therapy resides in enabling T cells to recognize virtually almost any antigen of choice, thus bypassing the requirement of MHC restriction for T cell recognition of tumor cells. While CAR-T cells targeting CD19 have had remarkable clinical success against B cell leukemia and lymphoma as demonstrated by their FDA approval in 2017, they are much less efficacious against solid tumors (Sterner & Sterner, 2021). Understanding how immunosuppressive factors of the TME impacts CAR-T cells is a major research focus.

By far, the most promising T cell-targeted immunotherapy is CBT, which has enabled the induction of highly durable anti-tumor immunity against several advanced cancer types (Kubli, Berger, Araujo, Siu, & Mak, 2021; Ribas & Wolchok, 2018). CBT agents consist of monoclonal antibodies targeting immune ‘checkpoint’ receptors, such as programmed death-1 receptor (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4). These immune checkpoint receptors are expressed on activated T cells to safeguard against overstimulation. In cancer, however, chronic antigen exposure induces the upregulation of PD-1 and CTLA-4 on T cells, as well as several other inhibitory receptors such as Tim-3 and Lag-3 (Y. Jiang, Li, & Zhu, 2015; Wherry & Kurachi, 2015). Signaling through these inhibitory receptors leads to T cell exhaustion, a state of dysfunction characterized by progressive loss of T cell effector function and impaired proliferation. Tumor cells in response to IFNγ signaling can upregulate the expression of PD-L1, the

ligand for PD-1, thereby directly suppressing T cell responses to favor tumor immune evasion (Garcia-Diaz et al., 2017). Thus, blockade of these immune checkpoint receptors via CBT prevents the transmission of inhibitory signals to T cells and allows for sustained T cell effector function against the tumor.

1.3. Immune Checkpoint Inhibitors: Origin, Success, and Limitations

The 1990s saw the rise of immune checkpoint receptor inhibition as a novel means to treat cancer. This was greatly facilitated by the development of specific monoclonal antibodies. Early studies using anti-CTLA-4 antibodies by Jeffrey Bluestone and James Allison independently established a role for CTLA-4 as a negative regulator of T cell activation (Krummel & Allison, 1995; Walunas et al., 1994). It is now known that in naïve T cells, CTLA-4 is expressed intracellularly. Upon T cell activation via TCR engagement and CD28 costimulation, CTLA-4 translocates to the cell surface and competes with CD28 for binding to the costimulatory ligands CD80 and CD86 expressed on APC (Chambers, Kuhns, Egen, & Allison, 2001; Seidel, Otsuka, & Kabashima, 2018). The degree of T cell activation is thus governed by the competition between these two receptors for ligand binding, though it should be noted that CTLA-4 has a higher affinity to CD80 and CD86 than CD28. While Bluestone investigated the role of CTLA-4 in controlling autoimmune diseases, Allison postulated that sustained T cell activation and proliferation as a consequence of CTLA-4 blockade might be beneficial against cancer (Chambers et al., 2001; Ribas & Wolchok, 2018). In 1996, Allison and colleagues published a seminal study demonstrating that therapeutic blockade of CTLA-4 could indeed induce the regression of established syngeneic tumors in mice (Leach, Krummel,

& Allison, 1996), which then prompted its evaluation in the clinic. In 2011, FDA approval for a monoclonal antibody targeting CTLA-4 called ipilimumab was granted for treatment of advanced melanoma and has since been extended for additional tumor indications.

The 1990s also witnessed the identification and establishment of PD-1 as a negative regulator of T cell activation through seminal work led by Tasuku Honjo (Ishida, Agata, Shibahara, & Honjo, 1992). Similar to CTLA-4, PD-1 expression is upregulated on activated T cells (Nishimura, Nose, Hiai, Minato, & Honjo, 1999; Ribas & Wolchok, 2018; Seidel et al., 2018). Binding of PD-1 to its ligands, PD-L1 and PD-L2, inhibits T cell proliferation and effector function and reduces T cell survival (Buchbinder & Desai, 2016; Ribas & Wolchok, 2018). PD-L1 can be broadly expressed by many cell types upon exposure to pro-inflammatory cytokines like IFNγ, whereas PD-L2 expression is restricted to APC (Garcia-Diaz et al., 2017). Tumor cell-intrinsic expression of PD-L1 in the TME enables direct suppression of effector T cell responses, allowing tumors to evade the immune response (Ribas & Wolchok, 2018). Blockade of PD-1, like CTLA-4, has also had remarkable success in the clinic, culminating in its rapid FDA approval in 2014 against refractory metastatic melanoma, which has since been expanded to include other indications (Vaddepally, Kharel, Pandey, Garje, & Chandra, 2020).

Although the outcome of both CTLA-4 and PD-1 ligation is inhibition of T cell function, the pathways are non-redundant. CTLA-4 and PD-1 regulate T cell activation at different stages of an immune response **(Figure 1.1)** (Buchbinder & Desai, 2016). CTLA-4 functions during the initial stages of naïve T cell activation (‘priming phase’), which occurs in the lymph nodes. In contrast, PD-1 functions at later stages of an immune response (‘effector phase’) and is believed to primarily impact previously activated T cells

in peripheral tissues (Keir, Butte, Freeman, & Sharpe, 2008). Because of these differences, these therapies are thought to target different T cell populations, and thus there is a potential for synergistic effects by combining them. Indeed, the combination of PD-1 and CTLA-4 blockade has proven to be quite potent in the clinic against advanced melanoma, leading to improved efficacy and increases in progression-free and overall survival (Khair et al., 2019; Larkin et al., 2019). A meta-analysis of 8 clinical trials of combination or monotherapy CBT with anti-CTLA-4 and anti-PD-1 across different tumor indications also showed an increased objective response rate for patients receiving combination CBT (K. Wu et al., 2019). For their contributions to the development of CBT, Allison and Honjo were jointly awarded the 2018 Nobel Prize for Physiology or Medicine.

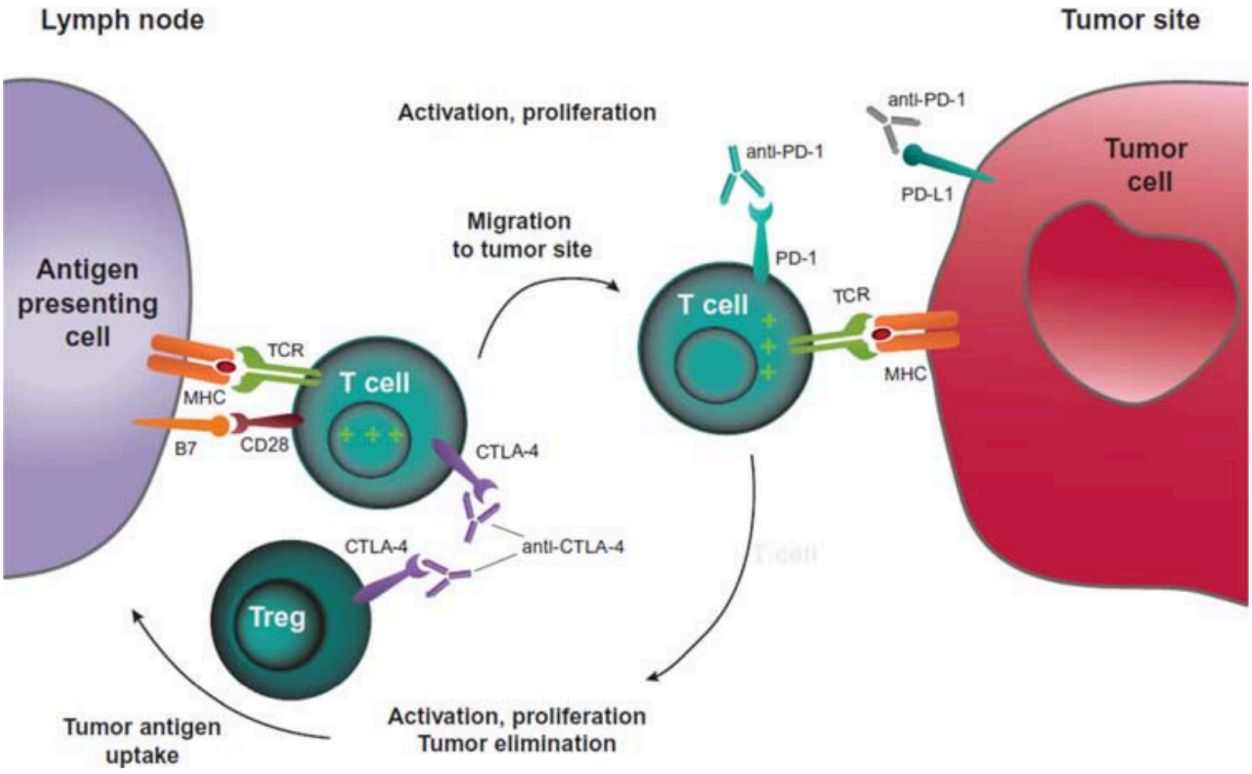

Figure 1.1. CBT therapy using anti-CTLA-4 and anti-PD-1 acts on different phases of the immune response. Anti-CTLA-4 enhances T cell activation during the priming phase of the immune response, whereas anti-PD-1 reinvigorates activated T cells during the effector phase of the immune response. Image from (Buchbinder & Desai, 2016).

Despite the remarkable clinical efficacy of CBT, the majority of cancer patients fail to respond and thus do not derive benefit from this therapy **(Figure 1.2).** This has fueled significant efforts towards identifying predictive biomarkers. As T cells are the cellular targets of CBT, one long-standing correlate of CBT response has been the pre-treatment presence of a T cell infiltrate in tumors (Ji et al., 2012; Tumeh et al., 2014). However, this association is not absolute, and among patients with a pre-existing T cell infiltrate, many still fail to respond to CBT or acquire resistance and progress while on treatment (Jenkins, Barbie, & Flaherty, 2018; O'Donnell, Long, Scolyer, Teng, & Smyth, 2017; Pitt et al., 2016; Restifo, Smyth, & Snyder, 2016; P. Sharma, Hu-Lieskovan, Wargo, & Ribas, 2017). One study highlighted the immense heterogeneity of tumor-infiltrating $CD8^+$ T cells in human lung and colorectal cancer patients, demonstrating that not all infiltrating T cells are reactive against tumor antigens (Simoni et al., 2018). These 'bystander' T cells are phenotypically distinct from tumor-specific T cells, lacking the expression of CD39 and other hallmarks of T cell exhaustion, and thus likely do not contribute to anti-tumor immunity (Simoni et al., 2018). As such, their abundance in tumors has been linked with poor response to anti-PD-1 treatment (Simoni et al., 2018). Indeed, clinical studies have shown that absolute T cell count does not correlate with response in melanoma patients treated with CBT (Wolchok et al., 2013), calling instead for a focus on the quality or profile of tumor-infiltrating T cells as the stronger predictor (Galon et al., 2006). Preclinical studies have demonstrated that the activation and recruitment of tumor-reactive effector T cells to the tumor is required for the success of CBT (Stefani Spranger et al., 2014). In support of this notion, the presence of a T cell activation gene signature in patient tumors has been positively correlated with clinical response to CBT in various tumor indications

(Herbst et al., 2014; Van Allen et al., 2015). Thus, a closer examination of the factors governing effective T cell activation against tumors and how they can be dysregulated may prove critical for understanding resistance to CBT.

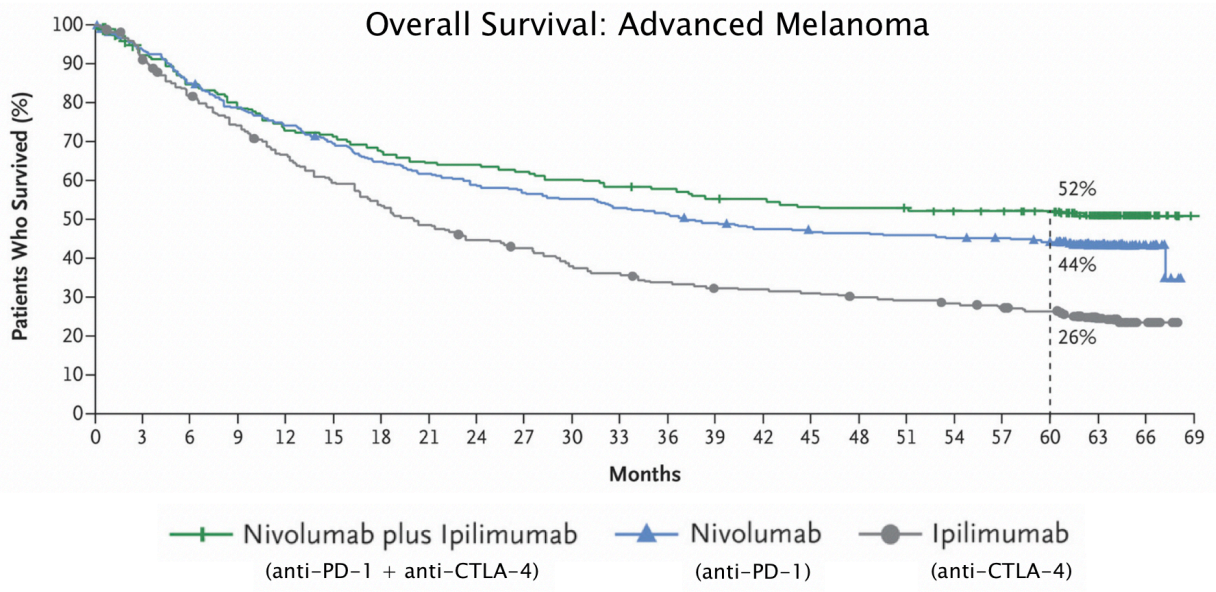

Figure 1.2. Overall survival of advanced melanoma patients treated with monotherapy or combination anti-CTLA-4 and anti-PD-1. Image adapted from (Larkin et al., 2019).

1.4. Expanding Beyond a T Cell-Centric View of Immunotherapy

Until recently, the field has primarily viewed immunotherapy through a T cell-focused lens. Prominent forms of immunotherapy discussed earlier, such as IL-2 cytokine therapy, ACT, and CBT have focused on enhancing anti-tumor T cell activation in a T cell-intrinsic manner. However, T cell activation is a convoluted process that requires multiple signals delivered by cell types called antigen-presenting cells (APC). Work from recent years have shed light on the contributions of APC to the induction of anti-tumor immunity, as well as how their functions may be subverted by the TME to promote tolerance and immunosuppression. Although the major types of APC, which include

macrophages, B cells, and DC, share the ability to internalize, process, and present antigens on MHC molecules, only DC are able to efficiently initiate T cell responses against these antigens, which occurs through a process termed 'priming' in lymph nodes. In the remainder of this introductory chapter, I will provide a high-level overview of our current understanding of the contributions of DC to anti-tumor T cell immunity.

2. The Role of Dendritic Cells in Anti-Tumor Immunity

2.1. Dendritic Cells and the Cancer-Immunity Cycle

The immunosurveillance of cancer and induction of anti-tumor immunity occurs through a multistep and iterative process that is referred to as the "Cancer-Immunity Cycle" (D. S. Chen & Mellman, 2013) **(Figure 1.3)**. The cycle begins with the release of antigens from dying tumor cells. Tumor cells are genetically unstable and can undergo genetic modifications, leading to mutated or aberrantly expressed proteins (i.e. neoantigens) that are foreign to the immune system (D. S. Chen & Mellman, 2013). These antigens are then captured and internalized by DC, which process and present the antigens on MHC molecules (D. S. Chen & Mellman, 2013). For immunity to be induced, it is critical that that the uptake of antigens by DC is accompanied by sensing of immunogenic signals that are released by dying tumor cells. These consist of damage-associated molecular patterns (DAMP) and danger signals that bind and activate pattern recognition receptor (PRR) pathways in DC (D. S. Chen & Mellman, 2013). In cancer, the predominant PRR pathway leading to DC activation is cGAS/STING sensing of tumor-derived cytosolic DNA (Woo et al., 2014). STING activation in DC induces a strong type-I-interferon (IFN-I) response that is positively regulated, leading to the induction of more IFN-I, the

upregulation of costimulatory molecules such as CD80 and CD86 and the chemokine receptor CCR7, and the production of pro-inflammatory cytokines like IL-12 (Corrales, Matson, Flood, Spranger, & Gajewski, 2017). These phenotypic and functional changes are hallmarks of DC maturation, and they do not occur in the context of antigen uptake without PRR engagement.

Once activated and loaded with antigen, DC then traffic to the nearest draining LN in a CCR7-dependent manner where they interact with naïve T cells. During these interactions, DC deliver three distinct but critical signals to activate or prime T cells: an antigen-specific signal via peptide-MHC (pMHC) (signal 1), a costimulatory signal (signal 2), and a cytokine signal (signal 3) (Smith-Garvin, Koretzky, & Jordan, 2009). Delivery of signal 1 in absence of other signals induces T cell anergy, a state of functional inactivation that induces tolerance, thus highlighting the importance of DC activation and maturation for immunogenic T cell priming (Schwartz, 2003). Successfully activated T cells then clonally expand via a proliferative burst and acquire effector functions (i.e. cytolytic ability via granzymes and perforins, and secretion of pro-inflammatory cytokines IFNγ and TNFα) (Smith-Garvin et al., 2009). They then leave the LN and infiltrate tumors following a gradient of CXCL9 and CXCL10 chemokines that is secreted by DC residing in the tumor (D. S. Chen & Mellman, 2013; S. Spranger, Dai, Horton, & Gajewski, 2017). Tumor-reactive cytotoxic T cells are then able to recognize and kill tumor cells, which causes the release of more tumor antigens and danger signals, thus restarting the Cancer-Immunity Cycle and amplifying the anti-tumor immune response (D. S. Chen & Mellman, 2013).

Loss of any of these functions by DC—antigen uptake, processing, and presentation, migration, cross-priming of T cells, and T cell recruitment—is highly

detrimental to anti-tumor immunity and enables tumor immune evasion. Given the importance of DC in driving anti-tumor T cell responses, it comes as no surprise that the presence of DC gene signatures in tumors correlates with a T cell-inflamed microenvironment and response to CBT, which will be further discussed in **Section 2.5**. For these reasons, it is imperative to improve upon our understanding of the role of DC in cancer to gain insights on how we can effectively harness them to strengthen T cell immunity and increase clinical responses to CBT and other immunotherapies.

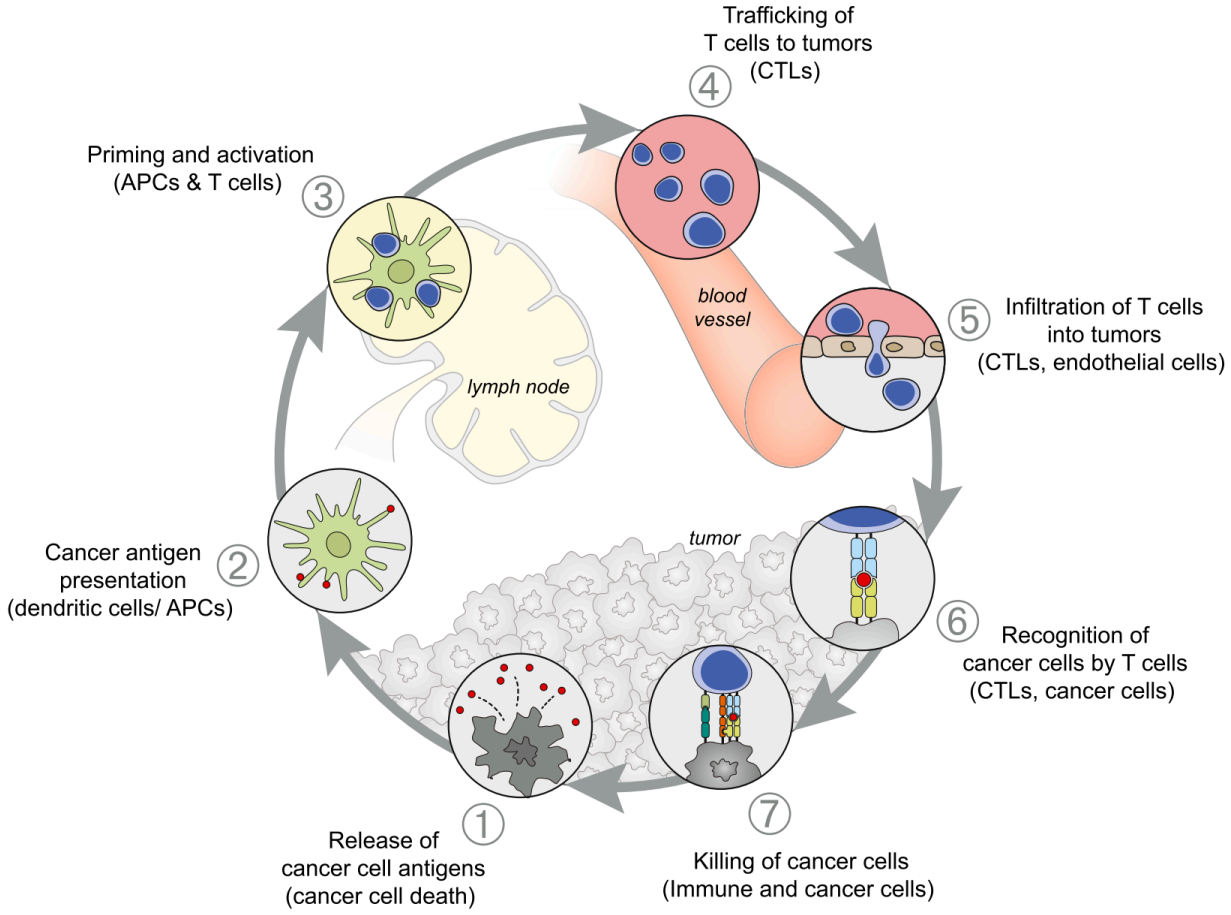

Figure 1.3. The Cancer-Immunity Cycle. The induction of productive anti-tumor immunity occurs through a multistep, iterative process involving many cell types. APCs, antigen-presenting cells; CTLs, cytotoxic T lymphocytes. Image from (D. S. Chen & Mellman, 2013).

2.2. Discovery of Dendritic Cells

The clonal selection theory, proposed by Frank Macfarlane Burnet in 1957, postulated that every lymphocyte expressed receptors with a pre-determined specificity to a unique antigen (Frank Macfarlane Burnet, 1957). Antigen binding to these receptors would trigger the lymphocyte to clonally expand, giving rise to a clonal pool of progeny cells with the same antigen specificity (Frank Macfarlane Burnet, 1957). Simply adding antigen to the lymphocytes, however, was not sufficient to induce clonal expansion. Thus, a prevailing enigma in the 1960s was elucidating the specific manner by which lymphocytes recognized their cognate antigen and become activated.

Early evidence that “splenic accessory cells” were required for lymphocyte activation derived from experiments performed by David E. Mosier, then a graduate student at The University of Chicago. Mosier was building upon seminal work from Robert Mishell and Richard Dutton at the Research Institute of Scripps Clinic (now known as The Scripps Research Institute), which was the first report of an *in vitro* primary antibody response generated by murine splenocytes against exogenously added sheep red blood cells (Mishell & Dutton, 1966). At the time, much attention was focused on the potential role of macrophages in inducing antibody formation by B cells, as they were known to phagocytose antigens (Askonas & Rhodes, 1965; Rittenberg & Nelson, 1960). Mosier performed the original Mishell-Dutton assay with one major modification: prior to adding the sheep red blood cells, he separated the murine splenocytes into “macrophage-rich” adherent and “lymphocyte-rich” non-adherent fractions based on differential binding to plastic dishes (Donald E. Mosier, 1967). Both cellular fractions were required to induce an antibody response against sheep red blood cells, which supported the existing view

that macrophages were essential contributors to the response (Donald E. Mosier, 1967). In his later work, however, Mosier revised this hypothesis, arguing instead that the cell type in the “macrophage-rich” fraction required for inducing immune responses was rare and therefore unlikely to be macrophages (D. E. Mosier & Coppleson, 1968).

While Mosier’s work postulated the existence of a new adherent cell type important for generating immune responses, it was Ralph Steinman, then a postdoctoral fellow in Zanvil Cohn’s lab at Rockefeller University, who performed the seminal work leading to the formal identification of DC (R. M. Steinman, Adams, & Cohn, 1975; R. M. Steinman & Cohn, 1973; Ralph M. Steinman & Cohn, 1974; R. M. Steinman, Kaplan, Witmer, & Cohn, 1979; R. M. Steinman, Lustig, & Cohn, 1974). Using phase-contrast light microscopy to visually inspect adherent murine splenocytes, Steinman documented a rare population of stellate cells with distinct morphological and functional features: “The nucleus is large, retractile, contorted in shape, and contains small nucleoli (usually two). The abundant cytoplasm is arranged in processes of varying length and width and contains many large spherical mitochondria” **(Figure 1.4)** (R. M. Steinman & Cohn, 1973). As the cells “continually extended and retracted their processes or dendrites”, thus resembling branches of a tree, Steinman referred to them as ‘dendritic cells’ from the Greek word *dendreon* for “tree” (Ralph M Steinman, 2007). In subsequent work, Steinman optimized a procedure to enrich for DC from murine splenocytes which enabled him to culture them *in vitro* and characterize them using several functional assays (R. M. Steinman et al., 1975; Ralph M. Steinman & Cohn, 1974; R. M. Steinman et al., 1979; R. M. Steinman et al., 1974). These experiments confirmed that DC were a truly novel cell type, lacking the surface differentiation markers of lymphocytes and the robust Fc

receptor-mediated endocytic capacity of macrophages (R. M. Steinman et al., 1975; Ralph M. Steinman & Cohn, 1974; R. M. Steinman et al., 1979; R. M. Steinman et al., 1974). In characterizing DC, Steinman found that they highly expressed major histocompatibility complex (MHC) proteins, such as Ia antigens (R. M. Steinman et al., 1979). Subsequent work by Steinman showed that DC are the predominant stimulatory cells in the mixed leukocyte reaction (MLR), which is an *in vitro* technique that models graft rejection and measures the allogeneic immune response induced from co-culture of leukocytes from different donors (R. M. Steinman, Gutchinov, Witmer, & Nussenzweig, 1983; R. M. Steinman & Witmer, 1978). He found that enriched DC were at least 100 times more potent in the MLR assay compared to bulk splenocytes, which contained only 1% DC (R. M. Steinman & Witmer, 1978). Ultimately, Steinman and his trainees would go on to demonstrate that DC were critical for mounting both T cell responses and antibody responses (Inaba, Steinman, Van Voorhis, & Muramatsu, 1983; Nussenzweig, Steinman, Gutchinov, & Cohn, 1980), as well as governing whether those responses would be immunogenic or tolerogenic (Bonifaz et al., 2002; Hawiger et al., 2001). He and his team further demonstrated that DC could be found in human peripheral blood and were thus translationally relevant cells (Van Voorhis, Hair, Steinman, & Kaplan, 1982).

Steinman's pioneering work on DC biology paved the way for subsequent studies cementing the role of DC as the critical APC type required for initiating adaptive immune responses. In recognition of his discovery of DC, Steinman was awarded several prestigious prizes, including the Gairdner Foundation Award in 2003, the Albert Lasker Award in 2007, and the Nobel Prize in Physiology or Medicine in 2011 (Banchereau et al.,

2011; Bashyam, 2007; Mellman & Nussenzweig, 2011; Nussenzweig & Mellman, 2011; Shortman, 2012; R. M. Steinman, 2007).

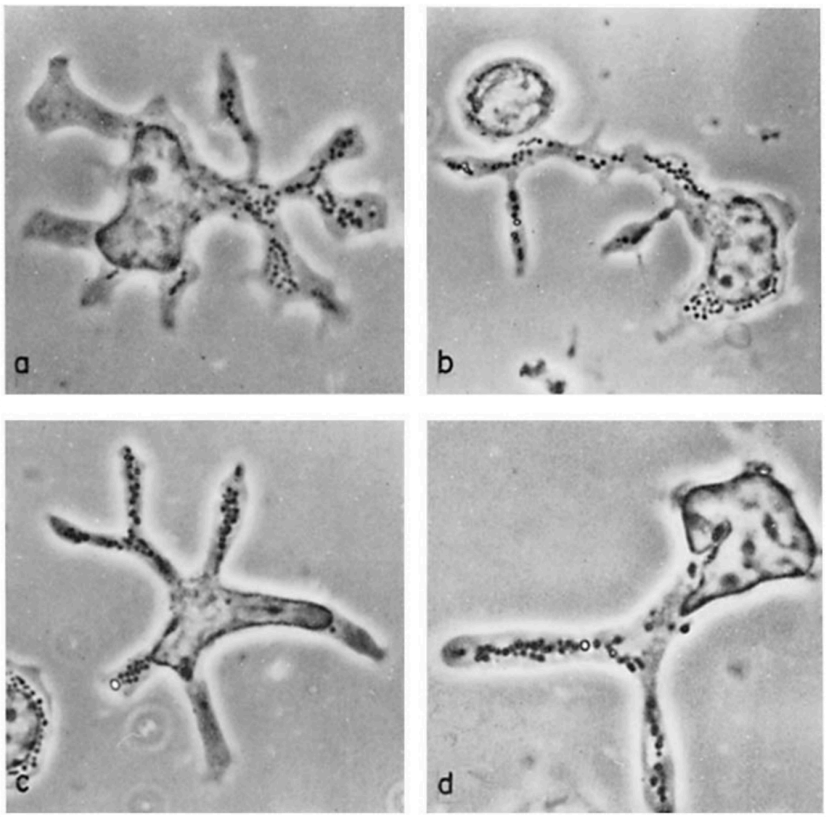

Figure 1.4. Phase-contrast microscopy images of glutaraldehyde-fixed DC isolated from murine spleen. Magnification: (a) X 4,500; (b) X 3,500; (c) X 3,200; (d) X 4,600. Image adapted from (R. M. Steinman & Cohn, 1973).

2.3. Ontogeny of Dendritic Cells

2.3.1. *Dendritic Cell Development*

For a time, DC were thought of as a single cell type found in lymphoid tissues. However, this notion was quickly challenged with the observation that Langerhans cells (LC) found in the epidermis shared many features with splenic DC, such as the stellate morphology and ability to induce T cell activation, thus indicating that the two may be related cell types (Schuler, Romani, & Steinman, 1985). Today, it is now known that DC

consist of several distinct subsets, and as the body's sentinel cells, they can be found in both lymphoid and nonlymphoid tissues.

All DC develop from hematopoietic stem cells (HSC) in the bone marrow (BM) and rely on Flt3 signaling for their development **(Figure 1.5)** (Anderson, Dutertre, Ginhoux, & Murphy, 2021). HSC give rise to multipotent progenitors that differentiate into lineage-restricted progenitors, the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP), which give rise to lymphocytes and myeloid cells, respectively (Akashi, Traver, Miyamoto, & Weissman, 2000; Anderson et al., 2021; Kondo, Weissman, & Akashi, 1997; Manz, Traver, Miyamoto, Weissman, & Akashi, 2001). While it is widely accepted that DC derive predominately from CMP, there are some reports that DC can differentiate from CLP (Manz et al., 2001; Shigematsu et al., 2004; L. Wu et al., 2001). CMP further give rise to the monocyte-DC progenitor (MDP) or granulocyte-macrophage progenitor (GMP) (Anderson et al., 2021). MDP are then thought to differentiate further into the common DC precursor (CDP), from which pre-DC arise (Naik et al., 2007; Onai et al., 2007). Pre-DC then leave the BM and infiltrate into lymphoid and non-lymphoid tissues where they further differentiate into specific DC subsets (Anderson et al., 2021).

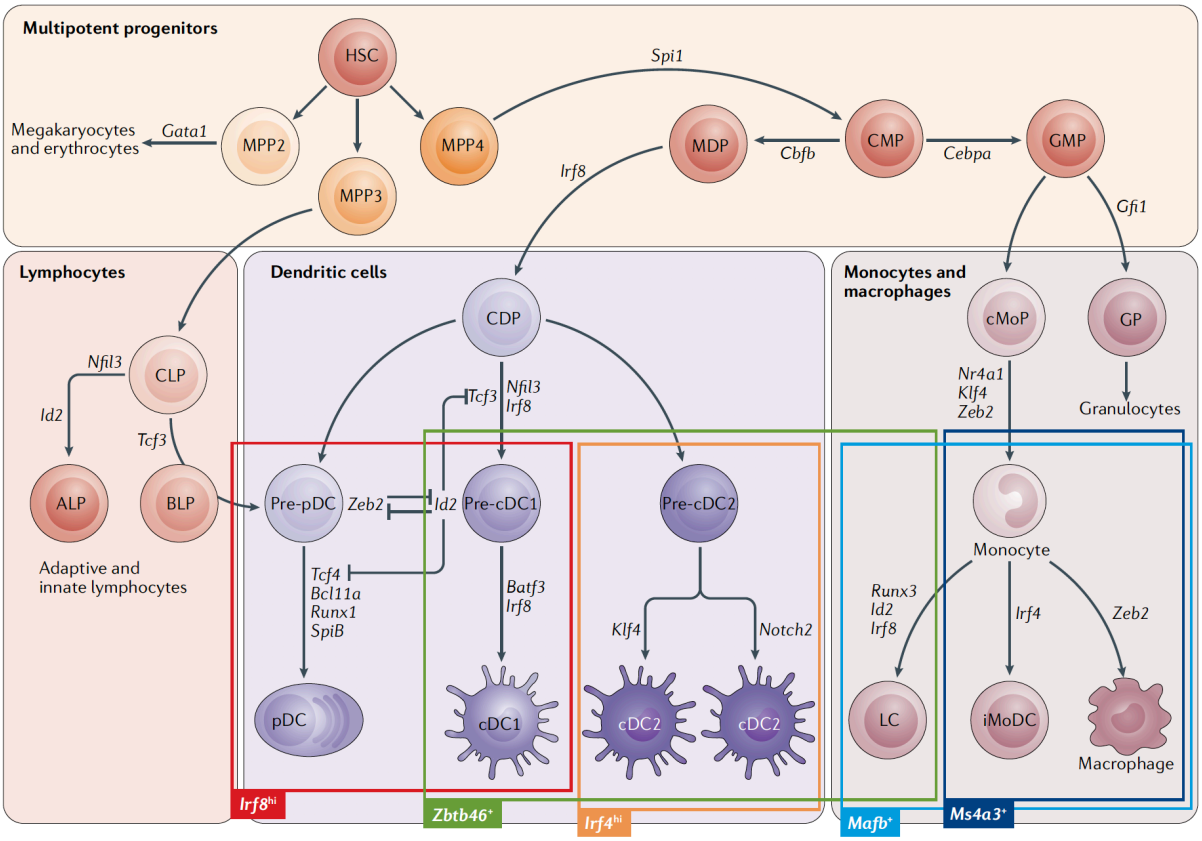

Figure 1.5. Model for murine DC development. In the BM, DC develop in a step-wise manner from HSC and have distinct transcription factor requirements and lineage restriction. cMoP, common monocyte progenitor; GMP, granulocyte-macrophage progenitor; GP, granulocyte progenitor; iMoDC, immature monocyte-derived DC; MDP, monocyte-DC progenitor. Image from (Anderson et al., 2021).

2.3.2. *Dendritic Cell Subsets*

The DC compartment is traditionally divided into three main populations: conventional DC (cDC; also referred to as classical or myeloid DC) comprising DC1 and DC2 subsets, plasmacytoid DC (pDC), and monocyte-derived DC (moDC) (Eisenbarth, 2019; Martin Guilliams et al., 2016; Guilliams et al., 2014; Miriam Merad, Priyanka Sathe, Julie Helft, Jennifer Miller, & Arthur Mortha, 2013; Mildner & Jung, 2014; Murphy et al., 2016). While these DC subsets derive from a common precursor, they have distinct developmental requirements, phenotypic traits, and functional specialization.

DC are canonically identified by their expression of CD45, MHC-II, and CD11c and the absence of all other immune cell lineage markers (M. Merad, P. Sathe, J. Helft, J. Miller, & A. Mortha, 2013). While the combination of these markers is sufficient to broadly capture all DC, it is limited in its specificity and will also result in contaminating myeloid cells, such as macrophages, which also express MHC-II and CD11c. Fortunately, greater specificity can be attained through the use of DC subset-specific markers or transcriptional signatures, as described below (Anderson et al., 2021; Guilliams et al., 2014; M. Merad et al., 2013).

cDC are driven by the transcription factor Zbtb46 and can be identified by their specific expression of CD26 (M. Guilliams et al., 2016; Murphy et al., 2016; Ansuman T. Satpathy et al., 2012). They comprise both DC1 and DC2 subsets and are generally thought of as the critical APC population required for immunosurveillance and induction of T cell immunity. DC1 are uniquely identified by their expression of CD8α in lymphoid tissues or CD103 in non-lymphoid tissues and by their expression of Clec9a and Xcr1 (Edelson et al., 2010; Hildner et al., 2008). They require the transcription factors Batf3 and IRF8 for their development. DC1 are highly adept at cross-presentation, a process whereby they present exogenously derived antigens on MHC-I complexes which enables them to directly interact with $CD8^+$ T cells (further discussed in **Section 2.4.3b**) (Hildner et al., 2008). For this reason, DC1 are characterized as robust $CD8^+$ T cell activators. Studies using $Batf3^{-/-}$ mice have supported this notion, revealing failed induction of $CD8^+$ T cell responses against viruses and cancer in the absence of DC1 (Hildner et al., 2008).

While the phenotype and functions of DC1 are relatively well-defined, the same cannot be said for DC2, which loosely refers to cDC expressing the markers CD11b and

Sirpα (M. Guilliams et al., 2016; Murphy et al., 2016; Ansuman T. Satpathy et al., 2012). DC2 comprise a heterogenous population of cells that have a limited ability to prime $CD8^+$ T cells but can robustly stimulate different $CD4^+$ helper T cell (T_H) responses. DC2 require the transcription factor IRF4 for development (Gao et al., 2013; Krishnaswamy et al., 2017; Tamura et al., 2005; Tussiwand et al., 2015; Williams et al., 2013), though there is evidence that IRF4-independent DC2 also exist (A. T. Satpathy et al., 2013). IRF4-driven DC2 can be further subdivided to KLF4-dependent DC2 and Notch2-dependent DC2, which are required for mounting T_H2 and T_H17 responses, respectively (Murphy et al., 2016; Nutt & Chopin, 2020).

pDC are defined by their expression of B220, Siglec-H, and Bst2, and they require the transcription factors IRF8, E2-2, and TCF4 for their development (Reizis, 2019). Compared to cDC, pDC express lower levels of MHC-II and are therefore unlikely to directly contribute to antigen-presentation and T cell priming (M. Merad et al., 2013). However, they are known to secrete large amounts of type-I-interferon (IFN-I) upon sensing foreign nucleic acids and thus play prominent roles in the setting of viral infection (Reizis, 2019).

MoDC are differentiated from monocytes that are recruited to sites of inflammation (Briseño et al., 2016; León, López-Bravo, & Ardavín, 2007; Menezes et al., 2016; Serbina, Salazar-Mather, Biron, Kuziel, & Pamer, 2003). While CD209a (DC-SIGN) has been reported as a specific marker for moDC, they remain difficult to distinguish from DC2 due to the high number of overlapping markers such as CD11b and Sirpα. Like cDC, moDC have been described as having the ability to prime T cell responses. However, a recent report suggests that this activity might actually be attributable to contaminating DC2

(Bosteels et al., 2020). Careful separation of moDC from cDC in this study revealed that moDC were poorly stimulatory and lacked the ability to migrate to secondary lymphoid organs (Bosteels et al., 2020).

2.3.3. *Functional States of Dendritic Cell Subsets*

Recent DC profiling studies indicate that the above-described DC subsets are generally conserved across species (Gerhard, Bill, Messemaker, Klein, & Pittet, 2021; Zilionis et al., 2019) and can be found infiltrating solid tumors (Broz et al., 2014; Laoui et al., 2016). To add further complexity, however, DC subsets can exist in distinct functional states depending on the inflammatory context. Activated mature cDC in peripheral tissues express higher levels of MHC-II and CD40, CD80, and CD86 costimulatory molecules. They also upregulate expression of the CCR7 chemokine receptor which binds to the lymphoid chemokines CCL19 and CCL21, thus entering a migratory state. For this reason, these DC are often referred to as “migratory DC” though the term “DC3” has also been used interchangeably (Zilionis et al., 2019). It was recently reported that activated cDC in lung tumors expressed an immunoregulatory program characterized by PD-L1, PD-L2, and IL-4Rα that dampened their ability to activate T cells (Maier et al., 2020). While the transcriptional profile of this population resembles that of migratory DC, they were referred to as “mature DC enriched in immunoregulatory molecules” (mregDC) to better reflect the immunoregulatory functions of these DC (Maier et al., 2020). The impact of distinct DC activation states on T cell responses warrant further investigation.

2.4. Dendritic Cell Functions

2.4.1. *Antigen Uptake*

As sentinel cells, DC utilize several distinct routes to take up antigens from their surroundings. Three main avenues of antigen internalization by DC include macropinocytosis, phagocytosis, and receptor-mediated endocytosis.

Macropinocytosis is a non-specific process of antigen uptake wherein large amounts of extracellular fluid are internalized by cells through the actin-dependent formation of vesicles called macropinosomes (Norbury, 2006). Macropinosomes can range in size from 0.2 to 5 μm. While other myeloid cells such as macrophages require the addition of exogenous stimuli (various growth factors or phorbol esters) to trigger macropinocytosis (Racoosin & Swanson, 1989, 1992), DC are able to do so constitutively (Sallusto, Cella, Danieli, & Lanzavecchia, 1995). Thus, the mechanism driving macropinocytosis by DC is likely to be distinct from other myeloid cells.

Phagocytosis refers to the uptake of particles that are usually larger than 0.5 μm such as dead cells and microbial pathogens (Uribe-Querol & Rosales, 2020). Unlike macropinocytosis, phagocytosis entails the internalization of specific antigens, and this specificity is governed by binding to antigen receptors. DC and other phagocytic cells express a variety of cell surface receptors to detect and eliminate foreign substances (Savina & Amigorena, 2007). These are typically categorized as non-opsonic and opsonic receptors (Uribe-Querol & Rosales, 2020). Non-opsonic receptors directly bind to pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP) and thus induce phagocytosis (Uribe-Querol & Rosales, 2020). They include C-type lectin family members, such as Dectin-1 and DC-SIGN, which recognize

glycosylated antigens on microorganisms (Uribe-Querol & Rosales, 2020). They also include receptors, such as TIM-1, TIM-4, CD36, and $\alpha_V\beta5$, which recognize phosphatidyl serine on the membranes of apoptotic cells. In contrast, opsonic receptors bind to particulate matter that are coated by opsonins, which are proteins that promote phagocytosis (Uribe-Querol & Rosales, 2020). Examples of opsonic receptors include Fc receptor or complement receptors, which bind to the Fc portion of antibodies and complement components, respectively (Uribe-Querol & Rosales, 2020). Binding of target particles to the phagocytic receptors induces the actin-dependent formation of pseudopodia which surrounds the particle, eventually fusing to form an intracellular vesicle called a phagosome (Uribe-Querol & Rosales, 2020). Phagosomes then fuse with lysosomes to form the phagolysosome where the contents are enzymatically degraded.

Similar to phagocytosis, receptor-mediated endocytosis also involves the internalization of specific antigens via receptor binding (Burgdorf & Kurts, 2008). However, in the case of receptor-mediated endocytosis, the antigens are typically soluble macromolecules such as metabolites, hormones, and proteins. For example, a prominent endocytic receptor in the innate sensing of tumor cells is the Clec9a receptor (also known as DNGR-1), which binds the filamentous form of actin (F-actin) that is exposed on damaged or dead cells (Canton et al., 2021; Cueto, del Fresno, & Sancho, 2020; Sancho et al., 2009; Sancho et al., 2008). Receptor-mediated endocytosis is generally dependent upon the formation of small clathrin-coated pits in the plasma membrane, though clathrin-independent endocytic pathways (i.e. via caveolins) also exist (Kaksonen & Roux, 2018). Once the pits are sufficiently invaginated, the plasma membrane then pinches together and forms clathrin-coated vesicles that are 60 to 120 nm in size (Kaksonen & Roux, 2018).

The subsequent uncoating of clathrin then enables these internalized vesicles to fuse with other nearby vesicles or endosomes for degradation or further trafficking (Kaksonen & Roux, 2018).

The ability to sample and internalize antigens is primarily a feature of immature DC. Upon maturation **(discussed in Section 2.4.2c)**, studies have shown that DC restrict their antigen uptake activities and increase the expression of surface MHC molecules to favor antigen-presentation. From a theoretical perspective, limiting antigen uptake post-maturation enables the DC to focus on presenting those antigens (typically DAMP or PAMP danger signals) that initially induced their activation so that they can effectively prime T cell responses against these foreign threats. The restricted ability of mature DC to internalize antigens can be attributed to two mechanisms: (1) the decreased expression of antigen receptors and (2) reduced levels of phagocytosis and macropinocytosis (Garrett et al., 2000; West, Prescott, Eskelinen, Ridley, & Watts, 2000). Studies have demonstrated a role for the Rho family GTPases, Cdc42 and Rac1, in mediating antigen internalization but the data around their contribution to regulating internalization levels is conflicting (Chi, Wang, Huang, Stamnes, & Chen, 2013; Garrett et al., 2000; West et al., 2000).

2.4.2. *Innate Immune Sensing*

As initiators of immunity, DC exert tremendous influence on the outcome of immune responses, instructing whether an immunogenic or tolerogenic adaptive immune response should be induced against a given antigen. This decision-making is dependent

on the environmental context where DC first encounter the antigen and involves DC-intrinsic sensing of IFN-I and danger signals via pattern recognition receptors (PRR).

2.4.2a. *Type-I-Interferon Sensing*

At steady-state, DC are described as immature and are characterized by low levels of MHC-II and costimulatory molecules. Immature DC in normal or healthy conditions have continuous endocytic activity, and they internalize and present predominantly self-derived antigens (Dalod, Chelbi, Malissen, & Lawrence, 2014; Dudek, Martin, Garg, & Agostinis, 2013; Mellman, 2013). It has been reported that some fraction of immature DC can undergo “partial” or “homeostatic” maturation (Lutz & Schuler, 2002), wherein they upregulate MHC-II molecules but not costimulatory molecules and can migrate to draining LN in a CCR7-dependent manner (Ohl et al., 2004). This homeostatic maturation program drives tolerogenic DC, however, and their main function is to tolerize T cells towards self-antigens (Dalod et al., 2014; Probst, Lagnel, Kollias, & van den Broek, 2003). Thus, the role of immature DC can be perceived as a safeguard against autoimmunity to suppress self-reactive T cells that may have escaped central tolerance (Dalod et al., 2014; Dudek et al., 2013; Lutz & Schuler, 2002). It comes as no surprise then that active suppression of DC maturation is frequently reported as a mechanism of immune evasion by tumors (Dudek et al., 2013).

A long-standing conundrum in the cancer immunology field has been how DC can induce immunogenic anti-tumor T cell responses given the lack of pathogenic factors in cancer. Clinical studies in metastatic melanoma initially established a link between the presence of an IFN-I gene signature and a T cell-inflamed TME (Harlin et al., 2009). IFN-

I are pleiotropic cytokines that are produced by somatic cells upon engagement of PRR which sense various danger signals. In mice, canonical IFN-I comprise 14 subtypes of IFNα and IFNβ1; other forms of IFN-I include IFN-ω, IFN-τ, IFN-δ, IFN-κ, and IFN-ε (Musella, Manic, De Maria, Vitale, & Sistigu, 2017). All of these IFN-I subtypes signal through a common heterodimeric IFNAR receptor via the JAK-STAT pathway, albeit with differential signaling strengths, leading to the production of more IFN-I and other interferon-stimulated genes (ISG) that modulate immune function (Cheon, Borden, & Stark, 2014; Schneider, Chevillotte, & Rice, 2014). IFN-I, specifically IFNα, was first discovered by Alick Isaacs and Jean Lindenmann in 1957 as an anti-viral agent (Isaacs & Lindenmann, 1957; Isaacs et al., 1957). The anti-cancer effects were not realized until years later in 1969 when Ion Gresser published a seminal report demonstrating the efficacy of IFN-I in controlling tumors in mice (Gresser & Bourali, 1969). This work and others paved the way for clinical studies, leading to FDA approval of IFNα in 1986 for the treatment of hairy cell leukemia (Golomb et al., 1986). Due to the toxicities associated with repeated high dose administration, enthusiasm for IFNα as a cancer treatment subsided rather quickly. This was in part driven by a lack of understanding of the mechanism of IFN-I (Aricò, Castiello, Capone, Gabriele, & Belardelli, 2019). As anti-cancer agents, IFN-I were initially thought to control tumors via their direct anti-proliferative and anti-angiogenic effects. Work from the past decade, however, has revealed that IFN-I also has a critical role in the innate immune sensing of cancer. A 2005 study demonstrated that IFN-I sensing by hematopoietic cells was required for protective anti-tumor responses against immunogenic tumors (Dunn et al., 2005). This was followed by two independent studies in 2011 showing that IFN-I sensing specifically in the DC

compartment was required for optimal anti-tumor $CD8^+$ T cell priming and the rejection of immunogenic tumors (Diamond et al., 2011; Fuertes et al., 2011).

2.4.2b. *Pattern Recognition Receptor Activation*

IFN-I can be produced by virtually all nucleated cells downstream of PRR activation. In the tumor immunology field, it is believed that STING-mediated cytosolic DNA-sensing in innate immune cells is the critical PRR pathway driving strong IFN-I responses (Corrales et al., 2017). In this pathway, binding of dsDNA to cyclic-GMP-AMP (cGAMP) synthase (cGAS) in the cytosol catalyzes the production of the endogenous STING ligand 2'3'-cGAMP. Binding of cGAMP to STING, which is localized on the endoplasmic reticulum (ER) membrane, induces conformational changes in STING that results in its translocation from the ER to the Golgi to recruit and activate TANK-binding kinase 1 (TBK1) (Corrales et al., 2017; Yu & Liu, 2021). TBK1 phosphorylates STING, which recruits IRF3 for phosphorylation by TBK1 (Corrales et al., 2017; Yu & Liu, 2021). Phosphorylated IRF3 then dimerizes and enters the nucleus to activate IFN-I transcription (Corrales et al., 2017; Yu & Liu, 2021). Mice that lack STING or IRF3 have been reported to have defective T cell priming against tumors due to the inability to drive strong IFN-I responses (Woo et al., 2014). Thus, STING agonism presents a promising therapeutic avenue to drive an IFN-I response in DC and thereby enhance anti-tumor $CD8^+$ T cell priming (Corrales et al., 2015; Iurescia, Fioretti, & Rinaldi, 2018).

In addition to STING pathway engagement, IFN-I can also be induced by the activation of other PRRs. Tumor-derived dsRNA, when taken up by DC, can trigger IFN-I production via binding to endocytic TLR3 or cytosolic RIG-I/MDA5 sensors (Corrales et

al., 2017). Two relatively recent independent studies demonstrated that tumor cell-intrinsic loss of RNA-editing enzymes such as ADAR1 in tumors leads to accumulation of dsRNA that induces a more inflamed TME via cytosolic dsRNA sensing (Ishizuka et al., 2019; H. Liu et al., 2019). This induced vulnerability can be harnessed to improve responses to immunotherapy and overcome CBT resistance (Ishizuka et al., 2019; H. Liu et al., 2019). Based on these findings, RIG-I and MDA5 agonists present another rational therapeutic opportunity to enhance innate immune sensing of tumors by DC (Elion & Cook, 2018; Iurescia et al., 2018; Iurescia, Fioretti, & Rinaldi, 2020; Y. Wu, Wu, Wu, Wang, & Liu, 2017). Beyond nucleic acid sensing, TLR4 sensing of high mobility group box 1 (HMGB1), a nuclear non-histone chromatin-binding protein released by necrotic cells, is another mechanism of innate immune activation against tumors (Corrales et al., 2017). However, there are conflicting studies on whether HMGB1 sensing activates or suppresses DC (Corrales et al., 2017), and thus, more investigation is needed.

2.4.2c. *Dendritic Cell Maturation*

Innate immune sensing of tumors via any of these PRR pathways triggers a process called DC maturation. DC maturation encompasses a number of phenotypic and functional changes that collectively enhance the ability of DC to induce an immunogenic T cell response. Phenotypically, mature DC are characterized by increased expression of MHC molecules, costimulatory molecules, such as CD80, CD86, and CD40, as well as the chemokine receptor CCR7 that is required for trafficking to the draining LN (Dalod et al., 2014; Dudek et al., 2013). Functionally, mature DC have reduced endocytic activities, enhanced antigen-processing and presentation abilities, and can secrete pro-

inflammatory cytokines (i.e. IL-12, IFN-I) and chemokines (i.e. CXCL9, CXCL10) (Dalod et al., 2014).

2.4.3. *Antigen Processing and Presentation*

DC communicate with T cells by presenting peptides derived from internalized antigens in the context of MHC. These pMHC complexes are recognized by the TCR of T cells and serves as the antigen-specific signal (signal 1) that is required for T cell activation **(also discussed in Section 2.4.4)** (Smith-Garvin et al., 2009). The TCR of cytotoxic $CD8^+$ T cells bind to peptides presented in the context of MHC class I (MHC-I) complexes, whereas the TCR of $CD4^+$ T cells recognize peptides bound to MHC class II (MHC-II) complexes (Smith-Garvin et al., 2009). Thus, antigen presentation to $CD8^+$ T cells or $CD4^+$ T cells occurs in two distinct pathways that is dictated by whether the antigen was derived from an endogenous or exogenous source.

Endogenously sourced antigens (i.e. self-proteins or viral proteins from infected cells) are processed through the endogenous pathway and presented on MHC-I molecules for recognition by $CD8^+$ T cells **(Figure 1.6A)** (Vyas, Van der Veen, & Ploegh, 2008). Briefly, ubiquitinylated intracellular proteins are cleaved into long peptides in the cytosol by the proteasome (or immunoproteasome under conditions of IFNγ exposure). These peptides are then transferred into the ER via the transporter associated with antigen processing (TAP), where they can be further trimmed by the ER aminopeptidase associated with antigen processing (ERAAP) (Smith-Garvin et al., 2009). Trimmed peptides, usually 8-9 amino acids long, are then loaded onto MHC-I complexes in the peptide-loading complex (PLC), which is composed of calreticulin, tapasin, and ERp57

(Smith-Garvin et al., 2009). Peptide-bound MHC-I complexes are stabilized and are thus released from the PLC and transported to the cell surface via the Golgi apparatus for presentation (Smith-Garvin et al., 2009). While all nucleated cells are capable of presenting endogenous antigens via this pathway, only DC can initiate a T cell response with their pMHC-I complexes **(discussed in Section 2.4.3a)**.

Exogenously sourced antigens are processed through the exogenous pathway and presented in the context of MHC-II molecules for recognition by $CD4^+$ T cells (Smith-Garvin et al., 2009). Briefly, internalized antigens are degraded in endosomes and lysosomes by acid-dependent proteases such as cathepsins (Smith-Garvin et al., 2009). Vesicles containing MHC-II complexes that were synthesized in the ER and stabilized by binding to an invariant chain (Ii) fuse with late endosomes and lysosomes (Smith-Garvin et al., 2009). The Ii chain in the MHC-II complex is then degraded and replaced by a peptide (20+ amino acids long) from the endosome or lysosome. The pMHC-II complexes are then presented on the surface (Smith-Garvin et al., 2009). Whereas all nucleated cells can present endogenous antigens via the MHC-I pathway, only professional antigen-presenting cells (DC, macrophages, B cells) can present internalized antigens on MHC-II complexes via the exogenous pathway to elicit $CD4^+$ T cell responses (Smith-Garvin et al., 2009).

2.4.3a. *Antigen Presentation in the Tumor Context*

In the cancer setting, the induction of $CD8^+$ T cell responses against exogenous tumor antigens is critical for anti-tumor immunity. This presents a conundrum wherein exogenously derived tumor antigens need to be presented in the context of MHC-I

molecules in order to mount tumor-reactive $CD8^+$ T cell responses. Through a specialized process termed cross-presentation, DC have the ability to efficiently present exogenous antigens on MHC-I complexes to activate $CD8^+$ T cell responses **(Figure 1.6B)** (Embgenbroich & Burgdorf, 2018). It is for this reason that DC are believed to be the most critical antigen-presenting cell type in inducing $CD8^+$ T cell immunity. While there is no doubt that cross-presentation is integral for mounting $CD8^+$ T cell responses against exogenous antigens, an alternative pathway exists that is termed cross-dressing (Nakayama, 2015). While cross-presentation entails antigen presentation by host cells using endogenously derived MHC-I complexes, cross-dressing refers to antigen-presentation by host cells using donor cell-derived pMHC-I complexes **(Figure 1.6C)** (Nakayama, 2015). As cross-presentation and cross-dressing are the most relevant pathways for inducing anti-tumor $CD8^+$ T cell responses in the context of cancer, the following section will focus on discussing the existing evidence for these two pathways.

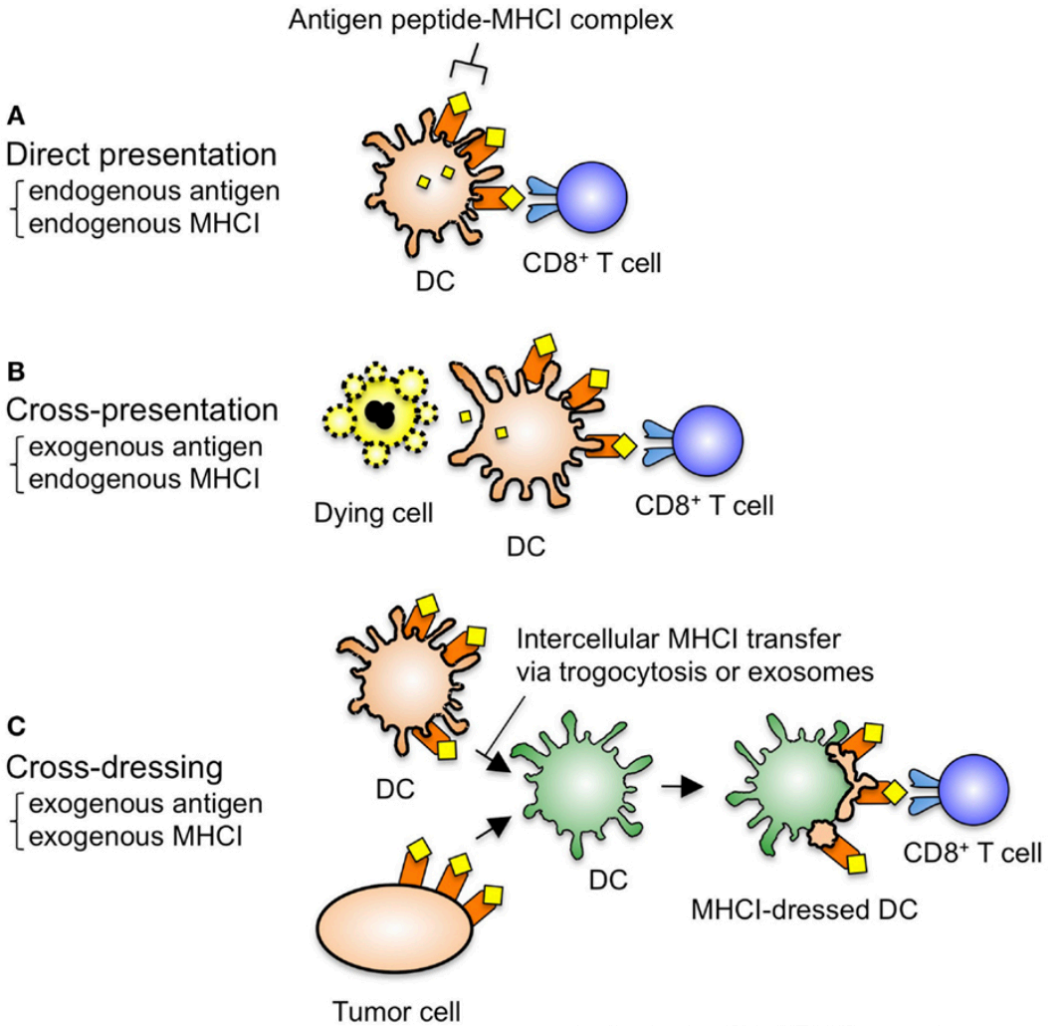

Figure 1.6. Mechanisms of antigen-presentation by DC to activate CD8⁺ T cells. (A) In direct-presentation, DC present endogenously derived antigens (i.e. self antigens or viral antigens in infected cells) on endogenous MHC-I to activate CD8⁺ T cells. **(B)** In cross-presentation, DC present exogenously derived antigens (i.e. dead cell debris) on endogenous MHC-I to activate CD8⁺ T cells. **(C)** In cross-dressing, DC acquire and present exogenous pMHC-I derived from adjacent cells to activate CD8⁺ T cells. Image adapted from (Nakayama, 2015).

2.4.3b. *Cross-Presentation*

Cross-presentation refers to the process by which exogenously derived antigens (i.e. cell-associated antigens) are processed and presented on MHC-I molecules. The phenomenon of cross-presentation was first reported in 1976 in graft rejection studies focusing on CD8⁺ T cell responses against minor histocompatibility antigens (Bevan,

1976). Minor histocompatibility antigens are small MHC-bound peptides that can elicit immunogenic responses in mice with different genetic backgrounds. Engraftment of splenocytes derived from donor C57BL/10 mice with MHC haplotype H-2^b into recipient Balb/c mice with MHC haplotype H-2^{dxb} (expressing both H-2^d and H-2^b MHC molecules) resulted in induction of $CD8^+$ T cell responses against C57BL/10 antigens presented in the context of H-2^d MHC molecules (Bevan, 1976). This observation suggested that minor histocompatibility antigens from transferred C57BL/10 splenocytes were internalized by host Balb/c DC, processed, and "cross-presented" in the context of the host's endogenous H-2^d MHC molecules (Bevan, 1976). The activation of $CD8^+$ T cell responses against cross-presented antigens is thus referred to as "cross-priming."

There is significant debate over the molecular mechanism(s) by which cross-presentation occurs. Two models for cross-presentation have been proposed: (1) the endosome-to-cytosol or cytosolic diversion pathway and (2) the vacuolar pathway **(Figure 1.7)** (Joffre, Segura, Savina, & Amigorena, 2012). In the endosome-to-cytosol pathway, internalized antigens are released from endosomes into the cytosol where they are cleaved by the proteasome, thus entering the endogenous MHC-I processing pathway as described earlier (Joffre et al., 2012). In the vacuolar pathway, antigens are degraded by cathepsins in the endolysosomal compartment (Joffre et al., 2012). Following fusion with vesicles containing MHC-I molecules, which can be newly synthesized from the ER or recycled from the cell surface, the peptides are loaded onto MHC-I molecules and subsequently transported to the cell surface for presentation. The relative contribution of the endosome-to-cytosolic and vacuolar pathways to cross-presentation is unclear, particularly *in vivo*. However, most studies report cross-presentation in the context of the

endosome-to-cytosol pathway, suggesting that this may be the more dominant pathway of cross-presentation. In support of this notion, $Tap^{-/-}$ mice which are unable to transfer peptides to the ER for MHC-I loading (via the endosome-to-cytosol pathway) have been shown to exhibit defective cross-priming responses against tumor antigens (Huang, Bruce, Pardoll, & Levitsky, 1996), thus indicating that this pathway is used for cross-presentation *in vivo*.

Cross-presentation efficiency is dictated by several factors. One significant factor is peptide stability during antigen-processing, given that overly rapid degradation by lysosomal proteases can destroy antigens before they can be loaded onto MHC-I molecules. Thus, having a slower degradation rate can help preserve antigens and facilitate their cross-presentation. Perhaps it is for this reason that DC are regarded as the most proficient cross-presenting cells. Compared to macrophages, DC have lower levels of lysosomal proteases such as cathepsins (Delamarre, Pack, Chang, Mellman, & Trombetta, 2005) and reduced kinetics of endosome maturation (Lennon-Duménil et al., 2002). Furthermore, different mechanisms have been reported whereby DC can prevent the acidification of endosomes to blunt the function of lysosomal proteases which are activated at acidic pH (Mantegazza et al., 2008; Savina et al., 2006).

While DC are generally recognized for their ability to cross-present antigens, the efficiency of cross-presentation varies quite considerably by DC subset. DC1 are the most potent cross-presenting DC subset in mice. This is supported by *in vivo* studies in $Batf3^{-/-}$ mice which demonstrate that in the absence of DC1, there is defective priming of $CD8^{+}$ T cell responses against viruses and immunogenic tumors (Hildner et al., 2008). Studies assessing the *ex vivo* cross-presentation ability of DC subsets isolated from murine

secondary lymphoid organs further confirm that DC1 are the most efficient cross-presenting DC subset. The ability of DC1 to cross-present is regulated by WD repeat- and FYVE domain-containing protein 4 (WDFY4), which is believed to be involved in endocytic vesicular targeting and localization, though the specific molecular mechanism has yet to be described (Theisen et al., 2018). Wdfy4$^{-/-}$ mice failed to mount CD8^{+} T cell responses against viruses and immunogenic tumors despite the presence of DC1 (Theisen et al., 2018). Interestingly, while WDFY4 was critical for DC1-mediated cross-presentation, it was dispensable for cross-presentation by moDC (Theisen et al., 2018). This observation suggests that different cell types may utilize distinct, non-overlapping mechanisms of cross-presentation to elicit CD8^{+} T cell responses, which may in part account for the range of cross-presentation efficiencies observed by different DC subsets. Thus, the contribution of different DC subsets to cross-presentation and cross-priming of CD8^{+} T cell responses likely varies depending on context and requires further investigation.

The route of antigen internalization can impact the likelihood that a given antigen is cross-presented (Blander, 2018; Burgdorf, Kautz, Böhnert, Knolle, & Kurts, 2007; Burgdorf & Kurts, 2008). Receptor-mediated endocytosis is generally believed to be more efficient than macropinocytosis or phagocytosis for cross-presentation, perhaps because endocytic receptors directly deliver antigens into early endosomes (Blander, 2018; Burgdorf et al., 2007; Burgdorf, Lukacs-Kornek, & Kurts, 2006; Moeller, Spagnoli, Finke, Veelken, & Houet, 2012). As mentioned earlier, in the tumor immunology field, Clec9a (DNGR-1) is believed to be critical for cross-presentation of tumor cell-associated antigens by DC1 (Canton et al., 2021; Cueto et al., 2020; Sancho et al., 2009; Sancho et

al., 2008). Signaling through Clec9a promotes rupture of endocytic vesicles, which enables the escape of vesicle contents into the cytosol for proteasome processing and MHC-I loading through the endocytic pathway (Canton et al., 2021). Targeting antigens to endocytic receptors such as Clec9a to promote their likelihood of being cross-presented is a therapeutic vaccination strategy for improving cross-priming responses (Caminschi, Maraskovsky, & Heath, 2012; Kreutz, Tacken, & Figdor, 2013; van Dinther et al., 2017).

There is increasing evidence that the specific context of antigen acquisition can also impact cross-presentation efficiency. For example, internalization of antigens derived from cells that underwent immunogenic cell death (i.e. necrotic cells) versus non-immunogenic cell death (i.e. apoptotic cells) are more likely to be accompanied by sensing of danger signals that will drive maturation of DC (Moretti & Blander, 2014). Innate immune sensing pathways and DC maturation were discussed in **Section 2.4.2**. DC maturation favors antigen presentation over antigen uptake, and so, this may also positively regulate cross-presentation.

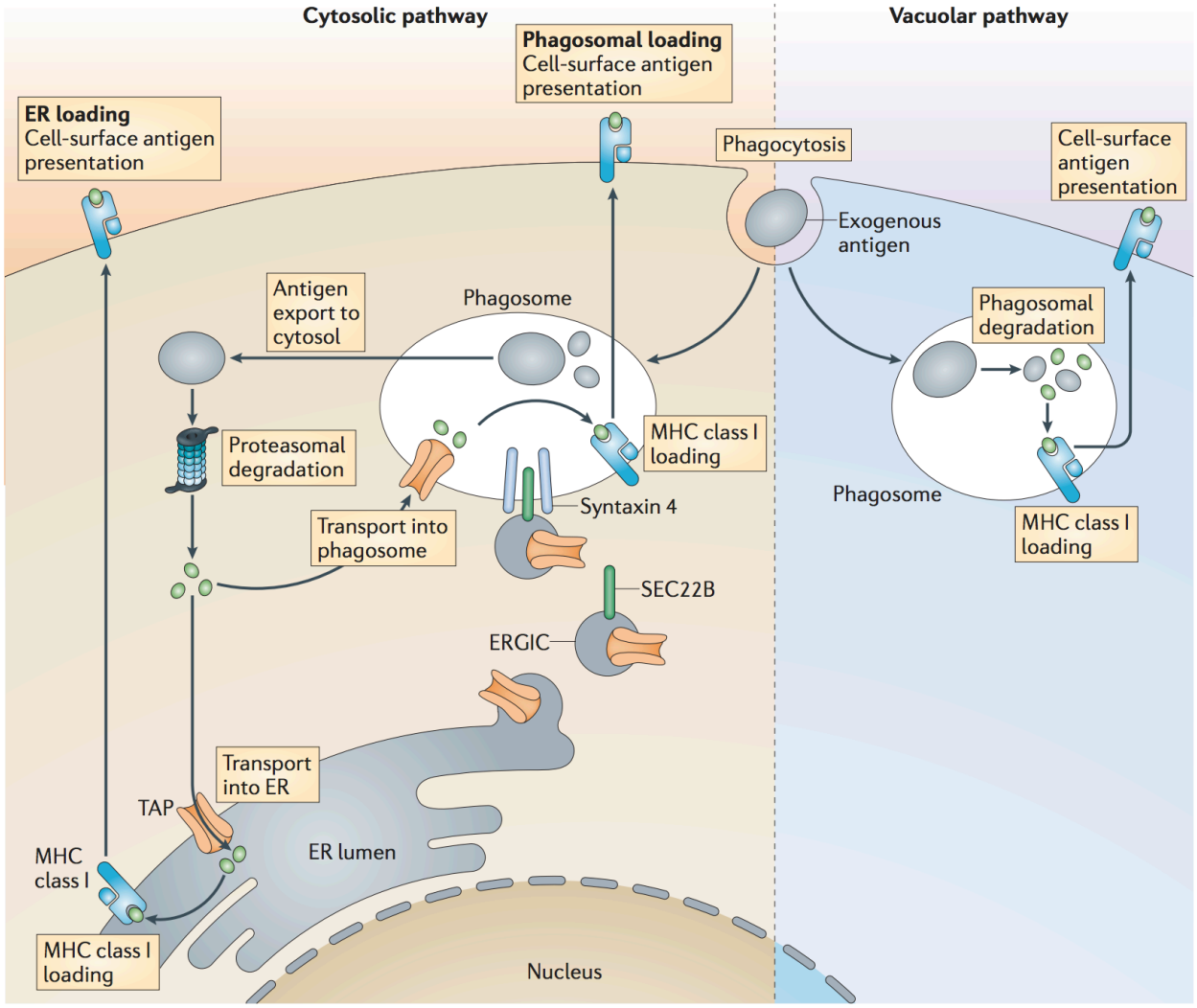

Figure 1.7. Cross-presentation pathways in DC. Two models for cross-presentation have been proposed. The cytosolic pathway *(left)* involves antigen export from phagosomes into the cytosol for processing and MHC-I loading via the endogenous pathway. The vacuolar pathway *(right)* entails processing and MHC-I loading in phagosomes. Image from (Joffre et al., 2012).

2.4.3c. *Cross-Dressing*

Cross-dressing as a form of antigen-presentation to $CD8^+$ T cells entails the acquisition and display of preformed pMHC-I complexes without the need for further processing. It was first described by Dolan *et al.* in the context of *in vitro* cocultures of MHC-mismatched cells (Dolan, Gibbs, & Ostrand-Rosenberg, 2006). When BM-DC derived from FVB mice with the MHC haplotype $H\text{-}2^q$ were cocultured with fibroblasts or

tumor cells with a different MHC haplotype (i.e. H-2^b), transfer of the H-2^b molecules could be detected on the surface of the H-2^q BM-DC (Dolan et al., 2006). Modifying the assay system such that the donor cells expressed the model T cell antigen OVA, the authors showed that H-2^q DC cross-dressed with H-$2K^b$:SIIN complexes were able to induce OTI T cell proliferation both *in vitro* and *in vivo* (Dolan et al., 2006). Wakim and Bevan demonstrated that cross-dressing could occur between DC and virally infected cells and thus contribute to priming of anti-viral $CD8^+$ T cells (Wakim & Bevan, 2011). Interestingly, cross-dressed DC from the Wakim and Bevan study were able to stimulate memory $CD8^+$ T cells but failed to activate naïve $CD8^+$ T cells, indicating that their main function may not reside in priming but rather in restimulating effector T cell responses (Wakim & Bevan, 2011). There is also increasing evidence that cross-dressed DC can contribute to the priming of anti-tumor $CD8^+$ T cell responses (Das Mohapatra et al., 2020; Squadrito, Cianciaruso, Hansen, & De Palma, 2018).

While the specific mechanism of cross-dressing remains elusive, there are two prevailing lines of thought, namely that the transfer of pMHC-I occurs during membrane exchange in a contact-dependent or contact-independent fashion. In the Dolan *et al.* and Wakim and Bevan studies discussed earlier, cross-dressing by DC required direct cell-to-cell contact (Dolan et al., 2006; Wakim & Bevan, 2011). Physically separating the DC from the donor cells via a transwell ablated the acquisition of pMHC-I by DC (Dolan et al., 2006; Wakim & Bevan, 2011). Membrane transfer resulting from direct cell-to-cell contact is commonly referred to as trogocytosis or cell nibbling, and is often accompanied by the transfer of cell surface molecules (Miyake & Karasuyama, 2021). Trogocytosis is not restricted to DC, and studies have shown that it can occur between many immune cell

types including T cells, APC, and natural killer cells (Miyake & Karasuyama, 2021; Nakayama, 2015; Nakayama, Hori, Toyoura, & Yamaguchi, 2021). While diverse immune cell types are able to engage in cross-dressing by trogocytosis, evidence suggests that only DC are able to induce T cell responses against antigens acquired by cross-dressing (Dolan et al., 2006). In the contact-independent mode, cross-dressing is mediated by the acquisition of exosome-like extracellular vesicles (EV) that contain pMHC-I. EV are secreted by many cell types and can be thought of as a form of communication between cells wherein proteins, nucleic acids, carbohydrates, and lipids are exchanged (Zeng & Morelli, 2018). DC cocultured with EV have been shown to elicit T cell activation *in vitro*. However, it is unknown how this occurs mechanistically. One hypothesis is that EV are simply attached to the DC cell surface and oriented in a way such that the pMHC-I complexes are accessible to T cells. Another hypothesis suggests the possibility of a bona fide fusion event between the membranes of EV and DC, wherein the EV becomes integrated into DC plasma membrane. Furthermore, the transfer of intracellular vesicles has been recently reported as a mechanism of antigen transfer between DC and is also accompanied by the transfer of pMHC-I to recipient DC (Ruhland et al., 2020). The surface presentation of pMHC-I derived from intracellular vesicles would suggest that some form of MHC-I recycling might be involved. More work is needed to understand how cross-dressing occurs mechanistically.

Unlike cross-presentation, which is an inherent function of DC1, the ability to acquire antigens via cross-dressing seems to be context dependent. DC1, DC2, moDC, and pDC have all been reported to partake in cross-dressing in various contexts including vaccination, infection, and cancer (Nakayama, 2015). There are a number of factors that

may mediate the ability of DC subsets to cross-dress. The nature of the donor cells, whether live or dying, is a potential influence, but this point is heavily debated. Dolan *et al.* demonstrated that DC primarily cross-dressed with pMHC-I complexes from dead cells rather than live cells (Dolan et al., 2006). In contrast, a more recent report suggested that cross-dressing is primarily a means of priming T cells against antigens derived from live cells (Das Mohapatra et al., 2020). It is further unknown whether cross-dressing constitutes a specific or non-specific phenomenon and whether it occurs at steady-state *in vivo.*

The contribution of cross-presentation and cross-dressing to the induction of T cell responses also requires more nuanced investigation. There are some reports showing that cross-presentation by DC1 is dispensable for $CD8^+$ T cell priming (Gilfillan et al., 2018; Ma et al., 2013; M. D. Sharma et al., 2018). For these cases, it will be interesting to evaluate whether cross-dressing is involved in mediating those responses. Dolan *et al.* hypothesized that cross-dressing might be particularly relevant for cross-priming of T cell responses when antigen levels are low (Dolan et al., 2006). They demonstrated that cross-dressing could still induce T cell responses against lowly abundant antigens, whereas cross-presentation was less effective (Dolan et al., 2006). It will be interesting to investigate differences in contexts where cross-presentation or cross-dressing may be more useful.

2.4.4. *Antigen Transport and T Cell Priming*

The DC functions described to this point—antigen uptake, innate immune signaling, IFN-I sensing, maturation, and antigen processing and presentation—prepares DC for

their ultimate role as potent T cell activators. In peripheral tissue sites, mature DC that are loaded with antigen upregulate a migratory program (migratory DC) characterized by expression of the G protein-coupled chemokine receptor CCR7, which binds the chemokine ligands CCL19 and CCL21 (J. Liu, Zhang, Cheng, & Cao, 2021; Yan et al., 2019). CCL19 is secreted by lymphoid-resident DC, while CCL21 is secreted by lymphatic endothelial cells (Yan et al., 2019). DC from mice lacking CCL19, CCL21, or CCR7 exhibit impaired trafficking to secondary lymphoid organs, which impacts the induction of adaptive immune responses (Förster et al., 1999; Gunn et al., 1999). Thus, CCL19 and CCL21 are essential for guiding $CCR7^+$ migratory DC from peripheral tissue sites to the draining LN, the main site of T cell priming. In the tumor context, $CCR7^+$ migratory DC are required for trafficking tumor antigen to the draining LN (Roberts et al., 2016). CCR7 expression in human tumors correlates with DC- and T cell-inflamed signatures and improved clinical outcomes (Roberts et al., 2016).

Migratory DC interact with naïve T cells in the T cell-rich zones of the draining LN, delivering three critical signals that are required for optimal T cell activation. Signal 1 is an antigen-specific signal obtained by TCR recognition of its cognate antigen, which is presented in the form of pMHC complexes by DC. This leads to the formation of a stable immunological synapse between the T cell and the DC, which initiates a cascade of intracellular molecular events in T cells known as TCR signaling (Courtney, Lo, & Weiss, 2018). The diversity of signal 1 is reflected not only by the range of antigenic peptides that can be recognized by the TCR, but also by differential signaling strength. Different TCRs have varying affinities for pMHC complexes. While it is generally perceived that a higher affinity TCR-pMHC interaction induces stronger T cell activation, there are many

conflicting reports (Gálvez, Gálvez, & García-Peñarrubia, 2019). Thus, more work is needed to understand the impact of TCR signaling strength on T cell activation. On the DC side, peptide affinity for MHC and the stability of the presented pMHC complexes are additional confounding factors that certainly also contribute to TCR signaling strength and T cell activation.

Signal 1 in the absence of other signals is insufficient to induce immunogenic T cell activation and instead drives a state of T cell inactivation termed anergy as described in **Section 2.1**. To overcome this fate, naïve T cells also require signal 2, which comprises costimulatory signals provided by DC (Smith-Garvin et al., 2009). The binding of CD80 and CD86 costimulatory molecules on mature DC to CD28 receptors on T cells reinforces T cell activation and promotes T cell proliferation, survival, and acquisition of effector function (Esensten, Helou, Chopra, Weiss, & Bluestone, 2016). While CD28 is constitutively expressed on both naïve and activated T cells, other costimulatory receptors (i.e. ICOS, OX40, 4-1BB) are induced following TCR activation (L. Chen & Flies, 2013). Signaling through these costimulatory receptors can fine-tune the phenotype and effector functions of activated T cells, thus diversifying the T cell response. As described earlier in **Section 1.3**, immune inhibitory receptors, such as CTLA-4 and PD-1, are also upregulated in response to T cell activation to counterbalance the immune response (Buchbinder & Desai, 2016). In general terms, the sum of costimulatory and inhibitory signals on T cells dictates their degree of activation.

Signal 3 provides differentiation signals to activated T cells in the form of cytokine signaling (Curtsinger & Mescher, 2010). DC can secrete cytokines, such as IL-12 and IFN-I, that further promotes T cell activation, proliferation, and survival, as well as T cell

acquisition of effector function such as cytolytic ability (i.e. granzymes and perforins) and secretion of pro-inflammatory cytokines (i.e. IFNγ and TNFα) (Thaiss, Semmling, Franken, Wagner, & Kurts, 2011). These cytokine signals can also derive from other cells in the priming environment, such as $CD4^+$ T cells, which are major producers of the T cell growth factor IL-2 and a host of other cytokines that can fine-tune the phenotype and function of activated $CD8^+$ T cells (Smith et al., 2004; Thaiss et al., 2011).

2.5. Dendritic Cells in Cancer

There is insurmountable evidence for the role of DC in driving anti-tumor T cell immunity. Studies from the past decade have established a requirement for the cross-presenting conventional DC subset, Batf3-driven DC1, in the generation of anti-tumor $CD8^+$ T cell responses. $Batf3^{-/-}$ mice lacking DC1 exhibit severe defects in anti-tumor T cell priming and are unable to reject immunogenic syngeneic tumors that are naturally controlled in WT mice (Hildner et al., 2008). Functionally, loss of DC1 effectively ablated cross-presentation of antigens, which is the canonical antigen-presentation pathway required for signal 1 of $CD8^+$ T cell activation (Hildner et al., 2008).

It is now known that the contributions of DC1 to anti-tumor immunity extend beyond T cell priming in the draining LN; they also have functions within the TME. There is evidence that DC1 in tumors can locally re-stimulate effector T cells *in situ* (Broz et al., 2014). A human study showed that stem-like $CD8^+$ T cells, which have self-renewal capacity and give rise to terminally differentiated effector T cells, are highly correlated with the presence of DC in intratumoral niches (Jansen et al., 2019). This observation suggests that intratumoral DC may play a role in maintaining a reservoir of self-renewing

$CD8^+$ T cells. Absence of these intratumoral niches in tumors correlate with reduced T cell infiltration in the tumor and more progressive disease (Jansen et al., 2019). Furthermore, a role for DC1 in effector T cell recruitment to the tumor has been recently reported (S. Spranger et al., 2017). DC1 in tumors are the predominant source of CXCL9 and CXCL10, which are the chemokines that are recognized by the CXCR3 receptor expressed on effector T cells (S. Spranger et al., 2017).

Given their critical contributions to the induction of anti-tumor immunity, it is unsurprising that DC1 exclusion is a mechanism of immune evasion by the tumor. Activated WNT/β-cat signaling in melanoma tumor cells has been shown to drive a non-T cell-inflamed TME and resistance to combination anti-PD-L1/anti-CTLA-4 therapy (S. Spranger, Bao, & Gajewski, 2015). Mechanistically, T cell exclusion was mediated by failed DC1 recruitment to the tumor due to impaired tumor cell-intrinsic CCL4 production (S. Spranger et al., 2015). Restoring DC via intratumoral transfer of Flt3-L-induced bone marrow-derived DC could restore tumor control and sensitivity to anti-PD-L1/anti-CTLA-4 therapy (S. Spranger et al., 2015).

Besides CCL4, the chemokines CCL5 and XCL1 have also been reported to be involved in the recruitment of DC1 and can be produced by natural killer (NK) cells. A study reported that activated COX1/2 signaling in melanoma tumors led to tumor cell-intrinsic production of prostaglandin-E2, which blunted NK cell recruitment to tumors (Böttcher et al., 2018). As NK cells expressed CCL5 and XCL1, this then caused a defect in the recruitment of DC1 to tumors leading to impaired anti-tumor priming and accelerated tumor outgrowth (Böttcher et al., 2018). Another study found that NK cells positively regulated DC1 abundance by producing Flt3-L, a cytokine required for the

development of DC1 (Böttcher et al., 2018). The presence of NK cells and DC1 in human melanoma samples correlated with anti-PD1 responses (Böttcher et al., 2018). Presence of the DC1 signature in tumors is also associated with increased overall survival (Broz et al., 2014). Thus, increasing DC1 numbers or enhancing their function presents a therapeutic strategy to treat cancer. Indeed, a preclinical study using the combination of systemic Flt3-L administration to increase pre-DC1 progenitors and intratumoral delivery of Poly(I:C) to drive their activation was able to enhance responses to anti-PD-L1 therapy in melanoma tumors (Salmon et al., 2016).

However, there are also paradoxical reports of DC1-infiltrated tumors being resistant to CBT. One recent study demonstrated that in the context of lung tumors, antigen uptake by DC1 induces an immunoregulatory program characterized by the expression of PD-L1, PD-L2, and the IL4Rα (Maier et al., 2020). This DC1 state, termed 'mature DC enriched in immunoregulatory molecules' (mregDC1), had dual regulatory and immunogenic roles (Maier et al., 2020). They could drive the differentiation of native T cells into regulatory T cells, but also induce the activation of $CD8^+$ T cells (Maier et al., 2020). Ablating the immunoregulatory functions of mregDC1 via IL-4 blockade led to induction of a stronger effector T cell response and reduced tumor burden (Maier et al., 2020). Interestingly, DC2 could also acquire a similar immunoregulatory program after antigen-uptake in lung tumors (Maier et al., 2020).

Relative to other DC subsets, the contributions of DC1 to anti-tumor immunity have been rather well-elucidated. This has been greatly facilitated by the presence of highly specific markers to identify them (i.e. Xcr1, Batf3, CD8α/CD103), as well as DC1-specific mouse models (i.e. $Batf3^{-/-}$, Xcr1-DTR) to interrogate their impact on immune responses.

The same cannot be said for other DC subsets, such as DC2 and moDC, which exhibit immense heterogeneity and overlapping markers. While more nuanced studies are required to dissect the specific contributions of these DC subsets to anti-tumor immunity, there is evidence that they can indeed be harnessed to drive protective anti-tumor T cell responses. Depletion of T_{reg} cells enabled two subsets of DC2 to activate $CD4^+$ conventional T cell (T_{conv}) responses against melanoma tumors in mice (Binnewies et al., 2019). Melanoma patients with high DC2 and low T_{reg} abundance in their tumors exhibited higher $CD4^+$ T_{conv} cells, which correlated with increased sensitivity to anti-PD-1 therapy (Binnewies et al., 2019). These findings were reminiscent of the observations made in a study of pancreatic ductal adenocarcinoma (Jang et al., 2017). Here, T_{reg} cells were also found to restrain the immunogenic functions of tumor-infiltrating DC (Jang et al., 2017). Mechanistically, this was due to T_{reg}-mediated suppression of costimulatory molecules CD80, CD86, and CD40 and increased expression of IDO on DC (Jang et al., 2017). T_{reg} depletion could restore DC functionality, leading to induction of a stronger effector T cell response and tumor control (Jang et al., 2017). As illustrated by these studies, there is significant potential of the DC compartment to contribute to anti-tumor immunity in ways that extend beyond DC1-mediated anti-tumor $CD8^+$ T cell priming. Thus, it will be important to study the roles of different DC subsets and activation states in the TME, as well as investigate ways to effectively engage them to drive maximal anti-tumor immunity and improve immunotherapy responses.

2.6. Comparison of Human and Murine Dendritic Cells

As we deepen our understanding on the contributions of DC subsets to anti-tumor immunity and identify new therapeutic opportunities using mouse models, a critical challenge will be assessing human relevance and translating our findings into clinic. While our knowledge of murine DC subsets is rather comprehensive, there is still much progress to be made on understanding human DC subsets. Unlike murine DC, human DC are not as readily accessible, and this has greatly impeded research efforts to better understand them (M. Guilliams et al., 2016; M. Merad et al., 2013; Patente et al., 2019). Most studies on human DC were performed using skin and peripheral blood (M. Merad et al., 2013; Patente et al., 2019). However, recent advances in transcriptional profiling and flow cytometric technologies have greatly facilitated cross-correlation studies between murine and human DC, revealing that the human DC compartment is also remarkably heterogeneous (M. Guilliams et al., 2016; See et al., 2017; Villani et al., 2017; Zilionis et al., 2019).

Human DC counterparts for the major murine DC subsets, DC1, DC2, pDC, and moDC, have been described **(Figure 1.8)** (Gerhard et al., 2021; M. Guilliams et al., 2016; See et al., 2017; Villani et al., 2017; Zilionis et al., 2019). These comparisons are based largely on functional similarities as phenotypic markers are often not shared between human and murine DC. Both cDC subsets as well as pDC can be found in peripheral blood and in lymphoid and non-lymphoid tissues in humans. Human DC1 are characterized by expression of BDCA-3 (CD141), Xcr1, and Clec9a, and they are driven by Batf3 and IRF8 transcription factors (M. Merad et al., 2013). Like murine DC1, they express high levels of TLR3 and produce high amounts of IL-12 and IFN-I upon activation

of this PRR pathway (Lauterbach et al., 2010). Furthermore, they exhibit a superior ability to prime $CD8^+$ T cells, relying on signaling via the Clec9a endocytic receptor for the cross-presentation of dead cell-associated antigens to T cells (Cueto et al., 2020; Huysamen, Willment, Dennehy, & Brown, 2008; Jongbloed et al., 2010). Human DC2 are marked by CD1c and Sirpα expression and are quite heterogeneous. They comprise two subsets, DC2-A and DC2-B (DC2 and DC3, respectively, in (Villani et al., 2017)). DC2-A express higher levels of CD11c, CD1c, and MHCII, while DC2-B express an inflammatory gene signature (Villani et al., 2017). The functional nuances between these two DC2 subsets are still being elucidated, but on a more global level, DC2 have been reported to drive T_H1, T_H2, and T_H17 responses (Leal Rojas et al., 2017; Segura et al., 2012), as well as $CD8^+$ T cell activation (Villani et al., 2017). Human pDC are identified by expression of CD123, BDCA-2 (CD303), and BDCA-4 (CD304), and they are driven by the transcription factor E2-2. They express high levels of TLR7 and TLR9 and can are major producers of IFN-I (M. Merad et al., 2013; Rhodes, Tong, Harman, & Turville, 2019). Human moDC can be differentiated from monocytes *in vitro*, and while they have been useful tools to study DC, their relevance *in vivo* has been questioned. Studies have shown that *in vitro* differentiated moDC do not faithfully recapitulate any of the canonical DC subsets, though they may be closely related to inflammatory DC that are context-dependent (Patente et al., 2019; Rhodes et al., 2019; Zilionis et al., 2019).

Transcriptional profiling studies of human blood and tissues have identified additional DC subsets beyond the ones discussed above. These subsets include AXL^+ $Siglec6^+$ (AS)-DC (also referred to as “transitional DC”) that can give rise to pDC or DC2 (Alcantara-Hernandez et al., 2017; Leylek et al., 2019; Villani et al., 2017), as well as

migratory DC (also referred to as DC3 in humans) that express CCR7 (Gerhard et al., 2021; Zilionis et al., 2019). An inflammatory CD163$^+$ CD14$^+$ DC subset (also termed DC3, but distinct from DC3 referring to migratory DC) that shares characteristics of DC2 and monocytes has also been reported to drive functional CD8$^+$ T cell responses (Binnewies et al., 2019; Bourdely et al., 2020; Dutertre et al., 2019; Villani et al., 2017). It will be of great interest to determine whether these cells represent *bona fide* DC subsets or are activation states of existing DC subsets and to assess whether they are involved in the anti-tumor immune response.

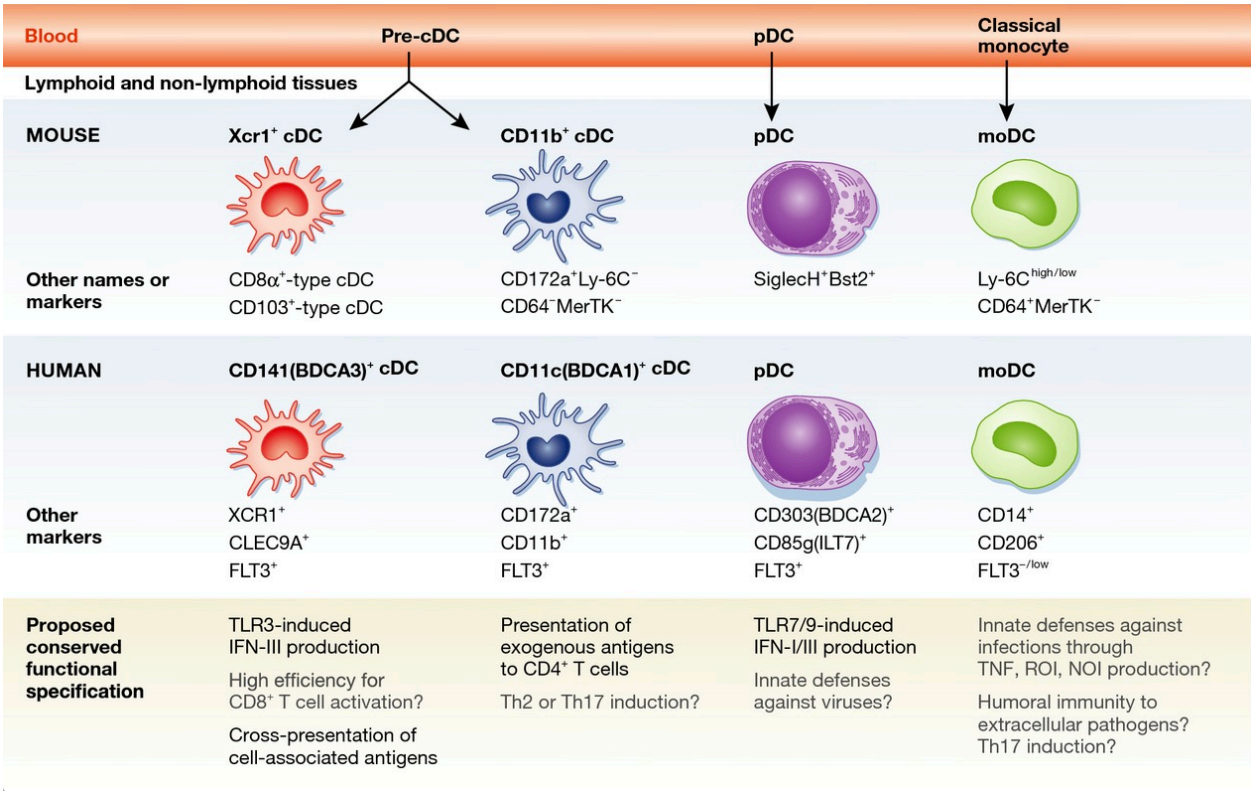

Figure 1.8. Comparison of the major DC subsets in mouse and human. The major murine DC subsets, DC1, DC2, pDC, and moDC, are conserved in humans but are defined by different markers (Dalod et al., 2014).

3. SUMMARY

Despite the tumultuous history of cancer immunotherapy, the recent clinical success of CBT has greatly illuminated the potential of harnessing the immune system to fight cancer. CBT has enabled unprecedented responses against advanced metastatic cancers, leading to increases in overall survival. However, its success is capped by its reach, as the majority of cancer patients fail to respond to CBT.

While a T cell infiltrate is associated with sensitivity to CBT, it is far too simplistic a view. A stronger correlate with response is the presence of T cell activation. As critical initiators of T cell responses, the role of DC in driving anti-tumor immunity must naturally be considered. DC are unique in that they bridge innate and adaptive immune responses. Depending on their activation status, they can dictate whether an immunogenic or tolerogenic T cell response is induced. Thus, they have significant influence on the outcome of anti-tumor immune responses.

Though the function of DC in antigen uptake, processing, and presentation and T cell priming is dogma, work from the past decade has highlighted the important contributions of DC, specifically the Batf3-driven DC1 subset, to productive anti-tumor T cell immunity. These functions extend beyond canonical T cell priming as DC1 are also required for recruiting effector T cells and sustaining their activation in the tumor. While we have a comprehensive understanding of the role of DC1 in anti-tumor immunity, the same cannot be said for other DC subsets whose contributions remain rather poorly described. To add another level of complexity, increasing evidence demonstrates that DC activation states can impact their function, highlighting that DC may exhibit greater plasticity than initially thought. From a therapeutic perspective, it will be important to

characterize these differential DC states and identify the signals that drive them. With the continual technological advances in flow cytometry and transcriptional profiling techniques, we are well-positioned to address these unknowns.

In Chapter 2, I will describe our efforts in identifying the functional DC states associated with productive anti-tumor T cell responses using a comparative model of a spontaneously regressing tumor and a progressing tumor. Specifically, I characterize an activation state of DC2 enriched with interferon-stimulated genes (ISG), which we termed 'ISG$^+$ DC.' In Chapter 3, I focus on identifying the signals mediating functional ISG$^+$ DC in regressor tumors and provide a broader context for the relevance of our findings. This dissertation concludes with Chapter 4, which presents a discussion of ongoing work and future directions and situates our findings in the context of the broader immuno-oncology field and beyond.

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CHAPTER 2

Cross-dressed CD11b$^+$ conventional dendritic cells contribute to protective anti-tumor CD8$^+$ T cell immunity

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Author Contributions
ED and SS conceptualized and designed the study. ED performed all experiments and analyzed the data. TBF generated CRISPR-modified cell lines and advised on immunofluorescence microscopy experiments. TBF and ED performed immunofluorescence image analyses. AB, SB, and ED performed computational analyses. This chapter is part of a revised manuscript under review that was written by ED with comments from other authors.

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ABSTRACT

Tumor-infiltrating antigen-presenting cells, such as dendritic cells (DC), have the capacity to shape anti-tumor T cell responses. While tremendous progress has been made in unraveling the role of Batf3-driven DC1 in the anti-tumor immune response, the contributions of other tumor-infiltrating DC subsets remain poorly understood. Furthermore, tumor-infiltrating DC exist in a range of functional states with differential impacts on anti-tumor immunity. In this study, we sought to identify and characterize the functionally relevant DC states associated with a productive anti-tumor T cell response. By comparing the DC infiltrate of spontaneously regressing tumors and progressing tumors, we identified a novel activation state of CD11b$^+$ conventional DC in tumors, which expressed an interferon-stimulated gene signature ('ISG$^+$ DC'). Through several complementary approaches, we demonstrate that ISG$^+$ DC drove protective anti-tumor CD8$^+$ T cell responses by cross-dressing with tumor-derived peptide-MHC complexes, thus bypassing the requirement for cross-presentation to induce CD8$^+$ T cell immunity.

INTRODUCTION

Cytotoxic CD8$^+$ T cell responses are critical for tumor clearance and the induction of anti-tumor immunity (Fridman, Pagès, Sautès-Fridman, & Galon, 2012). The generation of tumor-specific CD8$^+$ T cells (priming) occurs in the tumor-draining lymph node through interactions of naive CD8$^+$ T cells with antigen-presenting cells (APC) (Chen & Mellman, 2013). During these interactions, APC present tumor-derived antigens on major histocompatibility complexes (MHC) and provide costimulation and cytokine signaling. While APC comprise diverse cell types, dendritic cells (DC) in particular have

long been considered to be the most effective at priming $CD8^+$ T cell responses given their propensity for antigen processing and presentation as well as their ability to transport antigens from peripheral tissue sites to the draining lymph nodes (Inaba, Young, & Steinman, 1987). For this reason, DC are poised to shape anti-tumor $CD8^+$ T cell immunity.

The DC compartment is heterogeneous but has traditionally been defined as comprising conventional DC (cDC) and plasmacytoid DC (pDC). The cDC compartment can be further subdivided into two populations, $CD8\alpha^+/CD103^+$ DC1 and $CD11b^+/Sirp\alpha^+$ DC2, with distinct developmental requirements and functional specialization (Eisenbarth, 2019; Guilliams et al., 2016; Guilliams et al., 2014; Miriam Merad, Priyanka Sathe, Julie Helft, Jennifer Miller, & Arthur Mortha, 2013; Mildner & Jung, 2014; Murphy et al., 2016). DC1 require the transcription factors IRF8 and Batf3 for development and are adept at cross-presenting cell-associated antigens to $CD8^+$ T cells (den Haan, Lehar, & Bevan, 2000; Edelson et al., 2010; Hildner et al., 2008; Iyoda et al., 2002; Schulz & Reis e Sousa, 2002; Tamura et al., 2005). By contrast, DC2 are driven by the transcription factor IRF4 and are more potent at stimulating $CD4^+$ T helper cells (Gao et al., 2013; Krishnaswamy et al., 2017; Tamura et al., 2005; Tussiwand et al., 2015; Williams et al., 2013). The inclusion of monocytic cells that are recruited to inflammatory sites and differentiate into DC-like cells (moDC) has further increased the diversity of the DC compartment (Briseño et al., 2016; León, López-Bravo, & Ardavín, 2007; Menezes et al., 2016; Serbina, Salazar-Mather, Biron, Kuziel, & Pamer, 2003).

Recent DC profiling studies indicate that these DC subsets are generally conserved across species (Gerhard, Bill, Messemaker, Klein, & Pittet, 2021; Zilionis et

al., 2019) and can be found infiltrating solid tumors (Broz et al., 2014; Laoui et al., 2016). Different tumor types harbor distinct compositions of DC (Laoui et al., 2016) which can conceivably impact the resultant anti-tumor T cell response. In murine tumor models, DC1 are regarded as the most critical DC subset driving anti-tumor immunity given their specialized ability to cross-present antigens and prime $CD8^+$ T cell responses (Broz et al., 2014; Hildner et al., 2008; Roberts et al., 2016). Accordingly, tumors harboring a greater DC1 infiltrate tend to be better controlled (Salmon et al., 2016; Spranger, Bao, & Gajewski, 2015) and presence of the DC1 signature in patient tumors is associated with improved survival and response to checkpoint blockade immunotherapy (Barry et al., 2018; Böttcher et al., 2018; Broz et al., 2014; Michea et al., 2018).

However, there is increasing evidence that tumor-infiltrating DC can exist in distinct functional states with tremendous implications for the anti-tumor immune response. In support of this notion, it was recently reported that activated DC1 in lung tumors (mregDC) expressed an immunoregulatory program characterized by PD-L1, PD-L2, and IL-4Rα that dampened their ability to activate T cells (Maier et al., 2020). Furthermore, while progress has been made in understanding the role and function of DC1, the same cannot be said for other DC subsets whose contributions to anti-tumor immunity remain poorly described. Notably, some reports demonstrate that under specific therapeutic settings, DC and APC subsets distinct from DC1 can become robust $CD8^+$ T cell activators (Ma et al., 2013; Sharma et al., 2018). These studies point to the untapped potential of the DC compartment that can be harnessed to enhance anti-tumor $CD8^+$ T cell immunity and calls for more nuanced investigation into the functional DC states driving anti-tumor immunity.

In this study, we sought to dissect the contributions of distinct DC states and their influence on anti-tumor T cell responses during a productive or dysfunctional anti-tumor immune response. By comparing the DC compartment of a spontaneously regressing tumor (productive response) and a progressing tumor (dysfunctional response), we identified a novel activation state of CD11b^{+} cDC expressing an interferon-stimulated gene signature (ISG^{+} DC) that was enriched in regressor tumors. Like DC1, ISG^{+} DC shared the ability to robustly activate CD8^{+} T cells. However, while DC1 cross-presented antigens, we found that ISG^{+} DC stimulated T cells by cross-dressing with pre-formed peptide-MHC (pMHC) complexes derived from tumor cells. Cross-dressed ISG^{+} DC could drive protective, systemic anti-tumor CD8^{+} T cell responses in mice lacking DC1.

RESULTS

The regression of MC57-SIY tumors is independent of Batf3-driven DC1.

To identify functionally relevant DC states associated with productive anti-tumor immune responses, we established a comparative model system of a spontaneously cleared regressor tumor (MC57-SIY fibrosarcoma; productive immune response) and a progressively growing tumor (MC38-SIY colon carcinoma; dysfunctional immune response) **(Figure 2.1A)**. These tumor lines are well-established in the immuno-oncology field and have been engineered to express the model T cell antigen SIYRYYGL (SIY) to enable analyses of tumor-specific T cell responses.

Our initial analysis focused on the cDC compartment (defined as CD45^{+} MHCII^{+} Ly6C^{-} F4/80^{-} CD11c^{+} CD24^{hi}) given its widely reported impact on anti-tumor immunity (Broz et al., 2014; Roberts et al., 2016; Spranger et al., 2015; Spranger, Dai, Horton, &

Gajewski, 2017). At day 7 post-tumor inoculation in wild-type (WT) C57BL/6 mice, we detected a greater proportion of CD103$^+$ DC1 in regressor MC57-SIY tumors, whereas the DC compartment was skewed towards CD11b$^+$ DC2 in progressor MC38-SIY tumors **(Figures 2.1B-2.1C)**. This phenotype was conserved in immunodeficient Rag2$^{-/-}$ mice, suggesting that the presence of T cells had minimal impact on DC composition in the tumors **(Figures 2.1D-2.1F)**. A time course study demonstrated that both DC1 and CD8$^+$ T cells accumulated in regressor MC57-SIY tumors, but these trends were not evident in progressor MC38-SIY tumors **(Figure 2.2)**. Although recent studies highlighted a role for natural killer (NK) cells in DC1 recruitment to the tumor (Barry et al., 2018; Böttcher et al., 2018), we found that antibody-mediated depletion of NK cells in WT mice had no effect on the numbers of DC1 that infiltrated the tumors **(Figure 2.3A)**, nor on the regression of MC57-SIY tumors **(Figure 2.3B)**.

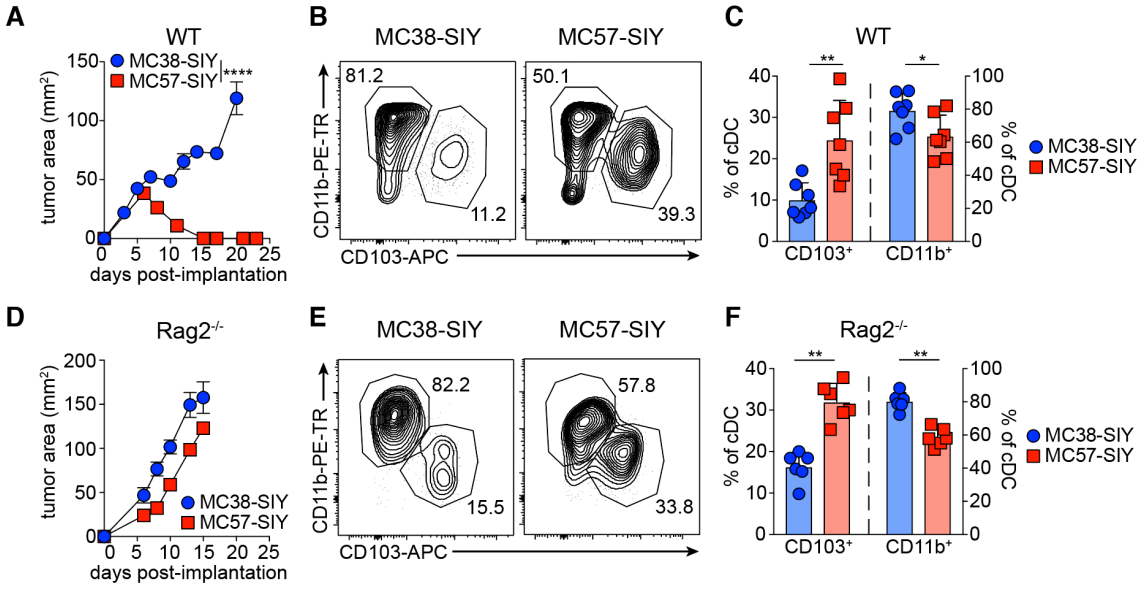


Figure 2.1. Regressor MC57-SIY fibrosarcoma tumors are highly infiltrated with CD103$^+$ DC1.
(A) Tumor outgrowth (mm^2) of MC38-SIY and MC57-SIY in WT mice. Representative data from one of three independent experiments are shown (n = 3-4 mice per group per experiment).

(B, C) Representative flow plot **(B)** and quantification **(C)** of CD103$^+$ DC1 and CD11b$^+$ DC2 (pre-gated on live CD45$^+$ MHCII$^+$ Ly6C$^-$ F4/80$^-$ CD11c$^+$ CD24^{hi}) in MC38-SIY and MC57-SIY tumors from WT mice at day 7 post-tumor inoculation. Data shown are pooled from two independent experiments (n = 3-4 mice per group per experiment).
(D) Tumor outgrowth (mm^2) of MC38-SIY and MC57-SIY in Rag2$^{-/-}$ mice. Representative data from one of three independent experiments are shown (n = 3-5 mice per group per experiment).
(E, F) Representative flow plot **(E)** and quantification **(F)** of CD103$^+$ DC1 and CD11b$^+$ DC2 in MC38-SIY and MC57-SIY tumors from Rag2$^{-/-}$ mice at day 15 post-tumor inoculation. Data shown are pooled from two independent experiments (n = 3 mice per group per experiment). *p<0.05, **p<0.01, ****p<0.0001; MWU test (C, F) or two-way ANOVA (A, D). Data are shown as mean ± s.e.m.

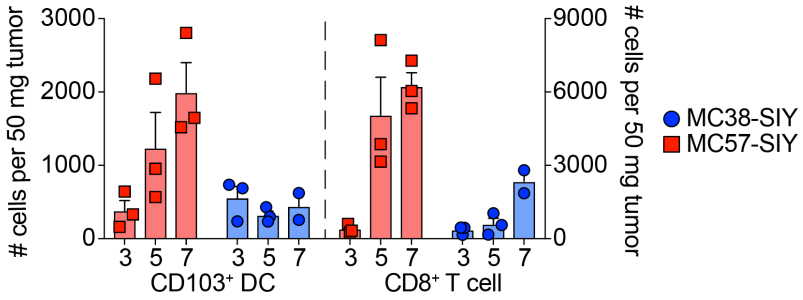


Figure 2.2. The regression of MC57 fibrosarcoma tumors is independent of Batf3-driven DC1.
Number of CD103$^+$ DC1 and CD8$^+$ T cells in 50 mg of MC38-SIY and MC57-SIY tumors at days 3, 5, and 7 post-tumor implantation in WT mice. Representative data from one of two independent experiments are shown (n = 3 mice per group per experiment). Data are shown as mean ± s.e.m.

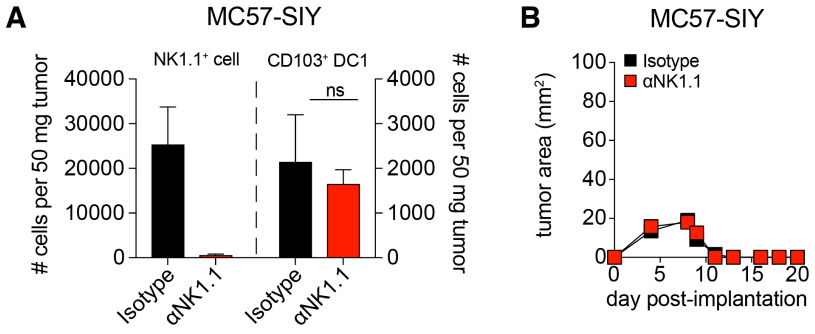
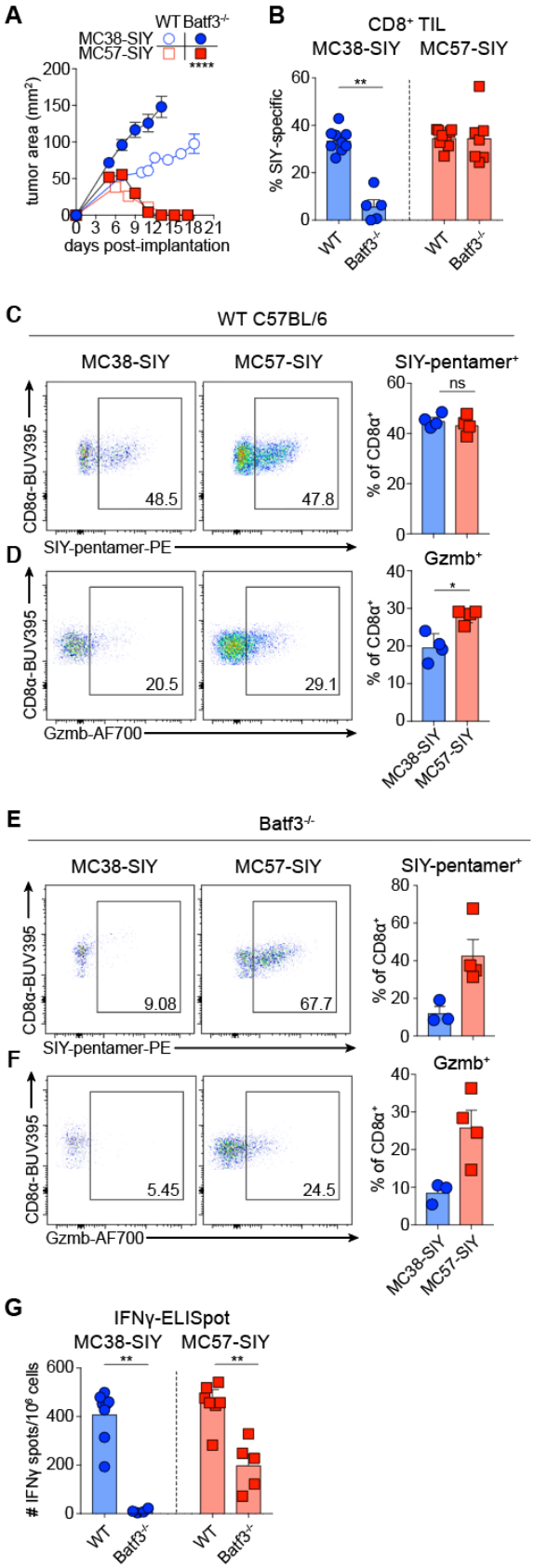


Figure 2.3. NK cells are dispensable for anti-tumor immunity against regressor MC57-SIY tumors.
(A) Number of NK1.1$^+$ cells and CD103$^+$ DC1 in 50 mg of MC57-SIY tumors at day 7 post-tumor implantation in WT mice treated with anti-NK1.1 to deplete NK cells or an isotype control.
(B) Tumor outgrowth (mm^2) of MC57-SIY in WT mice treated with anti-NK1.1 to deplete NK cells or an isotype control.
(A-B) Representative data from one of two independent experiments are shown (n = 4 mice per group per experiment). ns = not significant; MWU test (A) or two-way ANOVA (B). Data are shown as mean ± s.e.m.

Our observation of increased DC1 presence in regressor MC57-SIY tumors prompted us to evaluate whether they were necessary for tumor rejection. We implanted both tumor cell lines into Batf3$^{-/-}$ mice, which lack DC1 due to disruption of the Batf3 transcription factor required for their development (Hildner et al., 2008). Consistent with published data that DC1 are critical for priming T cell responses (Broz et al., 2014; Fuertes et al., 2011; Hildner et al., 2008), the growth of progressor MC38-SIY tumors in Batf3$^{-/-}$ mice was accelerated compared to WT mice **(Figure 2.4A)**. In contrast, we observed that regressor MC57-SIY tumors were rejected in Batf3$^{-/-}$ mice with similar kinetics as in WT mice **(Figure 2.4A)**, suggesting that their regression was independent of DC1. Analyses of the local T cell infiltrate within tumors from Batf3$^{-/-}$ mice further supported this notion, indicating that only MC57-SIY tumors, but not MC38-SIY tumors, harbored SIY-specific and granzyme B-expressing CD8^{+} T cells **(Figures 2.4B-2.4F).** These observations also held true at the systemic level: IFNγ-Enzyme-Linked ImmunoSpot (IFNγ-ELISpot) assays on splenocytes from tumor-bearing mice confirmed that systemic anti-tumor T cell responses against MC38-SIY tumors were completely ablated in Batf3$^{-/-}$ mice **(Figure 2.4G)**. In stark contrast, systemic T cell responses against MC57-SIY tumors were preserved in Batf3$^{-/-}$ mice albeit reduced by 58% when compared to the responses in WT mice **(Figure 2.4G)**. These data indicate that DC1 are not the only stimulatory APC driving anti-tumor CD8^{+} T cell responses in MC57-SIY tumors.

DC1 are thought to selectively express the Clec9a endocytic receptor, and signaling through this receptor promotes the cross-presentation of dead cell-associated antigens (Sancho et al., 2009; Sancho et al., 2008; Zelenay et al., 2012; Zhang et al., 2012). Using Clec9a$^{-/-}$ mice as a complementary model wherein DC1 are present but

functionally impaired, we affirmed that Clec9a-mediated cross-presentation by DC1 is not required for the rejection of MC57-SIY tumors **(Figure 2.5A)**. One possibility that would bypass the need for cross-presentation is tumor control by $CD4^+$ T cells (Mumberg et al., 1999). To assess whether regression of MC57-SIY is dependent on $CD8^+$ or $CD4^+$ T cells, we depleted each T cell subset alone or in combination and identified that tumor control was driven by $CD8^+$ T cells **(Figure 2.5B)**. These data indicate that cross-priming of $CD8^+$ T cells is an essential component of anti-tumor immunity but that in certain contexts it can be induced independent of cross-presenting DC1.


A
WT Batf3-/-
MC38-SIY
MC57-SIY
****
tumor area (mm2)
200
150
100
50
0
0 3 6 9 12 15 18 21
days post-implantation
B
CD8+ TIL
MC38-SIY MC57-SIY
% SIY-specific
60
40
20
0
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WT
Batf3-/-
WT
Batf3-/-
C
WT C57BL/6
MC38-SIY
MC57-SIY
SIY-pentamer+
CD8α-BUV395
48.5
47.8
SIY-pentamer-PE
ns
% of CD8α+
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40
20
0
D
Gzmb+
CD8α-BUV395
20.5
29.1
Gzmb-AF700
*
% of CD8α+
40
30
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MC38-SIY
MC57-SIY
E
Batf3-/-
MC38-SIY
MC57-SIY
SIY-pentamer+
CD8α-BUV395
9.08
67.7
SIY-pentamer-PE
% of CD8α+
80
60
40
20
0
F
Gzmb+
CD8α-BUV395
5.45
24.5
Gzmb-AF700
% of CD8α+
40
30
20
10
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MC38-SIY
MC57-SIY
G
IFNγ-ELISpot
MC38-SIY MC57-SIY
IFNγ spots/10^6 cells
600
400
200
0
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WT
Batf3-/-
WT
Batf3-/-

Figure 2.4. The regression of MC57 fibrosarcoma tumors is independent of Batf3-driven DC1.
(A) Tumor outgrowth (mm^2) of MC38-SIY and MC57-SIY in WT or $Batf3^{-/-}$ mice. Representative data from one of three independent experiments are shown (n = 3-4 mice per group per experiment).
(B) Quantification of SIY-specific $CD8^+$ T cells in MC38-SIY and MC57-SIY tumors from $Batf3^{-/-}$ mice at day 7 post-tumor implantation. Data shown are pooled from two independent experiments (n = 2-5 mice per group per experiment).
(C, D) Representative flow plot *(left)* and quantification *(right)* of SIY-specific $CD8^+$ T cells **(C)** and $Gzmb^+$ $CD8^+$ T cells **(D)** in MC38-SIY and MC57-SIY tumors from WT mice at day 7 post-tumor implantation.
(E, F) Representative flow plot *(left)* and quantification *(right)* of SIY-specific $CD8^+$ T cells **(E)** and $Gzmb^+$ $CD8^+$ T cells **(F)** in MC38-SIY and MC57-SIY tumors from $Batf3^{-/-}$ at day 7 post-tumor implantation.
(C-F) Representative data from one of two independent experiments are shown (n = 3-4 mice per group per experiment).
(G) ELISpot quantification of IFNγ-producing splenocytes from WT and $Batf3^{-/-}$ mice bearing MC38-SIY or MC57-SIY tumors at day 5 post-tumor inoculation. Data shown are pooled from two independent experiments (n = 3-4 mice per group per experiment). *p<0.05, **p<0.01, ****p<0.0001, ns = not significant; MWU test (B-G) or two-way ANOVA (A). Data are shown as mean ± s.e.m.

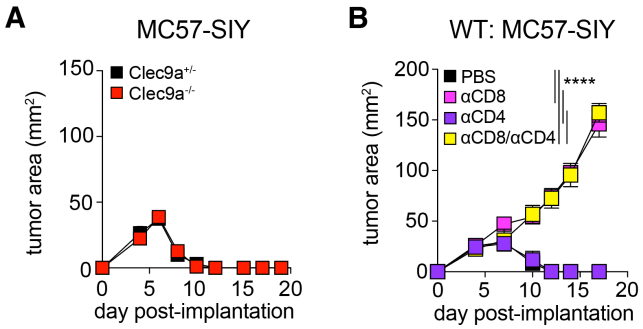

Figure 2.5. The regression of MC57 fibrosarcoma tumors is independent of Clec9a-mediated cross-presentation and $CD4^+$ T cells but requires $CD8^+$ T cells.
(A) Tumor outgrowth (mm^2) of MC57-SIY in $Clec9a^{+/-}$ and $Clec9a^{-/-}$ mice. Representative data from one of three independent experiments are shown (n = 4 mice per group per experiment).
(B) Tumor outgrowth (mm^2) of MC57-SIY in WT mice treated with anti-CD8 to deplete $CD8^+$ T cells, anti-CD4 to deplete $CD4^+$ T cells, anti-CD8 and anti-CD4 to deplete both $CD8^+$ and $CD4^+$ T cells, or PBS as a control. Representative data from one of two independent experiments are shown (n = 4 mice per group per experiment). ****p<0.0001; two-way ANOVA (B). Data are shown as mean ± s.e.m.

Identification of a DC cluster characterized by a strong IFN-I gene signature in regressor MC57 tumors.

We next aimed to identify the APC type(s) mediating the induction of protective immunity against regressor MC57-SIY tumors in Batf3$^{-/-}$ mice. To this end, we established a functional *ex vivo* co-culture assay using naïve 2C T cell receptor (TCR) transgenic CD8^{+} T cells that recognize the model antigen SIY presented on the MHC class I (MHC-I) molecule H-2K^{b} **(Figure 2.6A)**. Myeloid APC were sorted from regressor MC57-SIY tumors in WT and Batf3$^{-/-}$ mice and co-cultured with 2C T cells that were labeled with a proliferation dye. In this assay, T cell activation was solely dependent upon spontaneous antigen presentation by APC *in vivo* as exogenous SIY peptide was not added to the APC:T cell co-cultures. In both WT and Batf3$^{-/-}$ settings, only CD11c^{+} DC, but not Ly6C^{+} monocytes or F4/80^{+} macrophages, were able to induce 2C T cell proliferation **(Figures 2.6B-2.6C)**. In the Batf3$^{-/-}$ setting, this observation implied the presence of stimulatory DC in the tumor that were distinct from DC1. To confirm whether CD11c^{+} DC were indeed required for anti-tumor immunity against MC57-SIY tumors, we generated CD11c-diptheria toxin receptor bone marrow chimeras (CD11c-DTR BMC), a mouse model where all CD11c^{+} cells expressed DTR. Specific depletion of CD11c^{+} cells via DT administration completely abrogated anti-tumor CD8^{+} T cell responses against MC57-SIY tumors **(Figure 2.6D)**, which confirmed an absolute requirement for CD11c^{+} DC in the induction of anti-tumor CD8^{+} T cell responses. A caveat of this model is that CD11c can be highly expressed on other cell types beyond DC, such as macrophages (M. Merad, P. Sathe, J. Helft, J. Miller, & A. Mortha, 2013). Thus, in order to definitively confirm that bona fide DC are required for anti-tumor immunity against MC57-SIY tumors, we also

evaluated the immune response in zDC-DTR BMC, a mouse model where only Zbtb46-dependent cDC expressed DTR (Meredith et al., 2012). In agreement with our observations from CD11c-DTR BMC, selective depletion of cDC by DT administration completely ablated functional tumor-reactive T cell responses against MC57-SIY tumors both locally **(Figures 2.7A-2.7B)** and at the systemic level **(Figure 2.7C)**. Collectively, these data provided strong rationale for narrowing our search for stimulatory cells within the intratumoral DC compartment.

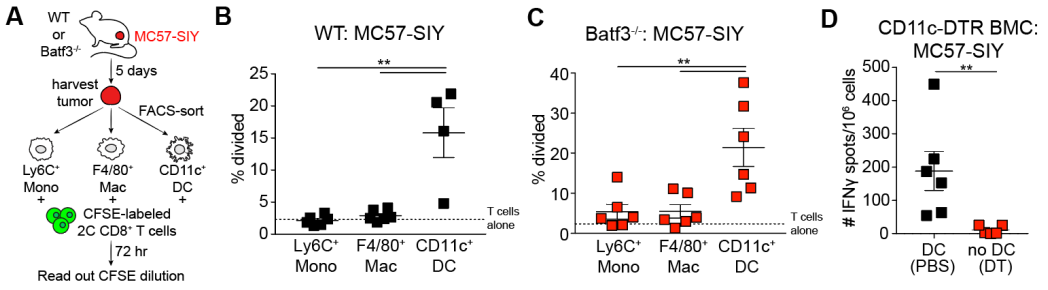

Figure 2.6. CD11c$^+$ DC comprise the predominant stimulatory APC compartment in regressor MC57-SIY tumors in both WT and Batf3$^{-/-}$ mice.
(A) Experimental design for **(B)** and **(C)**.
(B, C) Percentage of 2C T cell proliferation after 72 hr co-culture with APC sorted from MC57-SIY tumors in WT **(B)** or Batf3$^{-/-}$ **(C)** mice at day 5 post-tumor inoculation. Representative data from one of two independent experiments are shown (n = 5 mice per experiment).
(D) Number of IFNγ-producing splenocytes from CD11c-depleted (DT-treated) or non-depleted (PBS-treated) CD11c-DTR BMC mice bearing MC57-SIY tumors at day 5 post-tumor inoculation. Data shown are pooled from two independent experiments (n = 3 mice per group per experiment). **p<0.01; MWU test (B-D). Data are shown as mean ± s.e.m.

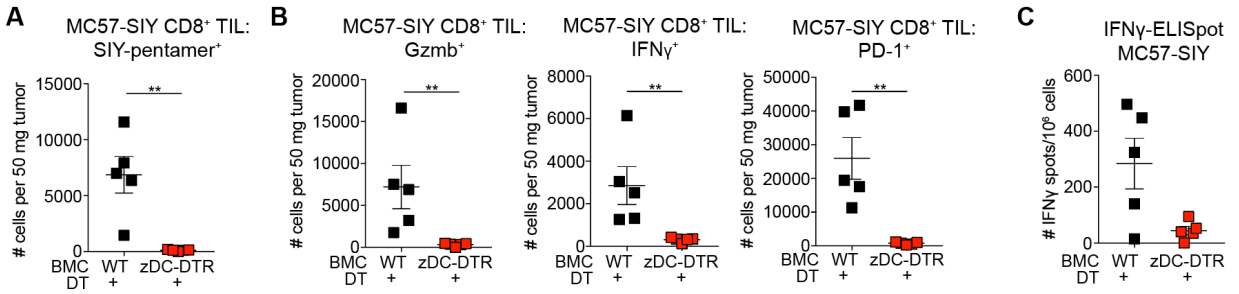

Figure 2.7. Zbtb46-dependent cDC are required to drive anti-tumor CD8$^+$ T cell responses against regressor MC57-SIY tumors.
(A) Number of IFNγ-producing splenocytes from cDC-depleted (zDC-DTR) or non-depleted (WT) BMC mice bearing MC57-SIY tumors at day 7 post-tumor inoculation.
(B) Number of Gzmb$^+$, IFNγ$^+$, and PD-1$^+$ CD8$^+$ T cells in 50 mg of MC57-SIY tumors at day 7 post-tumor implantation in DT-treated WT (control) or zDC-DTR (cDC-depleted) BMC mice.
(C) Number of IFNγ-producing splenocytes from DT-treated WT (control) or zDC-DTR (cDC-depleted) BMC mice bearing MC57-SIY tumors at day 7 post-tumor inoculation.
(A-C) Data shown are from one experiment (n = 5 mice per group per experiment). **p<0.01; MWU test (A-C). Data are shown as mean ± s.e.m.

To identify the functionally relevant DC states driving anti-tumor immunity in regressor tumors, we turned to single-cell RNA-sequencing (scRNA-seq) as an unbiased approach. We performed scRNA-seq of the CD45$^+$ immune infiltrate of regressor MC57-SIY tumors in Rag2$^{-/-}$ mice. The use of Rag2$^{-/-}$ mice enabled us to enrich for more cells as MC57-SIY tumors could grow progressively in this mouse model. Based on the expression of a canonical DC signature (*H2-Ab1*, *Flt3*, *Itgax*) **(Figures 2.8A-2.8B)** and the absence of marker genes corresponding to other lineages **(Figure 2.8A; Table S1)**, we identified a global DC cluster that was then computationally isolated and re-analyzed at a higher resolution **(Figure 2.8A)**. From this initial analysis, we observed a contaminating macrophage cluster expressing *Adgre1*, *Mafb*, and *C5ar1* genes that was excluded during a second round of filtering **(Figures 2.8B-2.8C)**. These analyses ultimately led us to the identification of seven distinct DC clusters **(Figures 2.9A-2.9B)**. Mapping the differentially expressed genes (DEG) of each DC cluster to the literature (Guilliams et al., 2016; Guilliams et al., 2014; Miriam Merad et al., 2013; Mildner & Jung,

2014; Murphy et al., 2016; Zilionis et al., 2019), we were able to identify several classically described subsets: DC1 (cluster 4: *Xcr1*, *Irf8*, *Batf3*), migratory DC (cluster 3: *Ccr7*, *Fscn1*, *Ccl22*), DC2 (cluster 1: *H2-Dmb2*, *H2-Eb1*, *Clec4a3*), moDC (cluster 0: *Lyz2*, *Cd209a*, *Ccr2*), and two distinct pDC clusters (cluster 5 pDC_A: *Tcf4*; and cluster 7 pDC_B: *Ccr9*, *Siglech*) **(Figure 2.9B; Table S2)**. To validate these assigned cluster identities, we scored the cells in our dataset for their expression of published DC subset-specific signatures from Zilionis *et al.* 2019 **(Table S3)** and found that our assignments largely agreed with the published signatures **(Figure 2.9C and 2.9E)** (Zilionis et al., 2019). Intriguingly, our DEG analysis identified one cluster that was enriched in IFN-stimulated genes (ISG) (cluster 2: *Cxcl10, Isg15*) for which we did not observe a comparable counterpart in the set of Zilionis DC signatures, although some cells in this cluster expressed the Zilionis DC2 signature **(Figure 2.9C)**. Based on its high ISG expression, we believe that cluster 2 likely represents an IFN-induced DC activation state and will refer to this cluster as 'ISG$^+$ DC.' Of note, a recent study identified an inflammatory cDC2 state (Inf-cDC2) in the context of viral infection that was induced by IFN-I (Bosteels et al., 2020), and we found that the expression of the Inf-cDC2 signature (generated in-house) was enriched in our ISG$^+$ DC cluster **(Figures 2.9D-2.9E; Table S3)**. The similarity in their transcriptional profiles might suggest that ISG$^+$ DC and Inf-cDC2 are related activation states; however, direct comparative studies are needed to determine whether this is indeed the case.

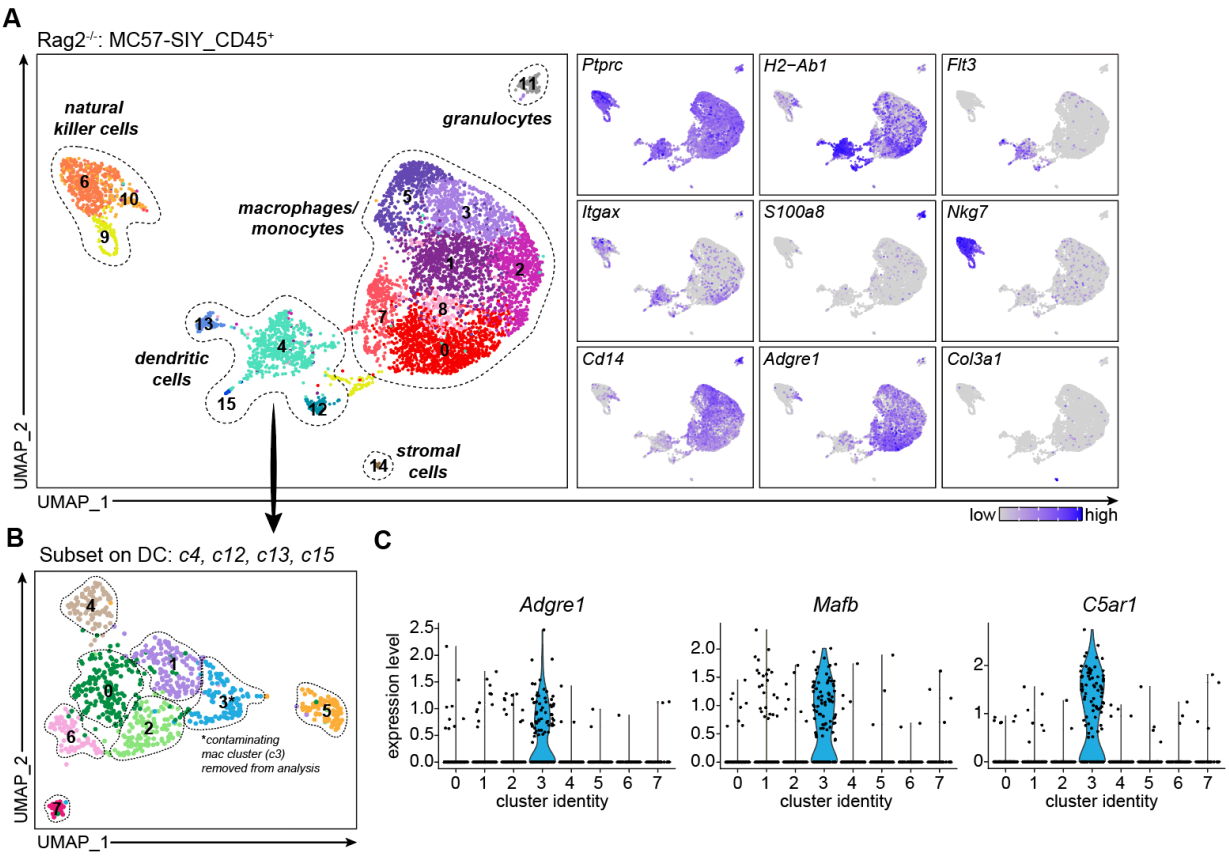

Figure 2.8. scRNA-seq of the CD45$^+$ infiltrate in regressor MC57-SIY tumors reveals several major immune cell types.
(A) *Left,* UMAP plot of scRNA-sequenced CD45$^+$ cells from pooled MC57-SIY tumors at day 7 post-tumor implantation in Rag2$^{-/-}$ mice (n = 5 mice). Each dot represents a single cell. Clusters corresponding to DC, monocytes/macrophages, granulocytes, stromal cells, and NK cells are indicated. *Right,* UMAP plots highlighting select marker genes used to broadly identify major immune cell clusters.
(B) UMAP plot of cells within the DC clusters (clusters 4, 12, 13, 15 from S2A). A contaminating mac cluster (cluster 3) was excluded from further downstream analysis.
(C) Violin plots showing the expression distribution of macrophage markers *Adgre1, Mafb,* and *C5ar1* in cluster 3. Each dot represents a single cell.

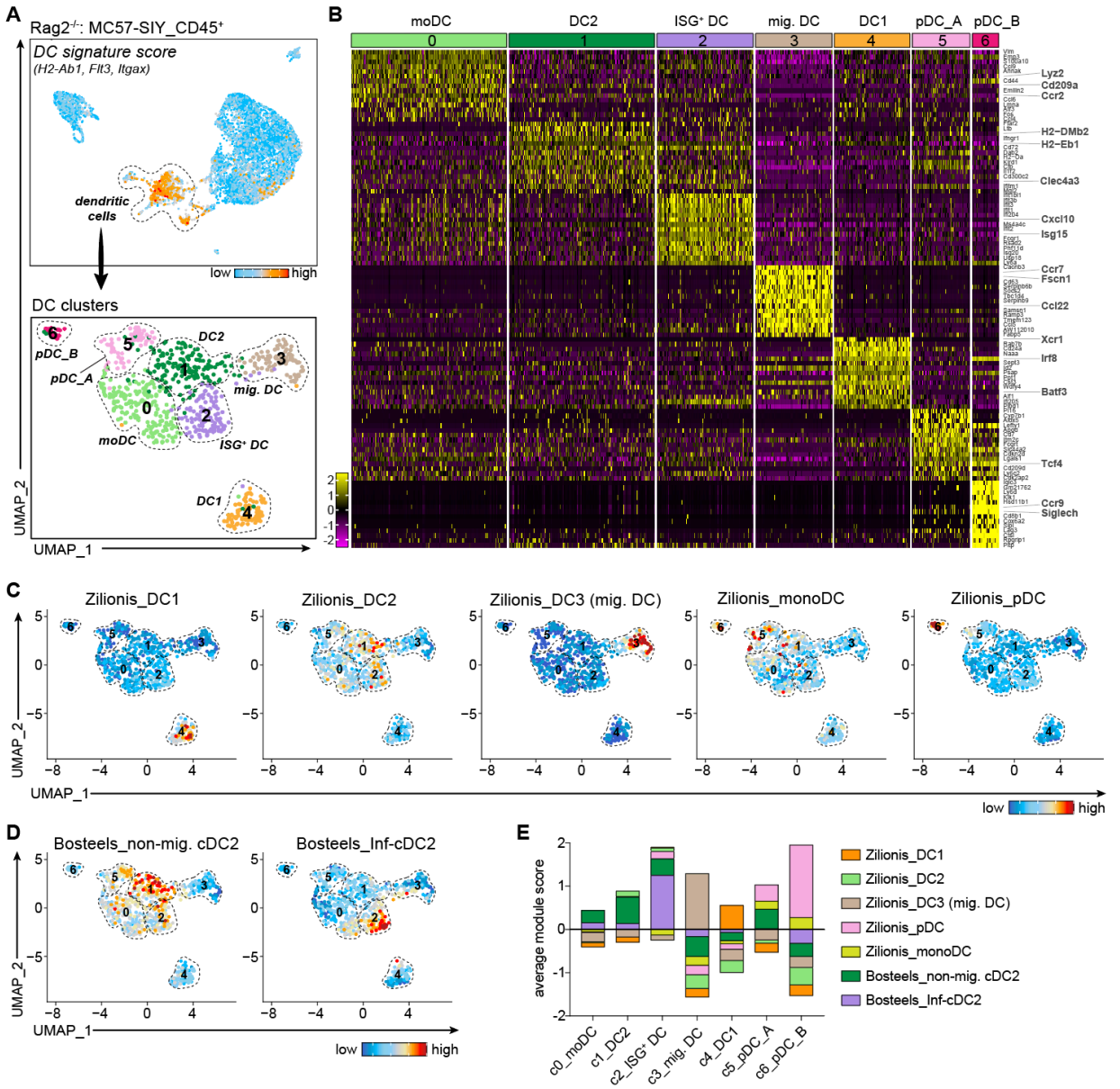

Figure 2.9. scRNA-seq of regressor MC57-SIY tumors identifies a DC cluster characterized by a strong IFN-I gene signature.
(A) *Top*, UMAP plot of cells from MC57-SIY tumors colored by expression module score of a DC signature (*H2-Ab1, Flt3, Itgax*). *Bottom*, UMAP plot of the cells contained within the highlighted DC cluste**r.**
(B) Heatmap showing top 15 DEG for each of the DC clusters identified in **(A)**.
(C, D) Feature UMAP plots of the DC clusters. Each cell is colored by its expression module score of the indicated literature-derived DC signature.
(E) Average expression score per cluster of the indicated literature-derived DC signatures.

We next aimed to determine whether induction of the ISG$^+$ DC state was required for anti-tumor immunity against our regressor model. To this end, we implanted MC57-

SIY cells into Ifnar1$^{-/-}$ mice, wherein host cells are deficient in IFN-I sensing. We observed failed tumor control **(Figure 2.10A)**, as well as reduced tumor-reactive T cell responses by IFNγ-ELISpot **(Figure 2.10B)**, thus confirming the necessity of IFNAR signaling for anti-tumor immunity. Previous studies have shown that T cell activation can be impacted by defects in T cell-intrinsic IFN-I sensing (Hervas-Stubbs et al., 2011; Huber & Farrar, 2011; Le Bon et al., 2006; Le Bon et al., 2003). To determine whether IFN-I sensing is specifically required in the CD11c^{+} DC compartment, we generated CD11c-DTR:Ifnar1$^{-/-}$ mixed bone marrow chimeras using WT or Ifnar1$^{-/-}$ hosts **(Figure 2.10C)**. DT administration in this model specifically ablated IFN-I sensing in the CD11c^{+} DC compartment, while other immune compartments were unperturbed. Implanting MC57-SIY tumors into this chimeric mouse model, we still observed significant reductions in the systemic anti-tumor T cell response by IFNγ-ELISpot when DC lacked the ability to sense IFN-I regardless of the *Ifnar1* status of the host mice **(Figure 2.10D)**. These data indicate that DC-intrinsic IFN-I sensing is required for the induction of a potent anti-tumor T cell response, thus confirming a role for the ISG^{+} DC state in anti-tumor immunity.

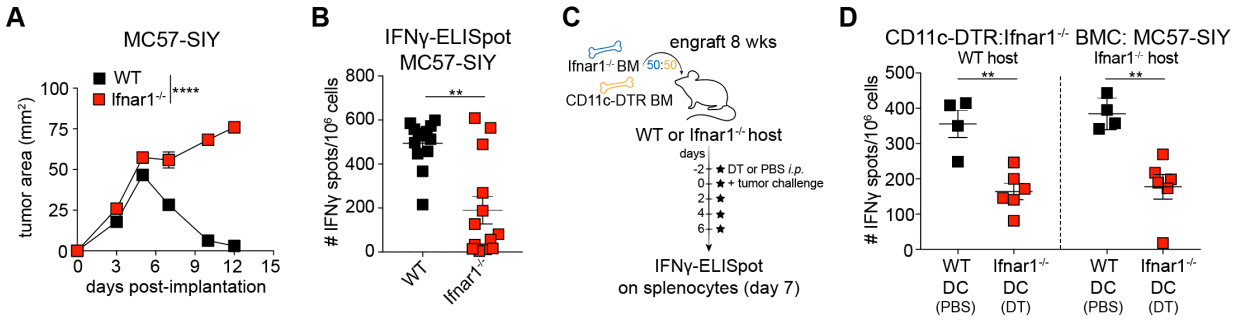

Figure 2.10. DC-intrinsic IFN-I sensing is required for optimal anti-tumor CD8^{+} T cell responses against regressor MC57-SIY tumors.
(A) Tumor outgrowth (mm^2) of MC57-SIY in WT or Ifnar1$^{-/-}$ mice. Representative data from one of three independent experiments are shown (n = 3-4 mice per group per experiment).

(B) ELISpot quantification of IFNγ-producing splenocytes from WT and Ifnar1$^{-/-}$ mice bearing MC57-SIY tumors at day 7 post-tumor inoculation. Data shown are pooled from three independent experiments (n = 3-5 mice per group per experiment).
(C) Experimental design for **(D)**.
(D) ELISpot quantification of IFNγ-producing splenocytes from CD11c-DTR:Ifnar1$^{-/-}$ mixed BMC mice (WT hosts, *left*; Ifnar1$^{-/-}$ hosts, *right*) bearing MC57-SIY tumors at day 7 post-tumor inoculation. Data shown are pooled from two independent experiments (n = 2-3 mice per group per experiment). **p<0.01, ****p<0.0001; MWU test (B, D) or two-way ANOVA (A). Data are shown as mean ± s.e.m.

ISG^{+} DC are present in Rag2$^{-/-}$, WT, and Batf3$^{-/-}$ mice.

In order to study the ISG^{+} DC state, we identified *Axl* (an IFN-inducible receptor tyrosine kinase) (Schmid et al., 2016) as a surface-expressed marker that significantly differentiated the ISG^{+} DC cluster from the remaining DC clusters **(Figure 2.11; Table S4)**. As the ISG^{+} DC cluster also expressed *Itgam* encoding for CD11b **(Figure 2.11)**, we used the co-expression of AXL and CD11b to identify ISG^{+} DC in the DC compartment by flow cytometry. Given that AXL can be expressed on other immune cell types, such as NK cells, monocytes, and macrophages, we took careful measures to exclude these cell types in our gating strategy to ensure a specific analysis of DC **(Figures 2.12A-2.12C)**. Using this gating strategy, we were able to identify the presence of ISG^{+} DC in regressor MC57-SIY tumors in Rag2$^{-/-}$ and WT mice, and importantly, in Batf3$^{-/-}$ mice **(Figures 2.12D-2.12I)**.

As ISG^{+} DC were originally identified in MC57-SIY tumors from Rag2$^{-/-}$ mice, we aimed to confirm that ISG^{+} DC from both immunocompetent and Rag2$^{-/-}$ mice expressed similar transcriptional signatures. To do so, we performed bulk RNA-sequencing (bulk RNA-seq) and derived transcriptional signatures for sorted DC populations from MC57-SIY tumors in Rag2$^{-/-}$ and WT mice **(Figure 2.13A and Table S3)**. Cells from the scRNA-seq dataset that scored highly for either the Rag2$^{-/-}$ ISG^{+} DC or DC1 signatures were significantly enriched ($p \leq 3.22 \times 10^{-8}$) in their corresponding clusters of our scRNA-seq

dataset **(Figures 2.13B-2.13E)**, which validated our flow panel and gating strategy to identify them. In line with our observations for the Rag2$^{-/-}$ ISG^{+} DC signature, cells that scored highly for the WT ISG^{+} DC signature were also significantly enriched (p=7.79x10^{-6}) in the c2_ISG^{+} DC scRNA-seq cluster **(Figures 2.13D-2.13E)**. In a pair-wise analysis against other clusters, the c2_ISG^{+} DC cluster consistently scored higher for both the Rag2$^{-/-}$ and WT ISG^{+} DC signatures **(Figure 2.13F)** with p≤7.47x10^{-7}. We further observed significant enrichment of the Rag2$^{-/-}$ ISG^{+} DC signature (p-adj≤0.05; $\log_2$FC cutoff=1) in the WT ISG^{+} DC signature by Gene Set Enrichment Analysis (GSEA) (Mootha et al., 2003; Aravind Subramanian et al., 2005) **(Figure 2.13G)**. Taken together, these analyses enabled us to conclude that ISG^{+} DC isolated from immunocompetent and Rag2$^{-/-}$ mice shared similar transcriptional profiles with each other and with the ISG^{+} DC originally identified from scRNA-seq.

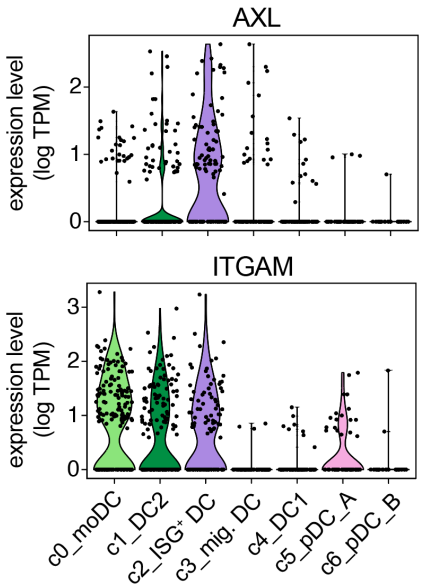

Figure 2.11. Co-expression of *Axl* and *Itgam* delineates the c2_ISG^{+} DC cluster from other DC clusters.
Violin plots showing the expression distribution of *Axl (top)* and *Itgam (bottom)* in the DC clusters. Each dot represents a single cell.

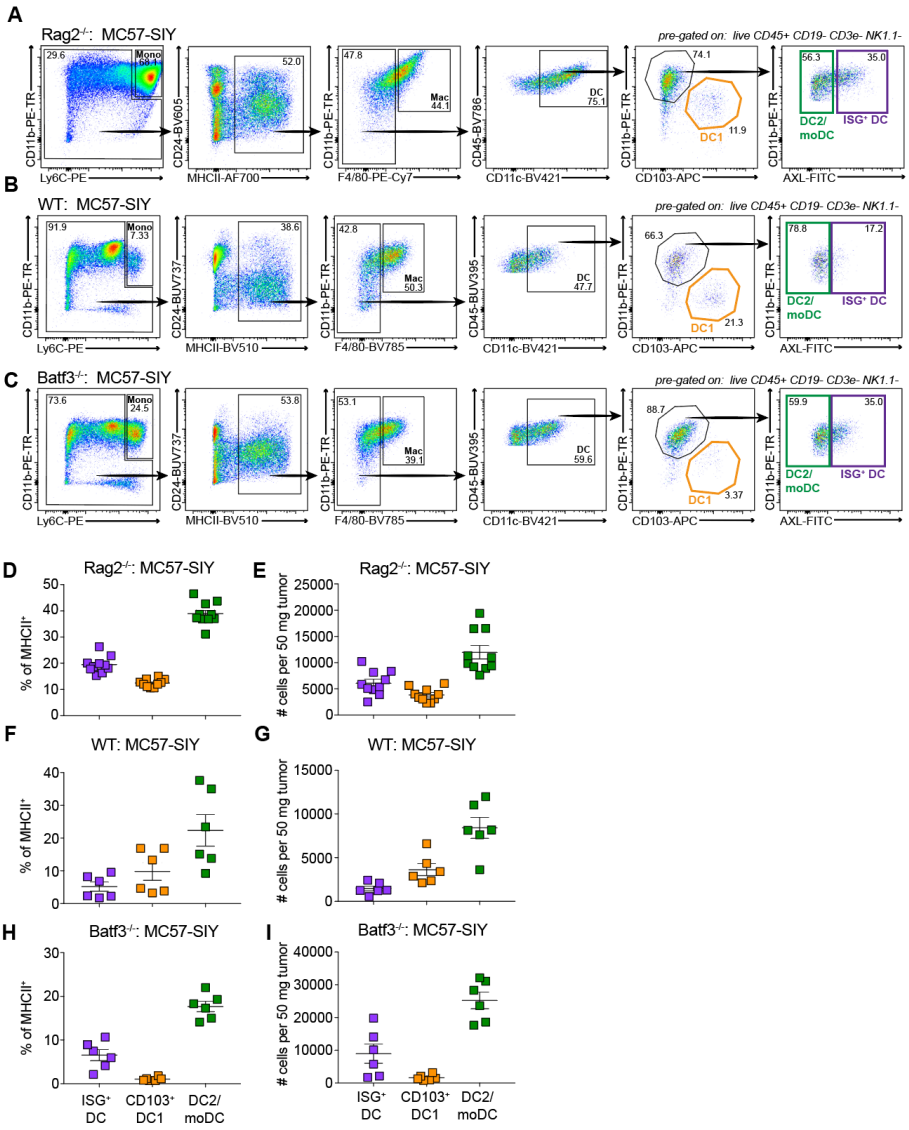

Figure 2.12. ISG$^+$ DC are present in regressor MC57-SIY tumors implanted in Rag2$^{-/-}$, WT, and Batf3$^{-/-}$ mice.

(A-C) Representative flow cytometry gating strategy for Ly6C$^+$ monocytes, F4/80$^+$ macrophages, CD11c$^+$ DC, ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC in Rag2$^{-/-}$ **(A)**, WT **(B)**, or Batf3$^{-/-}$ **(C)** mice, pre-gated on live CD45$^+$ CD19$^-$ CD3e$^-$ NK1.1$^-$ cells.

(D-I) Quantification of ISG$^+$ DC, CD103$^+$ DC1 and DC2/moDC in MC57-SIY tumors as a percentage of MHCII$^+$ cells or as absolute numbers per 50 mg tumor in Rag2$^{-/-}$ **(D, E)** and WT **(F, G)** mice at day 7 post-tumor inoculation, and in Batf3$^{-/-}$ mice **(H, I)** at day 11 post-tumor inoculation. Data shown are pooled from two independent experiments (n = 3-5 mice per group per experiment). Data are shown as mean ± s.e.m.

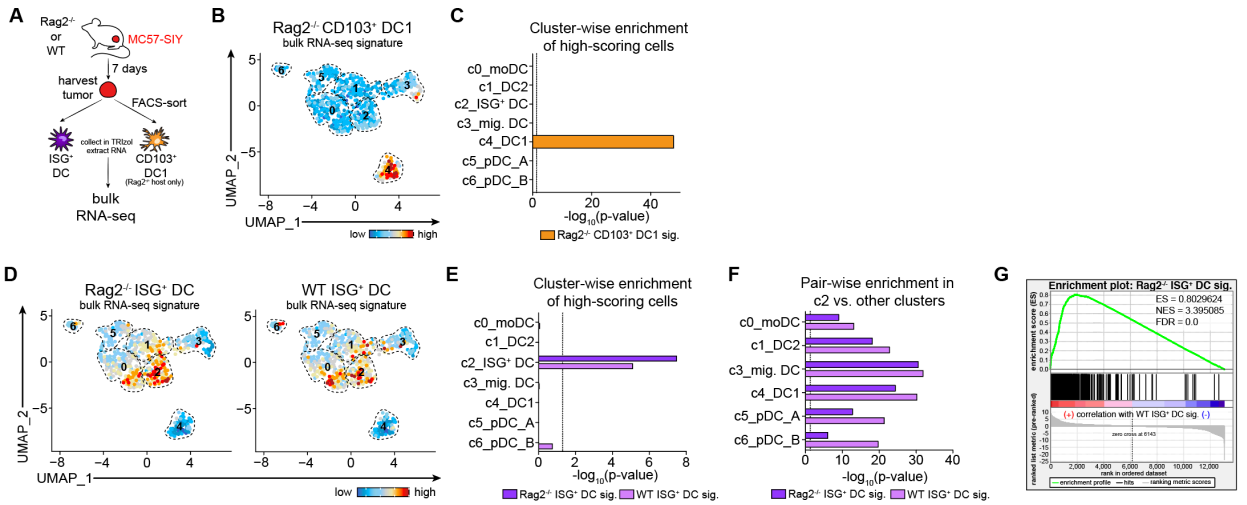

Figure 2.13. ISG$^+$ DC are present in regressor MC57-SIY tumors implanted in Rag2$^{-/-}$, WT, and Batf3$^{-/-}$ mice.
(A) Experimental design for **(B-G)**.
(B) Feature UMAP plots of the DC clusters. Each cell is colored by its expression module score of the bulk RNA-seq-derived Rag2$^{-/-}$ CD103$^+$ DC1 signature.
(C) Cluster-wise enrichment of cells that scored highly for Rag2$^{-/-}$ CD103$^+$ DC1 signature in scRNA-seq clusters. Dotted line denotes significance threshold (p=0.05, hypergeometric test).
(D) Feature UMAP plots of the DC clusters. Each cell is colored by its expression module score of the indicated Rag2$^{-/-}$ or WT bulk RNA-seq-derived ISG$^+$ DC signature.
(E) Cluster-wise enrichment of cells scoring highly for the Rag2$^{-/-}$ or WT ISG$^+$ DC signatures in scRNA-seq clusters. Dotted line denotes significance threshold (p=0.05, hypergeometric test).
(F) Enrichment of Rag2$^{-/-}$ or WT ISG$^+$ DC signatures in c2_ISG$^+$ DC cluster of scRNA-seq dataset in pairwise comparison with other clusters. Dotted line denotes significance threshold (p=0.05, Mann-Whitney Wilcoxon test).
(G) GSEA plot showing highly significant enrichment of Rag2$^{-/-}$ ISG$^+$ DC signature in WT ISG$^+$ DC signature. ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate.
(B-G) Data shown are from two independent experiments (n = 5 mice per group per experiment).

ISG$^+$ DC comprise an activation state of CD11b$^+$ DC2 that can be found in human tumors.

To characterize ISG$^+$ DC and how they phenotypically compare to other DC subsets, we assessed expression of a panel of myeloid cell markers. Given the difficulty of distinguishing DC2 from moDC by flow cytometry due to overlapping markers (Guilliams et al., 2014; Miriam Merad et al., 2013), we will refer to them collectively as DC2/moDC for completeness. Consistent with our collective sequencing analyses, we observed that ISG$^+$ DC were phenotypically distinct from DC1, migratory DC, and pDC, lacking expression of CD24 and CD103, CCR7, and Siglec H, respectively **(Figures**

2.14A-2.14B). Rather, they more closely resembled DC2/moDC, expressing high levels of CD11b and Sirpα **(Figures 2.14A-2.14B)**. Given their transcriptional similarity to Inf-cDC2 **(Figures 2.9D-2.9E)**, we assessed the expression of Inf-cDC2 markers and observed that ISG$^+$ DC also expressed CD64 and MAR-1 **(Figure 2.14B)**, as well as the cDC-specific marker CD26 **(Figure 2.14B)**. This observation suggests that ISG$^+$ DC might comprise a specific activation state of conventional DC2. To confirm the ontogeny of ISG$^+$ DC, we performed a classical fate-mapping experiment wherein we transferred sorted CD45.1$^+$ granulocyte-macrophage progenitors (GMP) or pre-DC precursors into MC57-SIY tumors and assessed their fates at day 3 post-transfer **(Figure 2.15A-2.15B)**. Only the transferred pre-DC but not GMP gave rise to ISG$^+$ DC **(Figures 2.15C-2.15G)**. We further affirmed this observation using zDC-DTR BMC mice. Selective depletion of cDC via DT administration resulted in an 83% reduction of ISG$^+$ DC numbers **(Figures 2.16A-2.16B)**, thus confirming their ontogeny as cDC. To probe whether the ISG$^+$ DC state encompassed DC2, we used IRF4$^{f/f}$xCD11c^{Cre} mice. In this mouse model, Cre-mediated deletion of IRF4 ablates DC2 development, which was validated by phenotyping splenic DC populations **(Figures 2.16C-2.16D)**. Implanting MC57-SIY tumors into IRF4$^{f/f}$xCD11c^{Cre} mice, we observed a 62% reduction in ISG$^+$ DC infiltrating regressor tumors in Cre-expressing mice compared to non-Cre-expressing littermates **(Figures 2.16E-2.16F)**. The remaining ISG$^+$ DC in Cre-expressing IRF4$^{f/f}$xCD11c^{Cre} mice is likely attributable to incomplete Cre recombination efficiency, although it is also possible that IRF4-independent DC2 may also contribute to the ISG$^+$ DC cluster. Nonetheless, these results indicate that the large majority of ISG$^+$ DC are indeed IRF4-driven DC2.

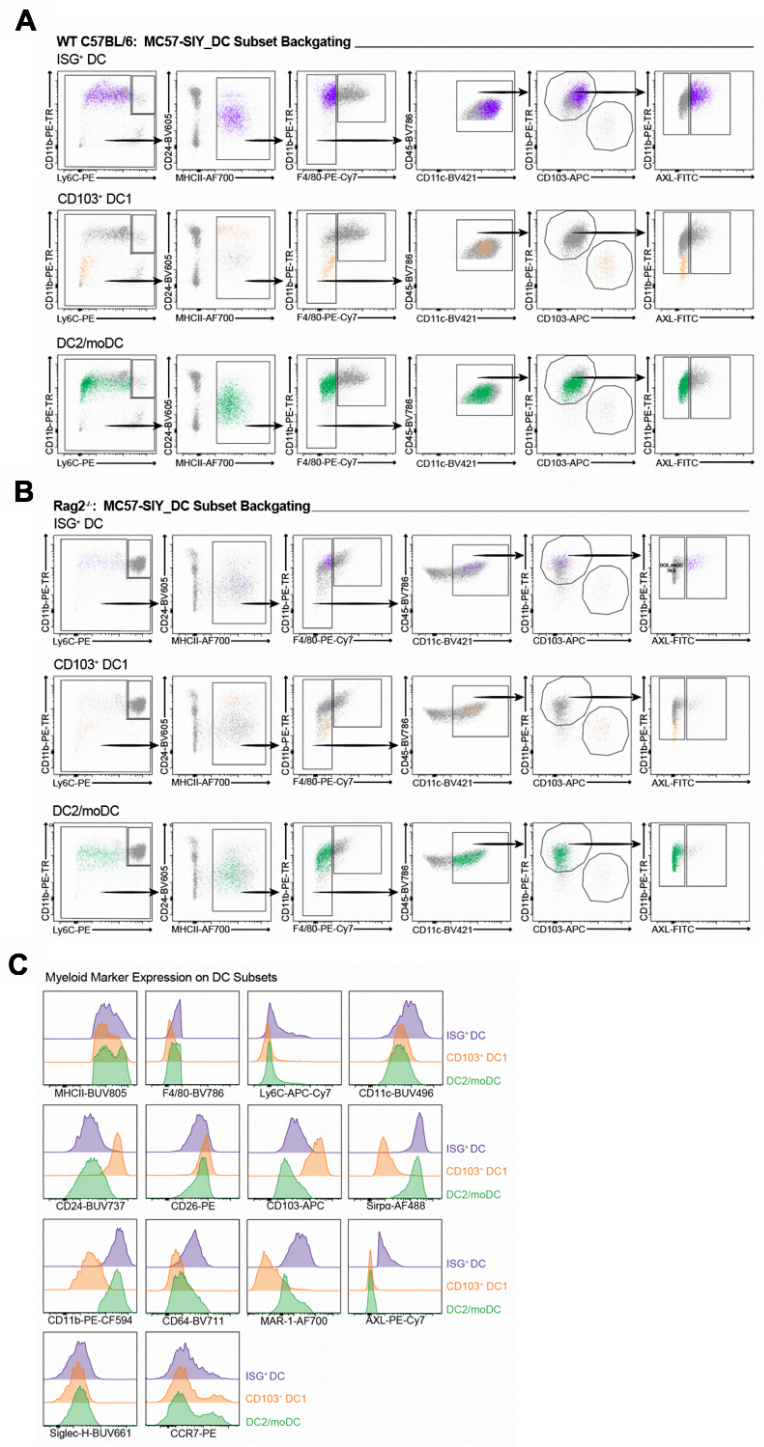

Figure 2.14. ISG^{+} DC are phenotypically distinct from DC1 but express several DC2-specific markers.
(A-B) Flow cytometry backgating of DC subsets in MC57-SIY tumors at day 7 post-implantation in WT **(A)** and Rag2$^{-/-}$ **(B)** mice.
(C) Flow cytometry histograms showing myeloid marker expression on ISG^{+} DC, CD103^{+} DC1 and DC2/moDC in MC57-SIY tumors at day 7 post-implantation in WT mice.
(A-C) Representative data from one of two independent experiments are shown (n = 3 mice per experiment).

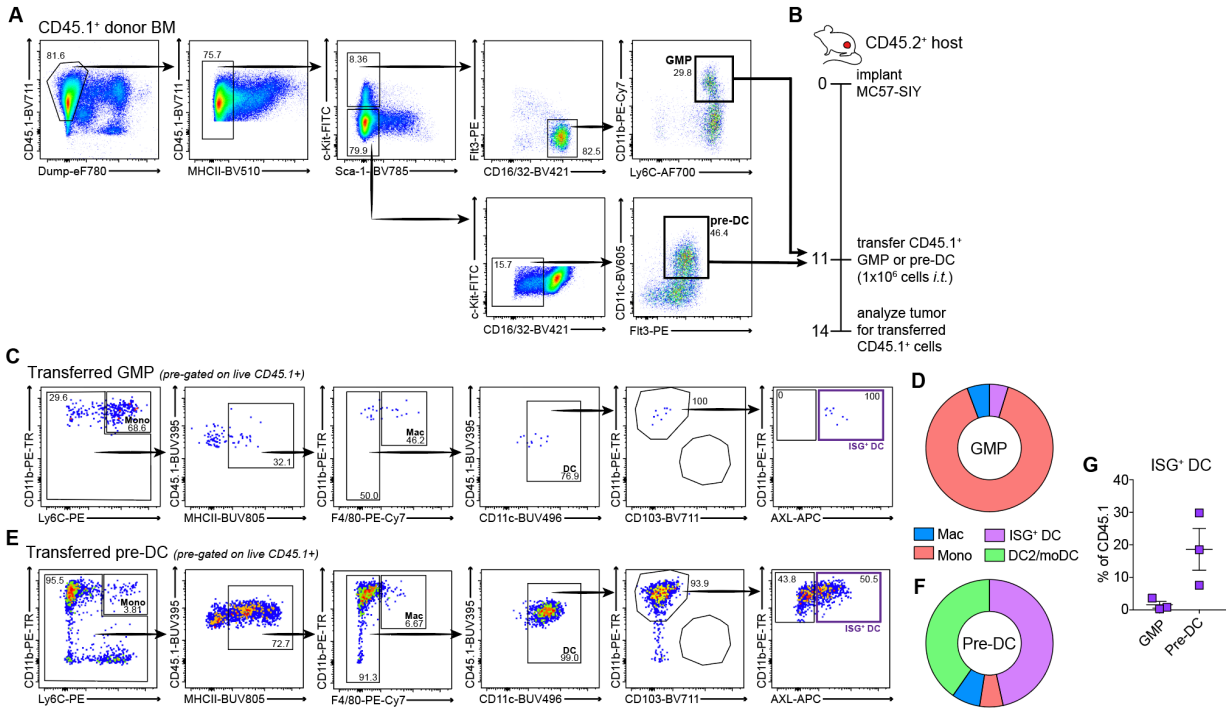

Figure 2.15. ISG$^+$ DC develop from pre-DC progenitors.
(A) Representative flow cytometry gating strategy for GMP and pre-DC in BM of donor WT CD45.1$^+$ mice.
(B) Experimental design for **(C-G)**.
(C) Representative flow cytometry gating strategy to examine fates of transferred CD45.1$^+$ GMP in MC57-SIY tumors at day 14 post-implantation in Rag2$^{-/-}$ mice.
(D) Pie chart depicting fates of transferred CD45.1$^+$ GMP at 3 days post-intratumoral transfer into MC57-SIY tumors.
(E) Representative flow cytometry gating strategy to examine fates of transferred CD45.1$^+$ pre-DC in MC57-SIY tumors at day 14 post-implantation in Rag2$^{-/-}$ mice.
(F) Pie chart depicting fates of transferred CD45.1$^+$ pre-DC at 3 days post-intratumoral transfer into MC57-SIY tumors.
(G) Frequency of ISG$^+$ DC derived from transferred GMP or pre-DC as a percentage of CD45.1$^+$ cells.
(C-G) Representative data from one of three independent experiments is shown **(C-F)**. Pooled data from three independent experiments are shown **(G)** (n = 3-5 donor mice and n = 1 recipient mice per group per experiment).

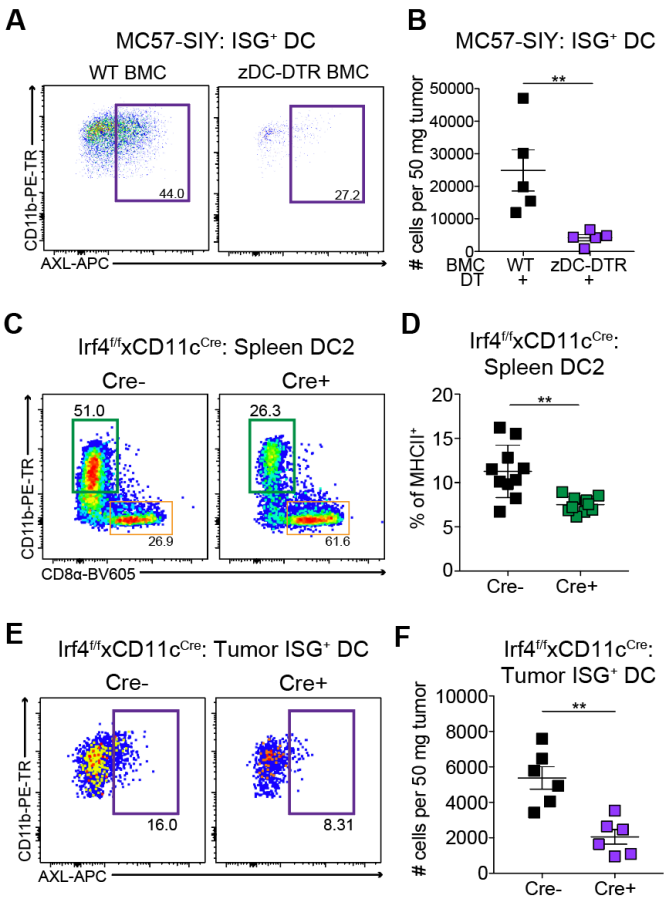

Figure 2.16. ISG$^+$ DC comprise CD11b$^+$ conventional DC, including IRF4-driven DC2.
(A) Representative flow plot of ISG$^+$ DC in MC57-SIY tumors at day 7 post-implantation in DT-treated WT (control) or zDC-DTR (cDC-depleted) BMC mice.
(B) Quantification of ISG$^+$ DC in MC57-SIY tumors as absolute numbers per 50 mg tumor in DT-treated WT (control) or zDC-DTR (cDC-depleted) BMC mice. Data shown are from one experiment (n = 5 mice per group per experiment).
(C) Representative flow plot of splenic cDC subsets in Cre- or Cre+ IRF4$^{f/f}$xCD11c^{Cre} mice.
(D) Quantification of splenic cDC subsets as a percentage of MHCII$^+$ cells in Cre- or Cre+ IRF4$^{f/f}$xCD11c^{Cre} mice. Data shown are pooled from two independent experiments (n = 4-6 mice per group per experiment).
(E) Representative flow plot of ISG$^+$ DC in MC57-SIY tumors at day 7 post-implantation in Cre- or Cre+ IRF4$^{f/f}$xCD11c^{Cre} mice.
(F) Quantification of ISG$^+$ DC in MC57-SIY tumors as absolute numbers per 50 mg tumor in Cre- or Cre+ IRF4$^{f/f}$xCD11c^{Cre} mice. Representative data from one of two independent experiments are shown (n = 4-6 mice per group per experiment). **p<0.01; MWU test (B, D, F). Data are shown as mean ± s.e.m.

Importantly, using data from Cheng *et al. Cell* 2021, a recent resource publication on human tumor-infiltrating myeloid cells (Cheng et al., 2021), we observed that cells that scored highly for the ISG$^+$ DC signature were significantly enriched (p=3.86x10^{-123}) in the c5_cDC2_ISG15 cluster **(Figure 2.17A)**, indicating that an ISG$^+$ DC-like cell population

can be found infiltrating human tumors. Furthermore, the c5_cDC2_ISG15 cluster was significantly enriched for the ISG$^+$ DC signature ($p \leq 1.57 \times 10^{-29}$) in a pairwise comparison against other clusters. Additionally, in our re-analysis of the Cheng *et al.* dataset restricted to tumor-derived cells, we observed a similar enrichment ($p = 3.04 \times 10^{-106}$) in the c5_cDC2_ISG15 cluster **(Figure 2.17B)**. It is worth noting, however, that the majority of cells in the Cheng *et al.* c5_cDC2_ISG15 cluster were derived from a single patient with renal cell carcinoma (Cheng et al., 2021), which is a relatively immunogenic cancer type (Heidegger, Pircher, & Pichler, 2019). As we also identified ISG$^+$ DC in the highly immunogenic MC57-SIY regressor tumor, it is conceivable that the presence of ISG$^+$ DC may be restricted to highly immunogenic tumors. This notion might explain why they are not frequently detected in the majority of previously published human tumor scRNA-seq datasets, which are primarily generated from late-stage or progressing tumors. Nonetheless, the observation of the ISG$^+$ DC signature in a cluster of human tumor-infiltrating cDC2 indicates that they may contribute to the anti-tumor immune response against human tumors.

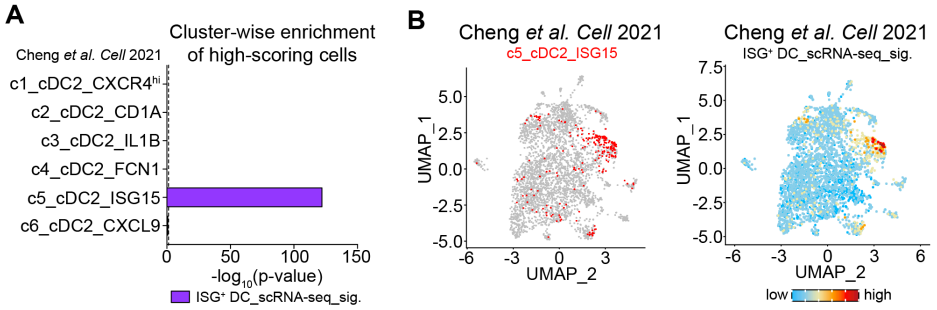

Figure 2.17. The ISG$^+$ DC signature is expressed by a cluster of DC2 infiltrating human tumors (Cheng *et al. Cell* 2021).
(A) Cluster-wise enrichment of cells from Cheng *et al. Cell* 2021 that scored highly for the scRNA-seq-derived ISG$^+$ DC signature. Dotted line denotes significance threshold (p=0.05, hypergeometric test).
(B) UMAP plot from re-analysis of human tumor-infiltrating cDC2 subsets from Cheng *et al. Cell* 2021. Cells that are found in the Cheng *et al.* c5_cDC2_ISG15 cluster are highlighted in red.

(C) UMAP plot of human tumor-infiltrating DC2 subsets from Cheng *et al. Cell* 2021. Each cell is colored by its expression module score of the scRNA-seq-derived ISG$^+$ DC signature. These cells overlap with cells that are in the Cheng *et al.* c5_cDC2_ISG15 cluster **(B)**.

ISG$^+$ DC acquire antigen by cross-dressing with tumor-derived pMHC-I complexes.

We next aimed to elucidate whether ISG$^+$ DC from regressor MC57-SIY tumors were capable of activating CD8$^+$ T cells. To this end, their stimulatory ability was evaluated using our previously established *ex vivo* co-culture assay **(Figure 2.18A)**. ISG$^+$ DC induced similar levels of 2C T cell expansion as DC1 and to a significantly higher degree than DC2/moDC as measured by replication indices **(Figure 2.18B)**. This observation suggests that in regressor MC57-SIY tumors, the stimulatory DC fraction primarily comprises DC1 and ISG$^+$ DC. As it is well-established that DC1 excel at cross-presenting cell-associated antigens to prime CD8$^+$ T cells compared to DC2 and other subsets (Broz et al., 2014; Edelson et al., 2010; Hildner et al., 2008; Iyoda et al., 2002), our observations from the co-culture assays prompted us to interrogate the mechanism of antigen-presentation used by ISG$^+$ DC to activate CD8$^+$ T cells. Three routes of antigen presentation used by DC to prime CD8$^+$ T cells have been reported: (1) direct presentation of intracellular antigens (exclusive to pathogen-infected DC); (2) cross-presentation of exogenously-derived antigens (i.e. dead cell debris); and (3) cross-dressing with pre-formed functional pMHC complexes derived from adjacent cells through membrane exchange (Embgenbroich & Burgdorf, 2018). Studies initially described the phenomenon of cross-dressing between virally infected and non-infected DC (Wakim & Bevan, 2011), but there is increasing evidence that cross-dressing as a means of antigen-presentation also occurs in the tumor setting and contributes to the induction of anti-tumor T cell responses (Das Mohapatra et al., 2020; Nakayama, Hori, Toyoura, & Yamaguchi, 2021;

Squadrito, Cianciaruso, Hansen, & De Palma, 2018). As cross-presentation and cross-dressing are most relevant in the cancer setting, we focused on these two pathways as the potential mechanism by which ISG$^+$ DC are activating CD8$^+$ T cells.

We have thus far demonstrated that systemic anti-tumor T cell responses against regressor MC57-SIY tumors are preserved in DC1-deficient Batf3$^{-/-}$ mice **(Figure 2.4)**. As the only other major stimulatory DC in the tumor **(Figure 2.18B)**, ISG$^+$ DC likely drive these responses in Batf3$^{-/-}$ mice. Therefore, by using the T cell response in Batf3$^{-/-}$ mice as a readout of activation by ISG$^+$ DC, we could infer their specific mode of antigen presentation. We first probed whether the preserved T cell responses in Batf3$^{-/-}$ mice were attributable to cross-dressing. MC57-SIY tumor cells lacking MHC-I expression were generated via CRISPR-Cas9-mediated deletion of β-2 microglobulin (β2M), which rendered them unable to transfer MHC-I complexes to ISG$^+$ DC during cross-dressing. We validated outgrowth of this line in WT mice **(Figure 2.19A)**. In contrast to MC57-SIY cells **(Figures 2.19B-2.19C, #1-2)**, implantation of MC57-SIY-β2M$^{-/-}$ cells into Batf3$^{-/-}$ mice led to complete loss of systemic anti-tumor T cell responses by IFNγ-ELISpot **(Figures 2.19B-2.19C, #3)**. This observation suggested that ISG$^+$ DC failed to induce a T cell response when they were precluded from acquiring pre-formed pMHC complexes from the tumor. One possible alternative explanation is the contribution from direct priming by the tumor cells themselves; however, our previous data demonstrating complete ablation of anti-tumor T cell responses in CD11c-DTR BMC mice **(Figure 2.6D)** and zDC-DTR BMC mice **(Figures 2.7A-2.7C)** effectively excludes this possibility. As an additional control to ensure that antigen from MC57-SIY-β2M$^{-/-}$ cells could be cross-presented in a WT host (i.e. by DC1), we implanted MC57-SIY-β2M$^{-/-}$ cells into WT mice

and indeed observed induction of a systemic T cell response **(Figures 2.19B-2.19C, #4)**. Taken together, these data led us to hypothesize that ISG$^+$ DC were activating CD8$^+$ T cells by cross-dressing with tumor-derived pMHC complexes.

We used several complementary approaches to validate that ISG$^+$ DC are indeed capable of cross-dressing. We first generated β2M$^{-/-}$ BMC mice wherein the host immune cells rather than the tumor cells lacked MHC-I molecules **(Figure 2.20A-2.20B)**. In this mouse model, cross-presentation is not possible due to the lack of host MHC-I and therefore CD8$^+$ T cell activation is dependent on cross-dressing by DC. As we would assess for cross-dressing by staining for tumor-derived H-2K^b, we performed extensive Fc receptor blocking (i.e. CD16/32, CD64) to ensure specific staining. Implanting MC57-SIY cells into β2M$^{-/-}$ BMC mice and profiling the tumor-infiltrating DC, we detected the highest levels of tumor-derived H-2K^b complexes on the surface of β2M$^{-/-}$ ISG$^+$ DC compared to other DC subsets **(Figures 2.20C-2.20D)**. Furthermore, the systemic anti-tumor T cell response in β2M$^{-/-}$ BMC mice as assayed by IFNγ-ELISpot was comparable to the response in WT BMC mice **(Figure 2.20E)**, thus providing additional evidence for the contribution of cross-dressing ISG$^+$ DC to anti-tumor immunity.

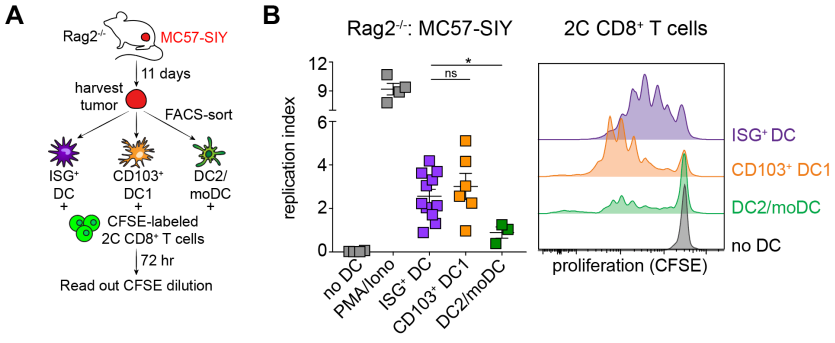

Figure 2.18. ISG$^+$ DC are capable of stimulating CD8$^+$ T cells *ex vivo*.
(A) Experimental design for **(B)**.
(B) *Left*, replication index of 2C T cells after 72 hr co-culture with ISG$^+$ DC, CD103$^+$ DC1 , and DC2/moDC. DC were sorted from Rag2$^{-/-}$ mice bearing MC57-SIY tumors at day 11 post-tumor inoculation. Data shown

are pooled from three independent experiments (n = 5 mice per experiment). *Right,* representative histogram of T cell proliferation peaks following co-culture with DC.
*p<0.05, ns = not significant; MWU test (B). Data are shown as mean ± s.e.m.

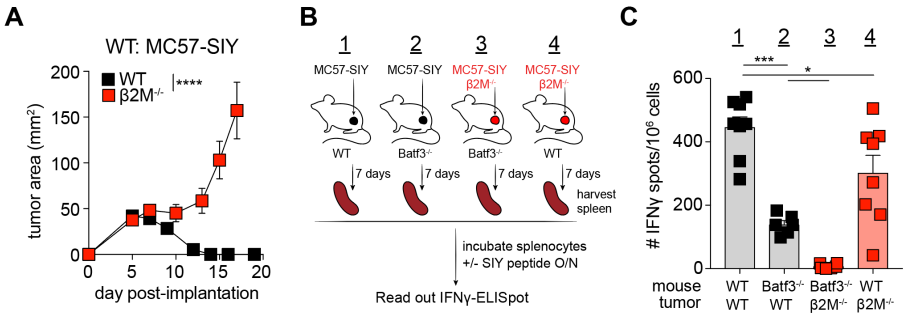

Figure 2.19. Precluding the donation of tumor-derived pMHC-I complexes for cross-dressing (via β2M$^{-/-}$ tumor cells) ablates anti-tumor CD8^{+} T cell responses in Batf3$^{-/-}$ mice.
(A) Tumor outgrowth (mm^2) of MC57-SIY and MC57-SIY-β2M$^{-/-}$ in WT mice. Representative data from one of two independent experiments are shown (n = 3 mice per group per experiment).
(B) Experimental design for **(C)**.
(C) Number of IFNγ-producing splenocytes from WT or Batf3$^{-/-}$ mice implanted with MC57-SIY or MC57-SIY-β2M$^{-/-}$ tumor cells at day 7 post-tumor inoculation. Data shown are pooled from three independent experiments (n = 3-4 mice per group per experiment). *p<0.05, ***p<0.001, ****p<0.0001; MWU test (B) or two-way ANOVA (A). Data are shown as mean ± s.e.m.

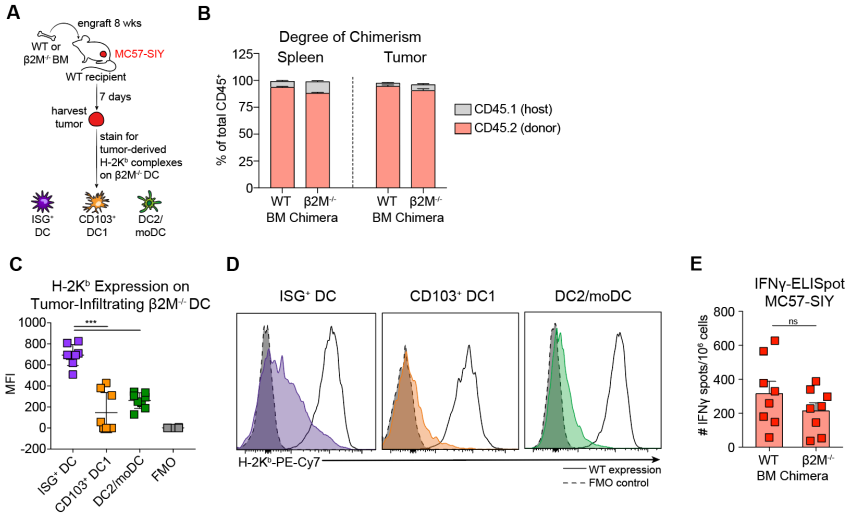

Figure 2.20. ISG^{+} DC can induce systemic anti-tumor CD8^{+} T cell responses by cross-dressing with tumor-derived pMHC-I complexes.
(A) Experimental design for **(B-E)**.
(B) Degree of chimerism in spleen and tumor tissues.
(C, D) Quantification **(C)** and representative flow histograms **(D)** of H-2K^{b} expression on ISG^{+} DC, CD103^{+} DC1, and DC2/moDC infiltrating MC57-SIY tumors in WT or β2M$^{-/-}$ BMC mice.
(E) Number of IFNγ-producing splenocytes from WT or β2M$^{-/-}$ BMC mice implanted with MC57-SIY tumor cells at day 7 post-tumor inoculation.

(B, C, E) Data shown are pooled from two independent experiments (n = 4 mice per group per experiment). ***p<0.01, ns = not significant; MWU test (B, C, E). Data are shown as mean ± s.e.m.

Cross-dressed ISG$^+$ DC can stimulate T cell proliferation *ex vivo* and induce protective systemic anti-tumor CD8$^+$ T cell immunity *in vivo*.

To further confirm that ISG$^+$ DC are cross-dressing, we established a complementary *in vivo* transfer assay, in which we implanted the C57BL/6-derived regressor tumor line (H-2^b) into MHC-I haplotype mismatched Balb/c mice (H-2^d) **(Figure 2.21A)**. For these experiments, the regressor MC57-SIY tumor line was engineered to express the ovalbumin-derived model antigen SIINFEKL (SIIN), which enabled us to detect the transfer of tumor-derived H-2K^b:SIIN complexes to Balb/c DC using the antibody 25-D1.16 (Porgador, Yewdell, Deng, Bennink, & Germain, 1997). Specificity of the 25-D1.16 antibody was validated using an isotype control and a SIIN-negative tumor cell line **(Figures 2.21B-2.21C)**. Following implantation of MC57-SIIN-SIY cells into Balb/c mice, we again detected the highest levels of tumor-derived H-2K^b:SIIN complexes on the surface of Balb/c ISG$^+$ DC, indicating that they are indeed most efficient at cross-dressing **(Figures 2.21B-2.21C)**. Of note, DC1 were able to acquire some appreciable amount of H-2K^b:SIIN complexes, but this was significantly lower compared to the levels on ISG$^+$ DC. To visualize the transfer of tumor-derived pMHC complexes *ex vivo*, we sorted ISG$^+$ DC from Balb/c mice bearing SIIN-negative MC57 tumors and co-cultured them *ex vivo* with MC57-SIIN-SIY tumor cells **(Figure 2.21D)**. Using immunofluorescence microscopy, we confirmed the presence of tumor-derived H-2K^b:SIIN complexes on Balb/c ISG$^+$ DC, indicating that ISG$^+$ DC are capable of acquiring pMHC from tumor cells *ex vivo* **(Figure 2.21E)**.

Having demonstrated that ISG$^+$ DC could cross-dress efficiently with tumor-derived pMHC, we next aimed to determine whether the acquired pMHC complexes were able to activate CD8$^+$ T cells. ISG$^+$ DC, DC1, and DC2/moDC were sorted from MC57-SIIN-SIY (H-2^b) tumors in Balb/c mice (H-2^d) and co-cultured with OTI TCR transgenic CD8$^+$ T cells **(Figure 2.21F)**. Due to the mismatched MHC haplotype between OTI T cells and Balb/c DC in this co-culture system, OTI T cells can only be activated by cross-dressed Balb/c DC that acquired pre-formed tumor-derived H-2K^b:SIIN complexes. Consistent with their higher degree of cross-dressing with H-2K^b:SIIN complexes (Figures 6B-6C), Balb/c ISG$^+$ DC induced the greatest OTI T cell activation as measured by replication indices **(Figures 2.21G-2.21H)**. By contrast, OTI T cell activation by Balb/c DC1 and DC2/moDC was weaker **(Figures 2.21G-2.21H)** in accordance with the lower levels of H-2K^b:SIIN complexes on these DC subsets **(Figures 2.21B-2.21C)**.

To assess whether CD8$^+$ T cells primed by cross-dressed ISG$^+$ DC could induce systemic immunity in the absence of DC1, we performed contralateral flank experiments. MC57-SIY tumor cells were first implanted the flank of Batf3$^{-/-}$ mice to initiate the anti-tumor immune response by ISG$^+$ DC. Six days later, we implanted secondary MC38-SIY tumor cells on the contralateral flank and evaluated their outgrowth **(Figure 2.22A)**. We observed that SIY-specific CD8$^+$ T cells induced by ISG$^+$ DC were able to robustly control the growth of MC38-SIY tumors, with a 90% decrease in average tumor burden compared to control at endpoint **(Figure 2.22B)**. While none of the mice in the control group were tumor-free at the end of the study, four out of 15 mice from the MC57-SIY group had completely eradicated their MC38-SIY tumors. We further determined that the eventual outgrowth of MC38-SIY tumors in all analyzed mice from the MC57-SIY group was due

to loss of the shared SIY antigen **(Figure 2.22C)**. Importantly, implanting MC57-SIY-β2M$^{-/-}$ tumor cells (lacking MHC-I for cross-dressing) in Batf3$^{-/-}$ mice **(Figure 2.22D)** completely failed to induce a protective systemic anti-tumor immune response, which enabled MC38-SIY tumors on the contralateral flank to grow out progressively similar to control **(Figure 2.22E)**. Collectively, these observations confirm that cross-dressed ISG^{+} DC functionally contribute to anti-tumor CD8^{+} T cell immunity.

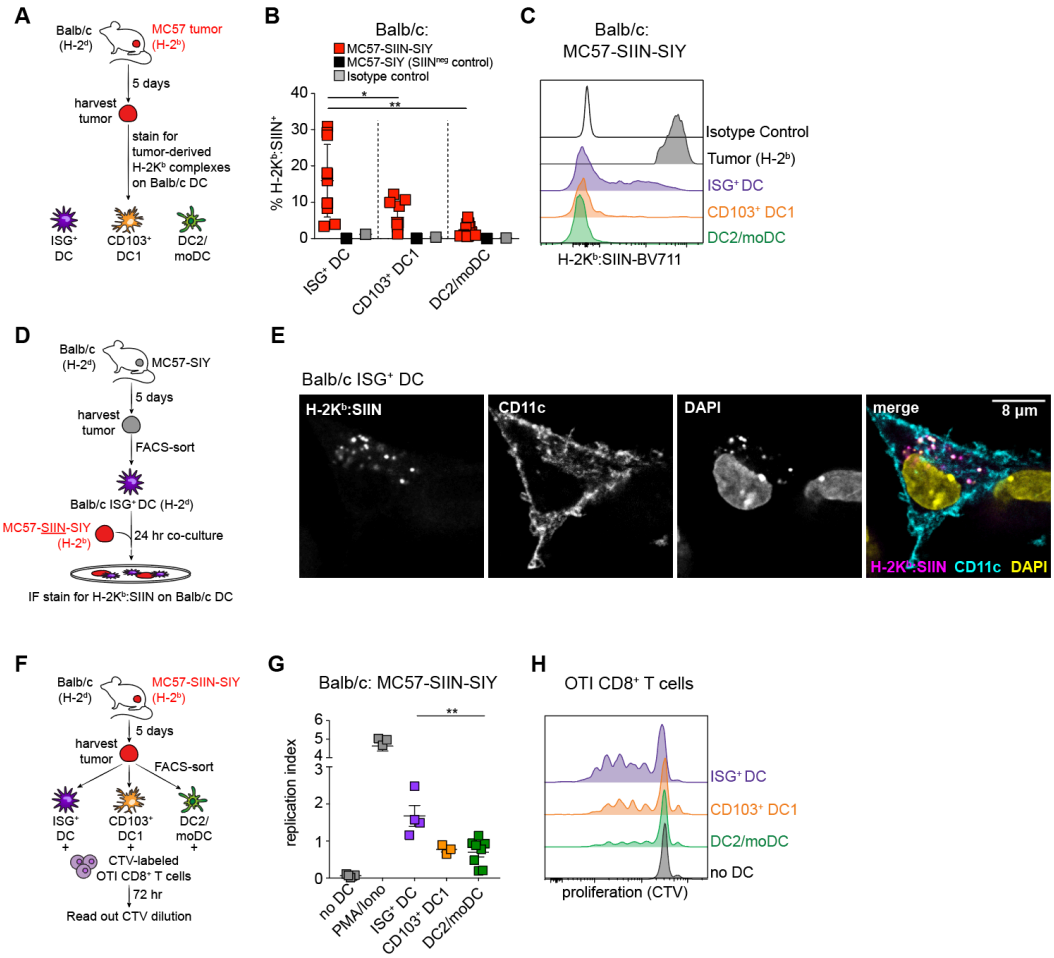

Figure 2.21. Balb/c ISG^{+} DC can cross-dress with tumor-derived H-2K^{b}:SIIN complexes *ex vivo* and activate OTI CD8^{+} T cells.
(A) Experimental design for **(B, C).**
(B, C) Percentage **(B)** and representative histogram **(C)** of H-2K^{b}:SIIN expression on CD11c^{+} DC subsets in Balb/c mice bearing MC57-SIIN-SIY or MC57-SIY (SIIN-negative "SIIN^{neg}" staining control) tumors at day 5 post-tumor implantation. Data shown are pooled from three independent experiments (n = 3-4 mice per experiment).

(D) Experimental design for **(E)**.
(E) Immunofluorescence image of H-2K^{b}:SIIN-cross-dressed Balb/c-derived ISG^{+} DC following a 24 hr co-culture with MC57-SIIN-SIY tumor cells. Representative data from one of two independent experiments are shown (n = 5 mice per experiment).
(F) Experimental design for **(G, H)**.
(G, H) Percentage **(G)** and representative histogram **(H)** of 2C T cell proliferation after 72 hr co-culture with ISG^{+} DC, CD103^{+} DC1, and DC2/moDC. DC were sorted from combined MC57-SIIN-SIY tumors from Balb/c mice bearing at day 5 post-tumor inoculation. Data shown are pooled from three independent experiments (n = 5 mice per experiment). *p<0.05, **p<0.01; MWU (B, G). Data are shown as mean ± s.e.m.

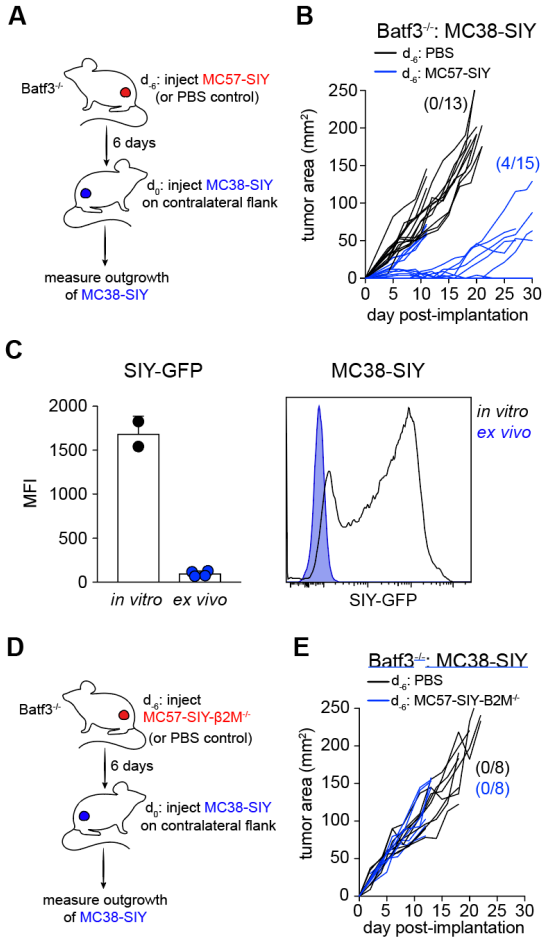

Figure 2.22. Cross-dressed ISG^{+} DC can drive protective systemic anti-tumor immunity in the absence of cross-presenting DC1.
(A) Experimental design for **(B)**.
(B) Tumor outgrowth (mm^{2}) of MC38-SIY in Batf3$^{-/-}$ mice that were pre-inoculated 6 days earlier with MC57-SIY or PBS on the contralateral flank. Numbers in parentheses indicate the number of tumor-free mice. Data shown are outgrowth curves for individual mice pooled from five independent experiments (n = 2-5 mice per group per experiment).
(C) Geometric MFI *(left)* and representative histogram *(right)* of SIY-GFP expression on the MC38-SIY cell line (*in vitro*) and outgrown MC38-SIY tumors isolated from Batf3$^{-/-}$ mice that had been pre-inoculated with MC57-SIY on the contralateral flank (*ex vivo*).
(D) Experimental design for **(E)**.

(E) Tumor outgrowth (mm^2) of MC38-SIY in $Batf3^{-/-}$ mice that were pre-inoculated 6 days earlier with MC57-SIY-$\beta2M^{-/-}$ or PBS on the contralateral flank. Numbers in parentheses indicate the number of tumor-free mice. Data shown are outgrowth curves for individual mice from two independent experiment (n = 4 mice per group per experiment). Data are shown as mean ± s.e.m.

DISCUSSION

In this work, we identified a novel activation state of conventional $CD11b^+$ DC, which we called ISG^+ DC, that was capable of driving anti-tumor $CD8^+$ T cell immunity. The contribution of ISG^+ DC to anti-tumor immunity could be best discerned using $Batf3^{-/-}$ mice lacking DC1. Whereas the absence of DC1 completely ablated anti-tumor $CD8^+$ T cell responses against progressor MC38-SIY tumors, those against regressor MC57-SIY tumors were still induced and capable of driving tumor rejection in $Batf3^{-/-}$ mice. We provide evidence that ISG^+ DC primed tumor-reactive $CD8^+$ T cell responses by cross-dressing with pre-formed tumor-derived pMHC-I complexes. The activation of ISG^+ DC presents a unique opportunity to bypass the requirement for cross-presenting DC1 to drive anti-tumor immunity.

ISG^+ DC are capable of stimulating anti-tumor $CD8^+$ T cell responses in the absence of DC1

The necessity of cross-presenting Batf3-driven DC1 in the induction of anti-tumor $CD8^+$ T cell responses has been well established (Broz et al., 2014; Hildner et al., 2008; Roberts et al., 2016; Salmon et al., 2016; Spranger et al., 2015; Spranger et al., 2017). Tumors that exclude or suppress DC1 function frequently evade the immune response and are refractive to immunotherapy (Broz et al., 2014; Salmon et al., 2016; Spranger et al., 2015; Spranger et al., 2017). Our progressor tumor model MC38-SIY colon carcinoma

exemplifies this paradigm wherein the absence of DC1 completely ablated anti-tumor $CD8^+$ T cell responses and led to accelerated tumor outgrowth. In a departure from this paradigm, however, we demonstrate using the spontaneous regressor tumor model MC57-SIY fibrosarcoma that anti-tumor $CD8^+$ T cell responses can still be maintained, albeit reduced, in the absence of DC1, thus implying the presence of additional stimulatory APC. Using several DC-specific mouse models, we show that conventional DC are the critical stimulatory APC cell type. By sequencing the $CD45^+$ infiltrate of regressor tumors, we identified and validated the existence of a DC activation state that was characterized by ISG expression (ISG^+ DC). ISG^+ DC from regressor MC57-SIY tumors were capable of stimulating 2C TCR-transgenic $CD8^+$ T cell activation *ex vivo* and could drive systemic anti-tumor immunity in $Batf3^{-/-}$ mice, as measured by delayed outgrowth of a secondary tumor expressing the shared SIY antigen on the contralateral flank.

The contrasting anti-tumor immune response in progressor MC38-SIY and regressor MC57-SIY tumors in $Batf3^{-/-}$ mice indicates that there is an opportunity to engage additional stimulatory APC beyond DC1 to drive anti-tumor immunity. Our finding that cell types distinct from DC1 can acquire T cell stimulatory potential fits with observations from other studies that used therapeutic interventions. One study reported that anthracycline-induced tumor control was still observed in $Batf3^{-/-}$ mice lacking DC1 and was dependent on the recruitment and differentiation of $CD11c^+$ $CD11b^+$ $Ly6C^{hi}$ cells (Ma et al., 2013). Another study demonstrated that combination immunotherapy with the PTEN inhibitor VO-OHpic and cyclophosphamide chemotherapy drove the differentiation of $Ly6C^+$ monocytic precursors into stimulatory $CD103^+$ DC1-like cells in a p53-dependent

manner (Sharma et al., 2018). Addition of a p53-agonist enabled the maintenance of Ly6C$^+$ CD103$^+$ cells which enhanced the response to immunotherapy (Sharma et al., 2018). Perhaps of greater relevance to our observations with ISG$^+$ DC in regressor MC57-SIY tumors, a study reported that Batf3-driven DC1 were dispensable for efficacy of Poly(I:C) immunotherapy due to the activation of CD11b$^+$ DC2, which they found acquired the ability to transport antigen to the draining LN and could stimulate CD8$^+$ T cell proliferation *ex vivo* (Gilfillan et al., 2018). As Poly(I:C) induces a strong IFN-I response, it is conceivable that ISG$^+$ DC may be contributing to anti-tumor immunity and efficacy of Poly(I:C) immunotherapy in this study. Uncovering the specific signals that drive stimulatory DC states, such as ISG$^+$ DC, carries significant therapeutic implications and is the focus of our investigation in Chapter 3.

ISG$^+$ DC comprise Zbtb46-dependent and IRF4-driven CD11b$^+$ cDC

Using a combination of DC subset-specific mouse models and classical progenitor transfer fate-mapping assays, we conclusively identified the ontogeny of ISG$^+$ DC as conventional DC, with IRF4-driven DC2 comprising the majority of the population. ISG$^+$ DC from regressor MC57-SIY tumors exhibited transcriptional similarity to Inf-cDC2, which was identified in the context of viral infection (Bosteels et al., 2020). Both ISG$^+$ DC and Inf-cDC2 were capable of stimulating CD8$^+$ T cell responses. While direct comparative studies are needed to determine their degree of relatedness, ISG$^+$ DC did express the Inf-cDC2 markers CD26, CD64, and MAR-1. The expression of CD64 and MAR-1 receptors on Inf-cDC2 was reported to be critical for the uptake of viral antigens by Inf-cDC2 in the form of immune complexes (Bosteels et al., 2020). While it is possible

that CD64 and MAR-1 may also contribute to antigen uptake by ISG$^+$ DC via Fc receptor-mediated endocytosis, this notion requires further evaluation, particularly given our observation that ISG$^+$ DC acquire tumor-derived antigens via cross-dressing.

More importantly, it will be critical to establish whether ISG$^+$ DC can be found in human tumors. Using the Cheng *et al. Cell* 2021 dataset (Cheng et al., 2021), we did observe enrichment of the ISG$^+$ DC signature in a subset of tumor-infiltrating human cDC2, which suggests that they do exist in the human tumor setting; however, their functional contribution to anti-tumor immunity in human cancer patients requires further inquiry. Notably, recent studies in humans identified the DC3 subset, which shared characteristics of DC2 and inflammatory monocytes and could activate CD8$^+$ T cell responses (Binnewies et al., 2019; Bourdely et al., 2020; Dutertre et al., 2019; Villani et al., 2017). It will be interesting to determine whether DC3 might be the human counterpart of ISG$^+$ DC and the implications for anti-tumor immunity.

ISG$^+$ DC cross-dress with tumor-derived pMHC-I to activate anti-tumor CD8$^+$ T cell responses

We demonstrate through several independent experiments that ISG$^+$ DC activate anti-tumor CD8$^+$ T cell responses by cross-dressing with tumor-derived pMHC-I complexes. The anti-tumor CD8$^+$ T cell responses raised against regressor MC57-SIY tumors in Batf3$^{-/-}$ mice were completely ablated when the tumor cells were precluded from donating MHC-I complexes for cross-dressing via β2M deletion. Using β2M$^{-/-}$ BMC mice or MHC-mismatched Balb/c mice, we could detect the highest levels of tumor-derived pMHC-I complexes on the surface of ISG$^+$ DC compared to other DC subsets, which

provided direct evidence of cross-dressing. We were able to further demonstrate that the transferred complexes were functional in an *ex vivo* co-culture assay, where we observed that ISG$^+$ DC could robustly stimulate OTI CD8$^+$ T cell proliferation in a manner that was correlative with their level of cross-dressing.

We offer a couple lines of reasoning to suggest why ISG$^+$ DC are particularly adept at this mode of antigen-presentation in MC57-SIY tumors. First, the phenomenon of CD8$^+$ T cell activation by cross-dressing depends on the acquisition of pMHC-I complexes from tumor cells. Accordingly, the number of pMHC-I complexes expressed on the surface of tumor cells is inherently an important factor for whether cross-dressing can occur. Given that ISG$^+$ DC are likely sensing IFN-I in the TME and that IFN-I is a positive regulator of MHC-I expression (Raval, Puri, Rath, & Saxena, 1998), it is conceivable that MC57-SIY cells may express higher levels of MHC-I, thereby increasing the probability of a cross-dressing event happening. Second, cross-dressing by DC was first reported by Wakim and Bevan in the context of viral infection, where IFN-I are key mediators of the anti-viral immune response (Wakim & Bevan, 2011). The downstream effects of IFNAR signaling in ISG$^+$ DC might explain their enhanced ability to cross-dress. We identified and used AXL solely as a phenotypic marker for ISG$^+$ DC. However, given that AXL is IFN-inducible and has been reported to be an endocytic receptor involved in antigen uptake (Schmid et al., 2016; Subramanian et al., 2014), it is plausible that AXL might be playing a role in the enhanced cross-dressing ability of ISG$^+$ DC.

Outlook and Future Directions

Our work broadens the current knowledge of functional DC states distinct from DC1 that are capable of driving anti-tumor $CD8^+$ T cell responses. It is becoming increasingly important to delineate the individual contributions of distinct DC states to the anti-tumor immune response as they may be non-overlapping. Our work is another example that the functional dichotomy between DC1 and DC2 is not black-and-white and changes under inflammatory conditions, wherein DC2 and other $CD11b^+$ conventional DC can acquire the ability to robustly activate $CD8^+$ T cells. The different modalities of antigen-presentation used by DC1 and ISG^+ DC, cross-presentation and cross-dressing respectively, can potentially have major implications for the resultant anti-tumor T cell response. The density of pMHC complexes on DC, for instance, has been described to impact memory and effector T cell responses (Bullock, Mullins, & Engelhard, 2003; Sykulev, Anikeeva, & Gakamsky, 2012). It is conceivable that cross-dressing as a means of antigen-presentation might yield a lower density of pMHC complexes on the surface of DC compared to direct or cross-presentation, which would therefore influence T cell priming. ISG^+ DC also express higher levels of costimulatory molecules compared to DC1, which can lead to further diversification of activated T cells. Additionally, the high expression of *Cxcl10* (an IFN-induced gene) by ISG^+ DC identified through scRNA-seq suggests that they may contribute to T cell recruitment, which is a function that has recently been ascribed to tumor-resident DC1 (Spranger et al., 2017). Accordingly, moving forward, it will be of great interest to investigate the range of T cell responses induced by DC1 or ISG^+ DC and evaluate their subsequent impact on anti-tumor immunity.

SUPPLEMENTAL TABLES

The following supplemental tables can be found at the link:

https://www.dropbox.com/sh/6km0bmlt16dlzap/AACiRFh5DeaJjaXcxHR7G66ha?dl=0

Table S1. Top 15 DEG for 15 clusters identified from scRNA-seq of the $CD45^+$ immune infiltrate of regressor MC57-SIY tumors in $Rag2^{-/-}$ mice.

Table S2. Top 20 DEG for the seven DC clusters subsetted from scRNA-seq of the $CD45^+$ immune infiltrate of regressor MC57-SIY tumors in $Rag2^{-/-}$ mice.

Table S3. DC subset gene signatures, literature-derived and experimental.

Table S4. ISG^+ DC marker genes and annotation of their subcellular localization.

Table S5. Antibodies and primer sequences.

MATERIALS AND METHODS

Mice. C57BL/6, Balb/c, and $Rag2^{-/-}$ mice were purchased from Taconic Biosciences. $Batf3^{-/-}$, $\beta2M^{-/-}$, $CD11c^{Cre}$, CD11c-DTR, $Clec9a^{-/-}$, $Ifnar1^{-/-}$, and $Irf4^{f/f}$ mice were purchased from Jackson Laboratories and bred in-house. $Irf4^{f/f}$x$CD11c^{Cre}$ mice were obtained by breeding $Irf4^{f/f}$ mice and $CD11c^{Cre}$ mice to specifically ablate Irf4 in the $CD11c^+$ compartment. T cell receptor transgenic (TCR-tg) 2C $Rag2^{-/-}$ and OTI $Rag2^{-/-}$ mice were bred and maintained in-house. Zbtb46-DTR (zDC-DTR) mice were a gift from the Cyster Lab at UCSF and the Mempel Lab at Harvard/MGH. All mice were housed and bred under specific pathogen free (SPF) conditions at the Koch Institute animal facility. $Ifnar1^{-/-}$ mice were initially housed and bred at the Koch Biology Building animal facility. Following rederivation, $Ifnar1^{-/-}$ mice were bred and maintained at the Koch Institute animal facility. For experiments with $Ifnar1^{-/-}$ mice, only female mice 6-8 weeks old were used. For all

other strains, mice were gender-matched and age-matched to be 6-12 weeks old at the time of experimentation. All experimental animal procedures were approved by the Committee on Animal Care (CAC/IACUC) at MIT.

Generation of cerulean-SIIN-SIY expression vector. The pLV-EF1α-IRES-puro vector (Addgene #85132) was digested with *BamHI* and *EcoRI* restriction enzymes (NEB) to linearize the vector. The cerulean-SIIN-SIY insert was generated using the Cerulean-N1 vector (Addgene #54742) linked to a codon-optimized sequence of the SIINFEKL (SIIN) and SIYRYYGL (SIY) peptides. The insert was then cloned into the linearized pLV-EF1α-IRES-puro vector (final construct referred to as 'pLV-EF1α-cerulean-SIIN-SIY-IRES-puro') using the In-Fusion cloning kit (Takara Bio), amplified, and sequenced for accuracy.

Generation of CRISPR knockout constructs. The px459-Cas9-puro vector (Addgene #62988) was digested with the *BbsI* restriction enzyme (NEB) to linearize the vector. CRISPR guides targeting exon 2 of murine β-2 microglobulin (β2M) and exons 1-3 of IRF3 were designed using Benchling **(Table S5)**. Forward and reverse oligos (Integrated DNA Technologies) for each guide were annealed together with a standard annealing protocol, cloned into the px459-Cas9-puro vector by T4 ligation (NEB), amplified, and sequenced for accuracy.

Tumor cell lines and tumor outgrowth studies. Parental and SIY-GFP expressing MC38 colon carcinoma, MC57 fibrosarcoma, 1969 fibrosarcoma, and B16 melanoma tumor cell lines were a gift from the Gajewski laboratory at The University of Chicago. The MC57

tumor line stably expressing cerulean-SIIN-SIY was generated by lentiviral transduction of the parental tumor line with the pLV-EF1α-cerulean-SIIN-SIY-IRES-puro construct and puromycin (Gibco) selected. Expression was confirmed using flow cytometry for cerulean-expressing cells. CRISPR-Cas9-mediated knockout tumor cell lines for β2M and IRF3 were generated by transient transfection with the pooled guide constructs and selected with puromycin for 48 hr. Cells surviving puromycin treatment were expanded, and the ablation of the target gene was confirmed by sequencing, qPCR, and/or western blot.

Tumor cell lines were cultured at 37°C and 5% CO_2 in DMEM (Gibco) supplemented with 10% FBS (Atlanta Biologicals), 1% penicillin/streptomycin (Gibco), and 1X HEPES (Gibco). Tumor cells were harvested by trypsinization (Gibco) and washed 3 times with 1X PBS (Gibco). Cells were resuspended in PBS, and $2x10^6$ tumor cells were injected subcutaneously into the flanks of mice. Subcutaneous tumor area measurements (calculated as *length* x *width*) were collected 2-3 times a week using digital calipers until the endpoint of the study.

In vivo depletion of cytolytic cells. To deplete Natural Killer (NK) cells, 50 μg of anti-NK1.1 (Bio X Cell) or an isotype control antibody (Bio X Cell) was injected intraperitoneally 2 days prior to tumor implantation and subsequently every 3-4 days thereafter for the duration of the study. To deplete T cells, 200 μg of anti-CD8 (Bio X Cell), anti-CD4 (Bio X Cell), combined anti-CD8/anti-CD4, or an equal volume of PBS was injected intraperitoneally 2 days prior to tumor implantation, and 100 μg was subsequently injected every 3-4 days thereafter for the duration of the study.

IFNγ-ELISpot. ELISpot plates (EMD Millipore) were coated overnight at 4°C with anti-IFNγ (BD Biosciences). Plates were washed and blocked with DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1X HEPES for 2 hr at room temperature (RT). Spleens were harvested from mice at day 5 or day 7 post-tumor inoculation and mashed through a 70 μm filter with a 1 mL syringe plunger to generate a single cell suspension. Red blood cells were lysed with 500 μL of ACK Lysing Buffer (Gibco) on ice for 2 min and splenocytes were washed 3 times with chilled PBS. For IFNγ-ELISpot assays using SIY peptide restimulation, $1x10^6$ splenocytes were assayed per well in the presence or absence of 160 nM SIY peptide. For IFNγ-ELISpot assays using irradiated parental tumor cell debris for restimulation **(Figures 3.10G-3.10H)**, $3x10^6$ splenocytes were assayed per well in the presence or absence of $1x10^5$ parental tumor cells that were irradiated a day prior with 4000 rad. As a positive control, splenocytes were incubated with a mixture of 100 ng/mL PMA (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich). Following an overnight incubation at 37°C and 5% CO_2, plates were developed using the BD mouse IFNγ-ELISpot kit, following manufacturer's protocol.

Generation of bone marrow (BM) chimeric mice. Host mice were irradiated with 500 rad, allowed to recover for 3 hr, and subsequently irradiated again with 550 rad. The next day, BM was harvested from the femur and tibia of donor mice, washed and resuspended in PBS, and $1x10^7$ cells were injected retro-orbitally into the irradiated host mice. For mixed BM chimeras, $1x10^7$ total cells of a 50:50 mixture of BM from donor mice was transferred. A period of 8 weeks was allowed for engraftment prior to the start of experiments.

Diphtheria toxin (DT)-mediated depletion. For depletion of DC in CD11c-DTR, CD11c-DTR:Ifnar1$^{-/-}$, and Zbtb46-DTR BM chimeras, 500 ng diphtheria toxin (DT) (Sigma-Aldrich) (or an equivalent volume of PBS for control mice) was injected intraperitoneally 2 days prior to tumor implantation and subsequently injected every other day thereafter for 7 days.

Tumor dissociation. Tumors were dissected from mice, weighed, and collected in 500 µL RPMI (Gibco) containing 250 µg/mL Liberase (Sigma-Aldrich) and 50 µg/mL DNase (Sigma-Aldrich). Tumors were minced with dissection scissors and incubated for 20 min at 37°C for enzymatic digestion. Following the digestion, tumor pieces were mashed through a 70 µm filter with a 1 mL syringe plunger to generate a single cell suspension. The dissociated cells were washed 3 times with chilled PBS containing 1% FBS and 2 mM EDTA (Gibco).

Flow cytometry and cell sorting. Prior to staining, cells were washed with FACS staining buffer (chilled PBS containing 1% FBS and 2 mM EDTA). Cells were stained for 15 min on ice with eBioscience Fixable Viability Dye eFluor 780 to distinguish live and dead cells and with anti-CD16/CD32 (clone 93, BioLegend) to prevent non-specific antibody binding. Cells were then washed once and cell surface proteins were stained for 30 min on ice with fluorophore-conjugated antibodies at the specified dilutions **(Table S5)**. For stains that used biotinylated primary antibodies, cells were washed twice and subsequently stained with a streptavidin-conjugated fluorophore for 30 min on ice. Following the surface

staining, cells were washed twice and analyzed directly or fixed with IC Fixation Buffer (eBioscience) for 20 min at RT for analysis the next day. To obtain absolute counts of cells, Precision Count Beads (BioLegend) were added to samples following manufacturer's instructions. Flow cytometry sample acquisition was performed on a BD LSRFortessa cytometer, and the collected data was analyzed using FlowJo v10.5.3 software (TreeStar). For cell sorting, the surface staining was performed as described above under sterile conditions, and cells were acquired and sorted into RPMI containing 10% FBS, 1% penicillin/streptomycin, and 1X HEPES using a BD FACSAria III sorter.

SIY-pentamer staining. To identify SIY-reactive $CD8^+$ T cells, samples were stained with a 1:100 dilution of a PE-conjugated SIY pentamer (ProImmune) for 30 min on ice. The pentamer was added during the surface staining step in the flow cytometry methods described above.

Progenitor transfer fate-mapping experiment. To expand pre-DC, $CD45.1^+$ C57BL/6 mice were injected every other day with 10 μg recombinant human Flt3L-Ig (Bio X Cell). BM was then harvested from the femur and tibia of mice by flushing the bones with RPMI using a 1 mL syringe. Cells were passed through a 70 μm filter, washed twice with PBS, and subjected to flow cytometry staining and sorting as described above. GMP were sorted as live $CD45.1^+$, $lineage^-$ (CD19, CD3e, NK1.1, MHCII, CD11c), $Sca\text{-}1^-$, $c\text{-}Kit^+$, $Flt3^-$, $CD16/32^+$, $CD11b^+$, $Ly6C^+$. Pre-DC were sorted as live $CD45.1^+$, $lineage^-$ (CD19, CD3e, NK1.1, MHCII), $Sca\text{-}1^-$, $c\text{-}Kit^-$, $CD16/32^-$, $Flt3^+$, $CD11c^+$. Equal numbers of GMP or pre-DC were injected *i.t.* into MC57-SIY tumor-bearing $CD45.2^+$ $Rag2^{-/-}$ host mice at day 11

post-tumor implantation. At 3 days post-transfer, MC57-SIY tumors were harvested and the fates of the transferred cells were analyzed by flow cytometry.

Staining for *in vivo* cross-dressing assay. Related to Figures 4E-4G, 5A-5C, and S5A-S5C. Surface flow staining for H-2K^b or H-2K^b:SIIN on the DC infiltrate of dissociated tumors was performed as follows: Cells were stained for 15 min on ice with eBioscience Fixable Viability Dye eFluor 780 and blocked with anti-CD16/CD32 (clone 93, BioLegend) and BV786 anti-CD64 (clone X54-5/7.1, BD Biosciences) to prevent non-specific antibody binding. Cells were then washed once and cell surface proteins were stained as described above. For detection of H-2K^b, PE-Cy7 anti-H-2K^b (clone AF6-88.5, BioLegend) was used at a 1:200 dilution. For detection of H-2K^b:SIIN, biotinylated anti-H-2K^b:SIIN (clone 25-D1.16, eBioscience) (Porgador et al., 1997) or a biotinylated isotype control (clone 2016875, eBioscience) was used at a 1:200 dilution, followed by a streptavidin-BV711 secondary at a 1:400 dilution. Specificity for H-2K^b or H-2K^b:SIIN staining was validated using a Fluorescence Minus One (FMO) control, an antigen-irrelevant tumor line, and/or an isotype control.

Cross-dressing visualization by immunofluorescence microscopy. MC57-SIY (H-2^b) tumor cells were implanted into the flanks of WT Balb/c mice (H-2^d). On day 5 post-tumor inoculation, Balb/c ISG$^+$ DC were sorted from dissociated tumors, as described previously. Coverslips in 6-well non-TC-treated plates were coated with 500 µL Poly-L-Lysine (Gibco) for 10 min at RT, washed 3 times with sterile water, and air-dried. Sorted Balb/c ISG$^+$ DC and MC57-SIIN-SIY tumor cells at a 1:1 ratio, or separately as controls, were plated on

the coverslips and cultured for 24 hr at 37°C and 5% CO_2 in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, 1X HEPES, 1X MEM Non-Essential Amino Acids, and 1X β-mercaptoethanol. After 24 hr, cells were fixed with 4% paraformaldehyde for 20 min at RT and gently permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min at RT. Coverslips were then blocked with 2.5% bovine serum albumin (Research Products International) and anti-CD16/CD32 (clone 93, BioLegend) for 20 min at RT. Primary antibodies at a 1:200 or a 1:400 dilution **(Table S4)** were added to the coverslips and incubated for 1 hr at RT. Coverslips were then washed 3 times with Dulbecco's PBS with calcium and magnesium (Gibco) for 5 min each. Secondary antibodies **(Table S5)** were added at 1:400 dilution and incubated for 20 min at RT. Following washes, coverslips were mounted onto glass slides using ProLong Gold Antifade with DAPI (Invitrogen). Slides were dried overnight, sealed with clear nail polish, and imaged using a Leica TCS SP8 confocal laser scanning microscope.

Ex vivo APC/DC-T cell co-culture assay. To obtain antigen-presenting cell compartments or specific DC subsets, cells were FACS-sorted from tumors as described above. To obtain $CD8^+$ T cells, TCR transgenic $CD8^+$ T cells were isolated from spleen and lymph nodes of naïve 2C or OTI TCR transgenic C57BL/6 mice using a $CD8^+$ T cell isolation kit (Miltenyi Biotec), following manufacturer's instructions. Isolated $CD8^+$ T cells were washed twice with PBS and stained with 2.5 μM CFSE (eBioscience) in PBS for 8 min at 37°C or 5 μM CellTrace Violet (Thermo Fisher Scientific) in PBS for 20 min at 37°C. The dye was then quenched with FBS, and the cells were washed 3 times with RPMI containing 10% FBS. For the co-culture, $5x10^5$ dye-labeled TCR transgenic $CD8^+$ T cells

and $1x10^5$ sorted antigen-presenting cells or DCs (5:1 T cell-DC ratio) were mixed and added to each well of a V-bottom tissue culture-treated 96-well plate in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, 1X HEPES, 1X MEM Non-Essential Amino Acids (Gibco), and 1X β-mercaptoethanol (Gibco). The cells were cultured at 37°C and 5% CO_2 for 72 hr at which point T cell proliferation was measured by dye dilution via flow cytometry as a proxy for T cell activation. To determine the replication indices per condition, gates for each individual proliferation peak were manually drawn in FlowJo to obtain cell counts per round of division. The replication indices per condition were then calculated as: total number of divided cells / total number of cells that underwent division. Expression of T cell activation markers and cytokines was also assessed by flow staining as described.

Single-cell RNA-sequencing and analysis. Live intratumoral $CD45^+$ cells from $Rag2^{-/-}$ mice bearing MC57-SIY tumors at day 7 post-tumor implantation were FACS-sorted as described above. Sorted cells were washed twice and resuspended at a final concentration of $1x10^3$ cells/μL in chilled PBS containing 0.04% BSA (Thermo Fisher Scientific). The cellular suspension was submitted to the Whitehead Institute Genome Technology Core for cDNA library preparation. Briefly, single cells were encapsulated into droplets using the 10X Genomics Chromium Controller, and the cDNA library was prepared using the Chromium Single Cell 3' Reagent Kits v2 (10X Genomics) following manufacturer's instructions. The resultant cDNA library was then sequenced by the MIT BioMicro Center using an Illumina HiSeq2000. Demultiplexing, mapping to the mm10 genome, and barcode and UMI counting were performed with 10X Genomics Cell Ranger

v3.0.1, and the resultant count matrix was loaded into Seurat v3.2.2 (Butler, Hoffman, Smibert, Papalexi, & Satija, 2018) for further processing. Cells expressing less than 200 genes or more than 4500 genes, as well as cells expressing more than 25% mitochondrial transcripts were excluded, which left 6262 cells for downstream analysis. The data was normalized using the Seurat LogNormalize function with the default scale factor of 10^4. The data was subsequently scaled using the Seurat ScaleData function and latent variables (number of UMIs and percentage of mitochondrial transcripts) were regressed out. The Seurat FindVariableGenes function was used to identify 2000 variable genes for principal component analysis (PCA). The Seurat FindClusters function, which implements the shared nearest neighbor (SNN) clustering algorithm, identified 15 clusters using the top 17 PCA components and a resolution of 0.8. The Seurat FindAllMarkers function was used to identify the differentially expressed genes (DEG) for each cluster compared to all other clusters with default parameters that required genes to be expressed in more than 25% of cells with a minimum 0.5-fold difference **(Table S1)**. To identify clusters, we manually compared the DEG lists of our clusters to reports in the literature.

Single-cell RNA-sequencing DC population analysis and ISG^+ DC surface marker identification. To examine DC at higher resolution, we computationally isolated cell clusters that expressed a canonical DC signature (*H2-Ab1* and *Itgax* and *Flt3*) using the Seurat SubsetData function (clusters 4, 12, 13, 15). The initial analysis using Seurat identified a contaminating macrophage cluster expressing *Adgre1, Mafb,* and *C5ar1* (cluster 3), which was subsequently excluded during another round of filtering. The remaining cells were then passed through the Seurat analysis pipeline as described

above which led to the identification of seven DC clusters (711 cells) using 2000 variable genes, the top 12 PCA components, and a resolution of 0.8 **(Table S2)**. To identify clusters, we manually cross-referenced the DEG lists of the DC clusters to the DC signatures recently reported in the literature (Guilliams et al., 2016; Miriam Merad et al., 2013; Mildner & Jung, 2014; Murphy et al., 2016; Zilionis et al., 2019). To validate our manual cluster assignments, we scored each cell in our dataset using the AddModuleScore function (Tirosh et al., 2016) for expression of DC subset gene signatures that were either published (Zilionis et al., 2019) or generated from an analysis of a publicly available dataset (GSM4505993) (Bosteels et al., 2020) **(Table S3)**. To identify surface markers for cluster 2 (ISG^+ DC) for downstream functional studies, we filtered the DEG list for cluster 2 and required that marker genes must (1) have a minimum avg_logFC threshold of 0.5; (2) have an adjusted p-value < 0.05; (3) be unique to cluster 2; (4) have an enrichment score < 0.5, defined as the ratio of percent expression in all other clusters (pct.2) vs. percent expression in cluster 2 (pct.1), (5) be surface-expressed; and (6) have a commercially available antibody **(Table S4)**.

Generation of Bosteels *et al.* DC signatures. To generate the Bosteels DC signatures for non-mig. cDC2 and Inf-cDC2, we downloaded the "CD45.1 WT derived cells from WT:WT chimeric mice" dataset from the Gene Expression. Omnibus database under accession number GSM4505993 (Bosteels et al., 2020). The dataset was analyzed with the Seurat package as described above, using the top 15 PCA components and a resolution of 0.4 to cluster the cells. Cluster identities were assigned by cross-referencing DEG from each cluster with the published marker genes (Bosteels et al., 2020). The top 20 DEG for non-

mig. cDC2 and Inf-cDC2 **(Table S3)** were used to generate the signatures used in the AddModuleScore analysis.

Bulk RNA-sequencing and analysis. ISG$^+$ DC and CD103$^+$ DC1 were FACS-sorted from MC57-SIY tumors in WT and Rag2$^{-/-}$ mice at day 7 post-implantation as described above using the gating strategies shown in **Figures 2.12A-2.12C**. Cells were sorted directly into TRIzol (Invitrogen), and RNA was isolated using a TRIzol-chloroform extraction. The RNA-containing aqueous layer was collected, purified using the RNeasy MinElute Cleanup Kit (Qiagen) following manufacturer's instructions, and submitted to the MIT BioMicro Center for library preparation (Clontech ZapR) and sequencing (Illumina NextSeq500). Paired-ended 38mer RNA-seq reads were pre-processed to trim five low-quality read positions from the second read (R2) of each pair, using the FASTX-Toolkit (Hannon Lab, CSHL). Reads were then mapped to the USCC mm9 mouse genome build (genome.ucsc.edu) using Bowtie v1.2.3 (Langmead, Trapnell, Pop, & Salzberg, 2009) and gene counts were quantified using RSEM v1.3.1 (Li & Dewey, 2011). Estimated expression counts generated by RSEM were used to detect differentially expressed (DE) genes (p-adj≤0.05) between pairwise conditions (Rag2$^{-/-}$ ISG$^+$ DC vs. Rag2$^{-/-}$ DC1; WT ISG$^+$ DC vs. Rag2$^{-/-}$ DC1) using DESeq2 v1.26.0 (Love, Huber, & Anders, 2014) with a 2X fold-change cutoff per comparison **(Table S3)**. Pairwise signature enrichment was analyzed using the pre-ranked mode in GSEA (A. Subramanian et al., 2005). The AddModuleScore function (Tirosh et al., 2016) in Seurat (Butler et al., 2018) was used to score cells from the scRNA-seq dataset for enrichment of the bulk RNA-seq-derived DC

signatures. Each cell in the scRNA-seq UMAP plot was then colored by its enrichment score for the bulk RNA-seq signatures.

Re-analysis of human dataset from Cheng *et al. Cell* 2021. A normalized expression matrix of scRNA-seq counts for the cDC2 dataset was retrieved from GEO (accession GSE154763) along with associated metadata per cell and processed with Seurat v3.2 (Stuart et al., 2019). A Seurat object was created such that the counts slot and data slot were populated with the library-size corrected counts and log-transformed normalized counts, respectively. The dataset was filtered (based on metadata annotation) to retain only cells from tumor samples. Only twenty patient samples with over 30 cells were selected and used for downstream analyses. Seurat's reference-based integration approach (Stuart et al., 2019) was used to hierarchically integrate samples, first per patient and then across patients. Dimensionality reduction with PCA and UMAP embeddings (with the top 35 principal components) were generated using the integrated dataset. The Seurat default "RNA" assay with log-transformed normalized counts was used for expression-based analyses.

Enrichment of ISG$^+$ DC signature in Cheng *et. al. Cell* 2021. Marker genes for the scRNA-seq-derived ISG$^+$ DC signature **(Table S3)** were translated from mouse to human symbols using the Broad GSEA chip file (MSigDB v7.2; https://data.broadinstitute.org/gsea-msigdb/msigdb/annotations_versioned/Mouse_Gene_Symbol_Remapping_Human_Orthologs_MSigDB.v7.2.chip) (Liberzon et al., 2011; A. Subramanian et al., 2005). First, all

cells in the Cheng *et al.* dataset were scored with the translated marker gene list using the AddModuleScore (Tirosh et al., 2016) function in Seurat. Per-cluster enrichment for high-scoring cells and pairwise enrichment between clusters were assessed as described in the section below. Subsequently, tumor-only cells in our re-analysis of the Cheng *et al.* dataset were scored in a similar fashion and likewise tested for enrichment per cluster.

Statistical tests for enrichment of external signatures in scRNA-seq DC clusters. Related to **Figures 2.13C-2.13F and 2.17A**. Statistical significance for the enrichment of high-scoring cells (standardized module score: > 2 for bulk RNA-seq signatures, > 2.5 for ISG^+ DC scRNA-seq signature) was assessed using an upper-tailed hypergeometric test of proportions (phyper, Stats R package; alpha = 0.05). To compare signature module scores for all cells within a given cluster with cells in every other cluster in a pairwise fashion, a two-sided Wilcoxon rank sum test (wilcox.test, Stats R package; alpha = 0.05) was used.

Data Availability. All data is available in the main text or the supplementary materials. The RNA-seq data has been deposited to the GEO database (GSE181939). Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Protective systemic immunity assay. $Batf3^{-/-}$ mice were implanted subcutaneously in the flank with $2x10^6$ MC57-SIY tumor cells or $2x10^6$ MC57-SIY-$\beta 2M^{-/-}$ tumor cells to induce the immune response by ISG^+ DC. As a control, a cohort of $Batf3^{-/-}$ mice was injected with

equal volume of PBS. Six days after the initial implantation with MC57 tumor cells or PBS control, $2x10^6$ MC38-SIY tumor cells were implanted on the contralateral flanks of the mice, and tumor outgrowth was measured as previously described.

Statistical analysis. All statistical analyses were performed using GraphPad Prism (GraphPad). All data are shown as mean ± s.e.m. Unless stated otherwise, statistical analyses were performed with MWU test (for comparison of two groups) or two-way ANOVA (for multiple comparisons) with *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns = not significant.

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CHAPTER 3

Tumor-derived type-I-interferon drives the stimulatory ISG$^+$ DC state

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ED and SS conceptualized and designed the study. ED performed all experiments and analyzed the data. TBF generated CRISPR-modified cell lines. EL generated recombinant IFNβ proteins in the laboratory of KDW. TD and LY performed the human and mouse tumor line screens and assisted with experiments. AB performed computational analyses. This chapter is part of a revised manuscript under review that was written by ED with comments from other authors.

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ABSTRACT

By comparing the dendritic cell (DC) infiltrate of progressor and regressor tumors, we identified a stimulatory activation state of CD11b$^+$ conventional dendritic cells (DC) expressing an interferon-stimulated gene signature which we called ISG$^+$ DC. ISG$^+$ DC could activate anti-tumor CD8$^+$ T cell responses in the absence of Batf3-driven DC1 by cross-dressing with tumor-derived peptide-MHC-I complexes. While ISG$^+$ DC from regressor tumors were stimulatory, we observed that those isolated from progressor tumors failed to elicit any CD8$^+$ T cell activation *ex vivo*. In order to understand why ISG$^+$ DC from progressor tumors were poorly stimulatory, we sought to identify the signals driving this activation state in regressor tumors. We determined that, unlike progressor tumor cells, regressor tumor cells constitutively produced type-I-interferon (IFN-I) at baseline. The high levels of IFN-I in the regressor tumor microenvironment drove an enhanced maturation state of ISG$^+$ DC, which enabled them to stimulate CD8$^+$ T cells. Ablation of regressor tumor-derived IFN-I led to loss of ISG$^+$ DC-mediated CD8$^+$ T cell priming. Conversely, exogenous addition of IFNβ or Poly(I:C), which induces IFN-I production, was sufficient to activate ISG$^+$ DC and rescue anti-tumor CD8$^+$ T cell responses against progressor tumors in the absence of DC1. Thus, the activation of ISG$^+$ DC presents a novel therapeutic opportunity to enhance CD8$^+$ T cell immunity and warrants further investigation.

INTRODUCTION

Type-I-interferons (IFN-I) are critical for the induction of effective and durable anti-viral and anti-tumor immunity (Musella, Manic, De Maria, Vitale, & Sistigu, 2017; Snell,

McGaha, & Brooks, 2017). Canonical IFN-I comprise a family of pleiotropic cytokines (14 subtypes of murine IFNα and one IFNβ) that signal in an autocrine and paracrine manner through a common heterodimeric IFNAR receptor via the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Cheon, Borden, & Stark, 2014; Musella et al., 2017; Snell et al., 2017). As IFNAR is ubiquitously expressed on all nucleated cells (Cheon et al., 2014; Musella et al., 2017; Snell et al., 2017), the effects of IFN-I can be far-reaching. IFNAR signaling leads to the production of more IFN-I and the regulation of more than 2,000 genes, including ~1,500 IFN-stimulated genes (ISG) and ~300 IFN-repressed genes (IRG) (Cheon et al., 2014; Schneider, Chevillotte, & Rice, 2014), which can shape the outcome of immune responses.

Given the immunostimulatory and anti-cancer effects of IFN-I, the presence of an IFN-I signature in tumors is generally viewed as a hallmark of a T cell-inflamed tumor microenvironment (TME) (Harlin et al., 2009; Spranger, 2016; Trujillo, Sweis, Bao, & Luke, 2018). The efficacy of several types of cancer treatments, including radiotherapy, chemotherapy, and immunotherapy, are also dependent upon induction of an IFN-I response in tumors (Budhwani, Mazzieri, & Dolcetti, 2018; Musella et al., 2017; Sistigu et al., 2014; Zitvogel, Galluzzi, Kepp, Smyth, & Kroemer, 2015). While IFN-I may promote tumor cell death via their direct anti-proliferative and anti-angiogenic effects (Sangfelt & Strander, 2001), there is insurmountable evidence that IFN-I sensing in the DC compartment is required to bridge innate immune sensing and priming of anti-tumor T cell responses (Diamond et al., 2011; Fuertes et al., 2011; Fuertes, Woo, Burnett, Fu, & Gajewski, 2013). These studies have relied on the use of IFNAR1$^{-/-}$:CD11c-DTR mixed bone marrow chimeras wherein IFN-I sensing can be specifically ablated in CD11c^{+} DC.

In particular, the importance of IFN-I for the cross-priming functions of a subset of conventional DC called Batf3-driven DC1 have been well-described (Diamond et al., 2011; Fuertes et al., 2011; Liang, Hannan, & Fu, 2021). IFN-I facilitates DC1-mediated cross-presentation by prolonging antigen retention via decreased endosomal acidification and by promoting DC1 survival (Lorenzi et al., 2011). IFN-I also enhances DC1 maturation via the upregulation of MHC-II and costimulatory molecules, such as CD40, CD80, and CD86, which is integral for successful T cell priming (Montoya et al., 2002; Simmons et al., 2012). Furthermore, IFN-I has been shown to upregulate CCR7 expression which is required for DC1 migration to the draining lymph node—the canonical site of T cell priming (Nguyen-Pham et al., 2011; Parlato et al., 2001). Thus, through these multifaceted effects, IFN-I enables DC1 to effectively mount anti-tumor $CD8^+$ T cell responses.

While the impact of IFN-I on DC1 has been well-characterized, much less is known about the effects IFN-I signaling on other DC subsets. In fact, since IFN-I promotes cross-priming (Fuertes et al., 2013) and DC1 are the predominant cross-priming DC subset (Broz et al., 2014; Edelson et al., 2010; Hildner et al., 2008), it is sometimes assumed that DC1 are the critical cellular targets of IFN-I. However, IFN-I may also mediate the functions of other DC subsets. A recently published study supports this notion (Bosteels et al., 2020). In the context of lung viral infection, IFN-I signaling was found to drive the differentiation of cDC2 to inflammatory cDC2 (Inf-cDC2) (Bosteels et al., 2020). Inf-cDC2 expressed features of macrophages (Fc receptors) and DC1 (IRF8), which enabled them to effectively prime anti-viral T cell responses using internalized antigen from Fc receptor-mediated endocytosis (Bosteels et al., 2020). While it remains to be seen whether Inf-cDC2 may also play a role in anti-tumor immunity, the notion that IFN-I signaling can alter

cellular phenotype and function is intriguing. It will be interesting to explore the impact of IFNAR signaling on distinct DC subsets in the TME and its downstream implications for anti-tumor immunity.

Given the role of IFN-I in bridging innate and adaptive immunity, much attention has been focused on identifying the specific innate immune sensing pathways that drive IFN-I production. IFN-I can be induced by virtually all nucleated cells upon sensing of danger signals via pattern recognition receptors (PRR). In the tumor context, it is believed that innate immune sensing of tumor-derived cytosolic DNA via the cGAS/STING pathway is the predominant driver of a strong IFN-I response (Woo et al., 2014; Zhu et al., 2019). Indeed, insufficient IFN-I signaling due to host STING inactivation has been shown to blunt T cell priming, leading to loss of tumor control (Woo et al., 2014). There is also evidence that innate immune sensing of tumor-derived dsRNA can similarly elicit IFN-I production (McBride, Hoebe, Georgel, & Janssen, 2006). While induction of a host IFN-I response is undeniably key for successful anti-tumor immunity, IFN-I can also be produced directly by tumor cells from activation of the same PRR pathways (Żeromski et al., 2019; Zhu et al., 2019). In fact, there is evidence that endogenous IFN-I production by tumors and intact tumor cell-intrinsic IFNAR signaling is critical for the efficacy of cancer treatments (Lan et al., 2019; Sistigu et al., 2014). Thus, a more nuanced understanding of the contribution of IFN-I derived from different cellular sources and the subsequent impact on anti-tumor immunity can yield refined insights into the role of IFN-I and anti-tumor immunity.

In Chapter 2, we described the identification of a novel activation state of $CD11b^{+}$ DC in spontaneously regressing tumors that expressed an interferon-stimulated gene

signature (ISG$^+$ DC). ISG$^+$ DC activated anti-tumor CD8$^+$ T cells by cross-dressing with tumor-derived peptide-MHC-I (pMHC-I) complexes and could drive protective systemic immunity even in the absence of DC1. Our finding has significant implications for the immuno-oncology field and beyond given the widely held belief that cross-presentation by DC1 is a requirement for T cell priming. In this chapter, we sought to dissect the signals mediating the stimulatory ISG$^+$ DC state, focusing our attention mainly on the role of IFN-I. We determined that unlike progressor tumor cells, regressor tumor cells exhibited constitutive IFN-I production at steady-state. The increased IFN-I in the regressor TME thus drove the ISG$^+$ DC state. Ablation of regressor tumor cell-derived IFN-I via CRISPR-Cas9-mediated deletion of the transcription factor IRF3 abrogated the ability of ISG$^+$ DC to mount anti-tumor T cell responses. Exogenous addition of IFNβ or Poly(I:C), which induces robust IFN-I production, was sufficient to induce ISG$^+$ DC and rescue T cell responses against progressor tumors in the absence of DC1. Our work establishes a rationale for the use of IFN-I or IFN-I-inducing agents to maximally engage the DC compartment to generate anti-tumor CD8$^+$ T cell immunity.

RESULTS

MC57-SIY tumor cells constitutively produce IFN-I at baseline.

Following up on our work described in Chapter 2, we aimed to understand how the functional ISG$^+$ DC state was induced in regressor MC57-SIY tumors. Our data thus far demonstrated that while anti-tumor T cell responses in regressor MC57-SIY tumors were driven by both DC1 and ISG$^+$ DC, those against progressor MC38-SIY tumors were solely dependent on DC1 **(Figure 2.4)**. This observation prompted us to interrogate whether

ISG$^+$ DC could be found in MC38-SIY tumors. While ISG$^+$ DC could be detected in progressor tumors, regressor MC57-SIY tumors were infiltrated with 5.6-fold higher numbers of ISG$^+$ DC on average compared to progressor MC38-SIY tumors **(Figure 3.1A)**. Furthermore, when sorted DC from progressor MC38-SIY tumors were evaluated in our *ex vivo* co-culture assay **(Figure 3.1B)**, only DC1 and not ISG$^+$ DC or DC2/moDC were stimulatory, which is consistent with our data indicating that T cell responses in this model are fully dependent on DC1 **(Figure 3.1C)**.

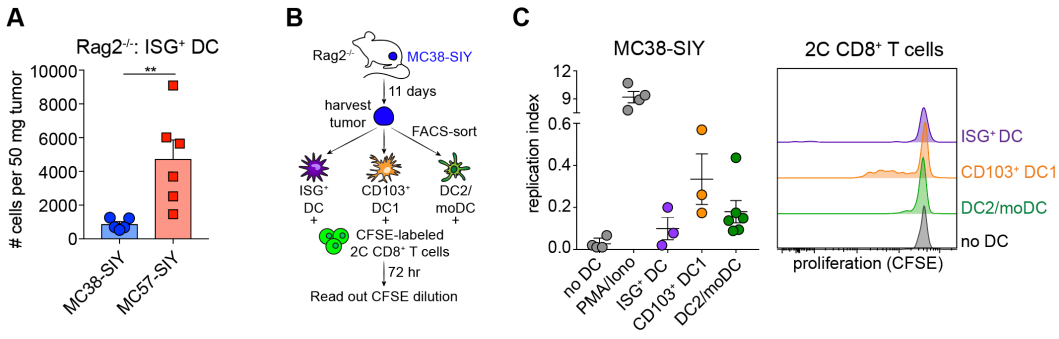

Figure 3.1. ISG$^+$ DC from progressor MC38-SIY tumors fail to stimulate 2C CD8$^+$ T cells *ex vivo*.
(A) Number of ISG$^+$ DC in 50 mg of MC38-SIY and MC57-SIY tumors at day 11 following tumor implantation in Rag2$^{-/-}$ mice. Data shown are pooled from two independent experiments (n = 2-3 mice per group per experiment).
(B) Experimental design for **(C)**.
(C) *Left*, replication index of 2C T cells after 72 hr co-culture with ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC. DC were sorted from combined MC38-SIY tumors from Rag2$^{-/-}$ mice at day 11 post-tumor inoculation. Data shown are pooled from two independent experiments (n = 5 per experiment). *Right,* representative histogram of 2C T cell proliferation peaks following co-culture with DC.
**p<0.01; MWU test (A, C). Data are shown as mean ± s.e.m.

The strong IFN response signature characterizing ISG$^+$ DC in Rag2$^{-/-}$ mice (Figures 2G) indicate that they are likely sensing IFN-I in the tumor. As described earlier, IFN-I proteins (i.e. IFNα/β) are secreted by many cell types upon PRR engagement (Fuertes et al., 2013; Musella et al., 2017; Zitvogel et al., 2015). In the tumor context, activation of STING-dependent cytosolic DNA-sensing in innate immune cells is believed to be the predominant PRR that drives IFN-I production (Fuertes et al., 2013; X. Liu et al., 2018;

Woo et al., 2014). To assess for the contribution of the host STING pathway to anti-tumor immunity against the regressor model, we implanted MC57-SIY cells into STING$^{-/-}$ mice but found that that the tumors still regressed with kinetics similar to those in WT mice **(Figure 3.2)**. This observation suggests that the IFN-I response in MC57-SIY tumors likely does not derive from STING-activated immune cells and implies either activation of other IFN-I-inducing PRR pathway(s) in immune cells or another cellular source.

Several studies have demonstrated that tumor cell-intrinsic IFN-I can be potent activators of anti-tumor immunity (Musella et al., 2017; Trujillo et al., 2018; Zitvogel et al., 2015). Accordingly, tumor cells can evolve to suppress cell-intrinsic IFNAR signaling to favor immune evasion (Albacker et al., 2017; Bidwell et al., 2012; Katlinskaya et al., 2016; Katlinski et al., 2017; Linsley, Speake, Whalen, & Chaussabel, 2014). The differences in ISG^{+} DC from regressor MC57-SIY and progressor MC38-SIY tumors in conjunction with our finding that anti-tumor immune responses against the regressor tumor are STING-independent prompted us to interrogate whether differential tumor cell-intrinsic IFNAR signaling was a contributing factor. We analyzed the expression of IFN-I and ISG transcripts and observed that MC57-SIY cells expressed higher transcripts of *Ifnb1*, *Irf7*, and *Isg15* compared to MC38-SIY cells at steady-state, indicating constitutive IFNAR signaling **(Figure 3.3A)**. As IFN-I are secreted cytokines, we next determined whether the amount of IFN-I present in tumor cell-conditioned media was enough to be sensed by DC to elicit a DC-intrinsic IFN-I response. To this end, we differentiated bone marrow-derived DC (BM-DC) *in vitro* from WT mice using Flt3-L and GM-CSF cytokines (Mayer et al., 2014) and stimulated them overnight with conditioned media from MC38-SIY or MC57-SIY cells. Consistent with their higher expression of IFN-related transcripts, the

MC57-SIY tumor-conditioned media strongly induced the expression of *Ifnb1*, *Irf7*, and *Isg15* transcripts in BM-DC, similar to levels induced by the STING agonist DMXAA **(Figure 3.3B)**. By contrast, the expression in BM-DC cultured in MC38-SIY conditioned media was more comparable to unstimulated BM-DC **(Figure 3.3B)**. To confirm the role of tumor-derived IFN-I, we generated MC57-SIY cells with CRISPR-Cas9-mediated deletion of the transcription factor interferon-regulatory factor 3 (IRF3), which rendered them incapable of producing IFN-I (Sato et al., 2000; Tamura, Yanai, Savitsky, & Taniguchi, 2008). We validated their IRF3$^{-/-}$ status by Western blot and qPCR and confirmed that MC57-SIY-IRF3$^{-/-}$ cells failed to upregulate IFN-I and ISG transcripts upon stimulation with DMXAA **(Figure 3.3C-3.3D)**. Importantly, when we cultured BM-DC with tumor-conditioned media derived from MC57-SIY-IRF3$^{-/-}$ cells, there was no induction of *Ifnb1*, *Irf7*, and *Isg15* transcripts in BM-DC **(Figure 3.3B)**, which confirmed that regressor MC57-SIY tumor cells indeed spontaneously produce IFN-I at steady-state.

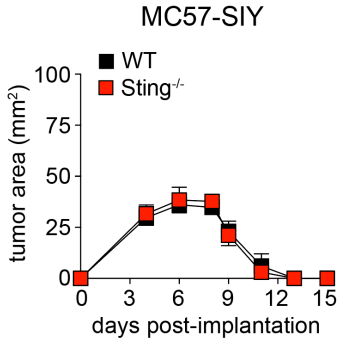

Figure 3.2. The regression of regressor MC57-SIY tumors is independent of STING activation.
Tumor outgrowth (mm^2) of MC57-SIY in WT or Sting$^{-/-}$ mice. Representative data from one of three independent experiments are shown (n = 3-5 mice per group per experiment). Data are shown as mean ± s.e.m.

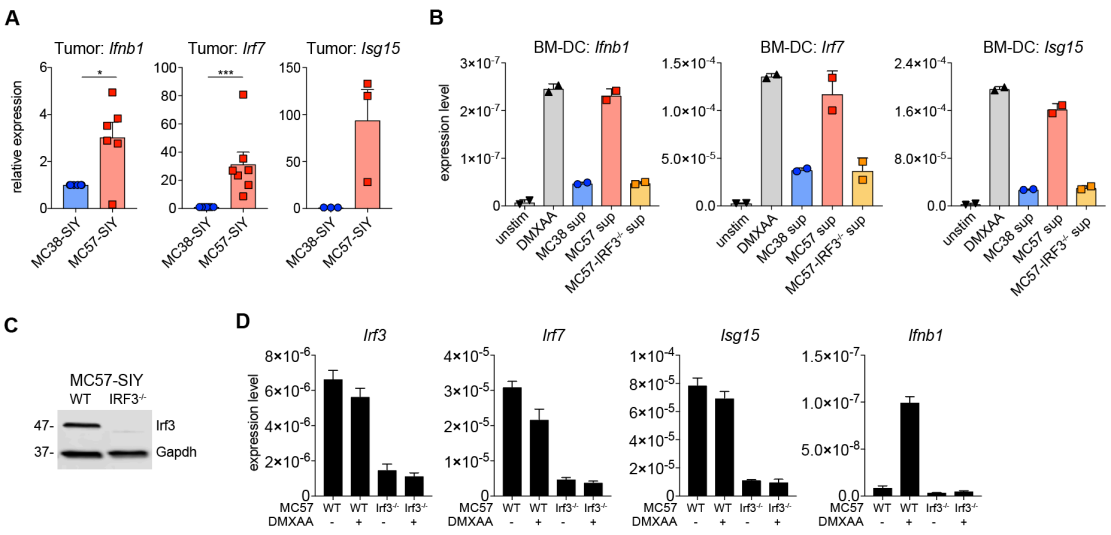

Figure 3.3. Regressor MC57-SIY tumor cells constitutively secrete IFN-I at baseline.
(A) Relative expression of *Ifnb1, Irf7, and Isg15* in MC38-SIY and MC57-SIY tumor cells at steady-state. Data shown are pooled from *Ifnb1* n = 6*; Irf7* n = 7*, Isg15* n = 3 independent experiments.
(B) Expression level of *Ifnb1, Irf7, and Isg15* in WT Flt3-L/GM-CSF-differentiated BM-DCs that were unstimulated or cultured for 24 hr with MC38-SIY, MC57-SIY, or MC57-SIY-IRF3$^{-/-}$ tumor-conditioned media. Data shown are pooled from two independent experiments.

<u>IFNAR signaling in MC57-SIY tumors drives the activation of ISG^{+} DC</u>.

To determine whether regressor MC57-SIY tumor-derived IFN-I could drive the functional ISG^{+} DC state, we assessed how ablation of IFN-I production via IRF3 deletion would impact anti-tumor immunity in WT and Batf3$^{-/-}$ mice. While we still observed rejection in WT mice that was likely mediated by DC1 **(Figure 3.4A)**, MC57-SIY-IRF3$^{-/-}$ tumors grew out progressively in Batf3$^{-/-}$ mice, which was in stark contrast to MC57-SIY tumors **(Figure 3.4B)**. As ISG^{+} DC are the predominant stimulatory DC in Batf3$^{-/-}$ mice, this observation suggests that tumor-derived IFN-I is indeed critical for driving ISG^{+} DC function. It is possible that IFN-I may be required for the differentiation or recruitment of ISG^{+} DC. When we analyzed the intratumoral DC compartment of MC57-SIY and MC57-SIY-IRF3$^{-/-}$ tumors, however, we did not observe significant differences in numbers or proportion for any DC subset, including ISG^{+} DC **(Figures 3.5A-3.5B)**. We validated

these observations using Ifnar1$^{-/-}$ mice. While we observed a trend towards reduced frequency of tumor-infiltrating DC in Ifnar1$^{-/-}$ mice compared to WT mice **(Figures 3.5C-3.5D)**, it was not significant, indicating that DC differentiation or recruitment are likely not the critical factors being impacted by IFN-I. Alternatively, IFN-I might be licensing ISG^{+} DC to stimulate T cells through their maturation enhancing effects (Hervas-Stubbs et al., 2011). In regressor tumors, ISG^{+} DC are the most mature DC, expressing the highest levels of costimulatory molecules such as CD86, CD80, and CD40 **(Figures 3.6A-3.6C)**. To determine whether their maturation state was driven by IFN-I, we generated WT:Ifnar1$^{-/-}$ mixed BMC mice **(Figure 3.7A)**. Indeed, the enhanced maturation of ISG^{+} DC, and to a lesser degree DC1 and DC2/moDC, was intrinsically dependent on IFNAR signaling, as indicated by higher expression of CD86 and MHCII on cells derived from WT BM compared to Ifnar1$^{-/-}$ BM **(Figures 3.7B-3.7C)**.

As costimulatory signaling is a critical component of successful T cell activation (Chen & Flies, 2013; Lenschow, Walunas, & Bluestone, 1996), we next assessed how the impaired maturation of ISG^{+} DC from MC57-SIY-IRF3$^{-/-}$ tumors would impact anti-tumor T cell responses in WT and Batf3$^{-/-}$ mice. While anti-tumor T cell responses were still induced in WT mice, likely by DC1, ISG^{+} DC from MC57-SIY-IRF3$^{-/-}$ tumors completely failed to mount anti-tumor T cell responses in Batf3$^{-/-}$ mice both locally and at the systemic level **(Figures 3.7D-3.7E)**. Together, these data indicate that in regressor MC57-SIY tumors ISG^{+} DC are activated by tumor-derived IFN-I to drive anti-tumor CD8^{+} T cell responses.

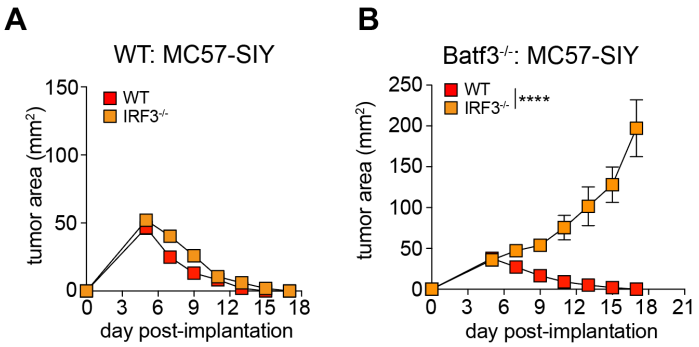

Figure 3.4. Ablation of regressor MC57-SIY tumor cell-derived IFN-I enables tumor escape in Batf3$^{-/-}$ mice.
(A, B) Tumor outgrowth (mm^2) of MC57-SIY or MC57-SIY-IRF3$^{-/-}$ in WT **(A)** or Batf3$^{-/-}$ **(B)** mice. Representative data from one of three independent experiments are shown (n = 3-4 mice per group per experiment). ****p<0.0001; two-way ANOVA (A, B). Data are shown as mean ± s.e.m.

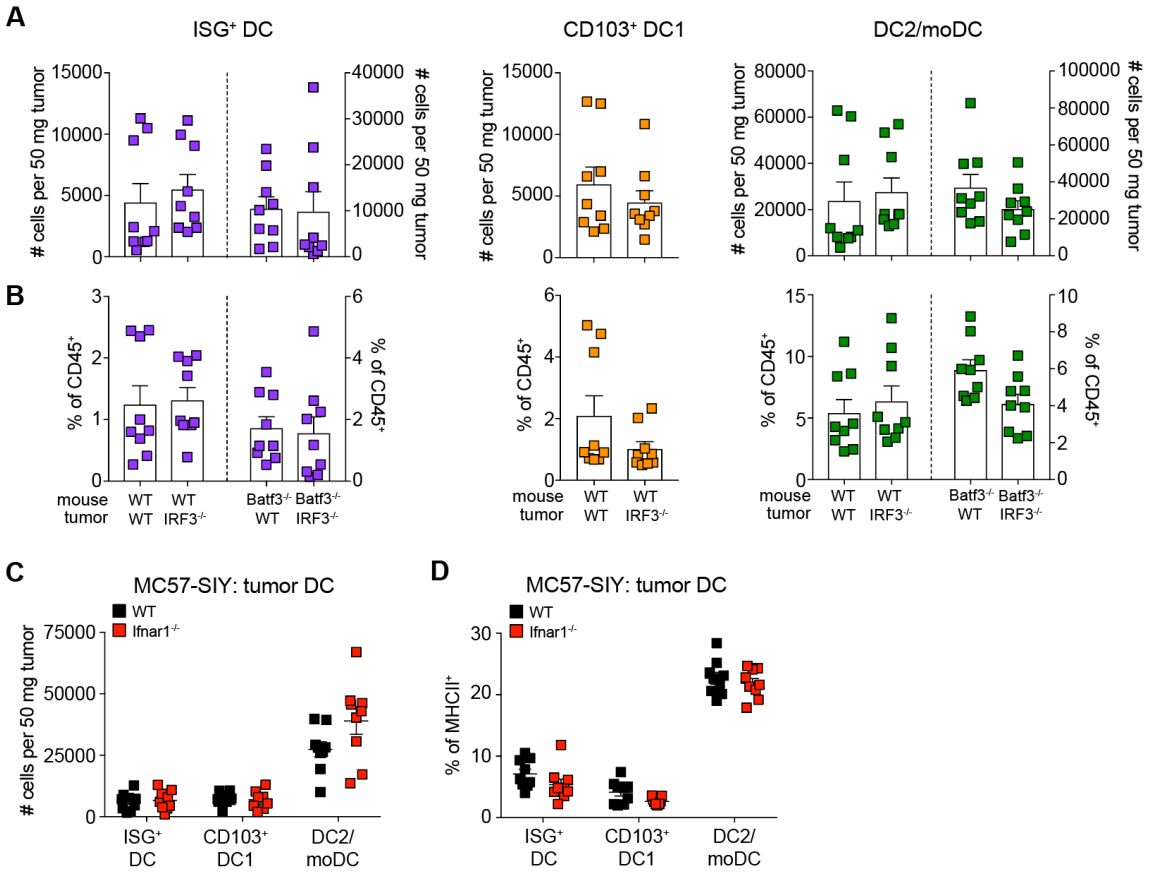

Figure 3.5. IFNAR signaling is not required for the differentiation or recruitment of ISG$^+$ DC.
(A) Number of ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC in 50 mg of MC57-SIY or MC57-SIY-IRF3$^{-/-}$ tumors at day 7 following implantation in WT or Batf3$^{-/-}$ mice.

(B) Percentage of ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC as a frequency of live CD45$^+$ cells in MC57-SIY or MC57-SIY-IRF3$^{-/-}$ tumors at day 7 following implantation in WT or Batf3$^{-/-}$ mice.
(A, B) Data shown are pooled from three independent experiments (n = 3 mice per group per experiment).
(C) Number of ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC in 50 mg of MC57-SIY tumors at day 7 following tumor implantation in WT or Ifnar1$^{-/-}$ mice.
(D) Percentage of ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC as a frequency of MHCII$^+$ cells in MC57-SIY tumors at day 7 following implantation in WT or Ifnar1$^{-/-}$ mice.
(C, D) Data shown are pooled from two independent experiments (n = 4-5 mice per group per experiment). MWU test (A-D). Data are shown as mean ± s.e.m.

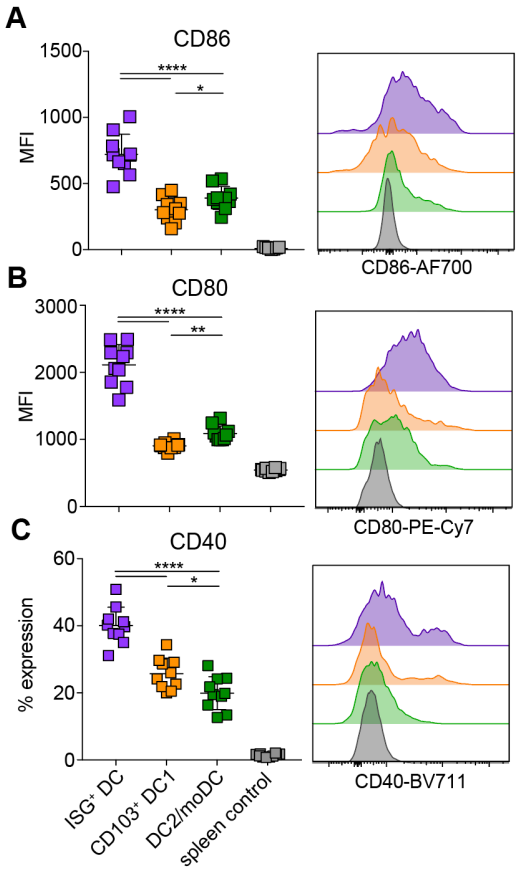

Figure 3.6. ISG$^+$ DC express the highest levels of costimulatory molecules.
(A) *Left,* geometric mean fluorescence intensity (MFI), and *right,* representative histogram of CD86 expressed by ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC from Rag2$^{-/-}$ mice bearing MC57-SIY tumors at day 11 post-tumor implantation.
(B) *Left,* geometric MFI, and *right,* representative histogram of CD80 expressed by ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC from Rag2$^{-/-}$ mice bearing MC57-SIY tumors at day 11 post-tumor implantation.
(C) *Left,* percent expression, and *right,* representative histogram of CD40 expressed by ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC from Rag2$^{-/-}$ mice bearing MC57-SIY tumors at day 11 post-tumor implantation.
(A-C) Representative data from one of two independent experiments are shown (n = 4-5 mice per experiment). *p<0.05, **p<0.01, ****p<0.0001; MWU test (A-C). Data are shown as mean ± s.e.m.

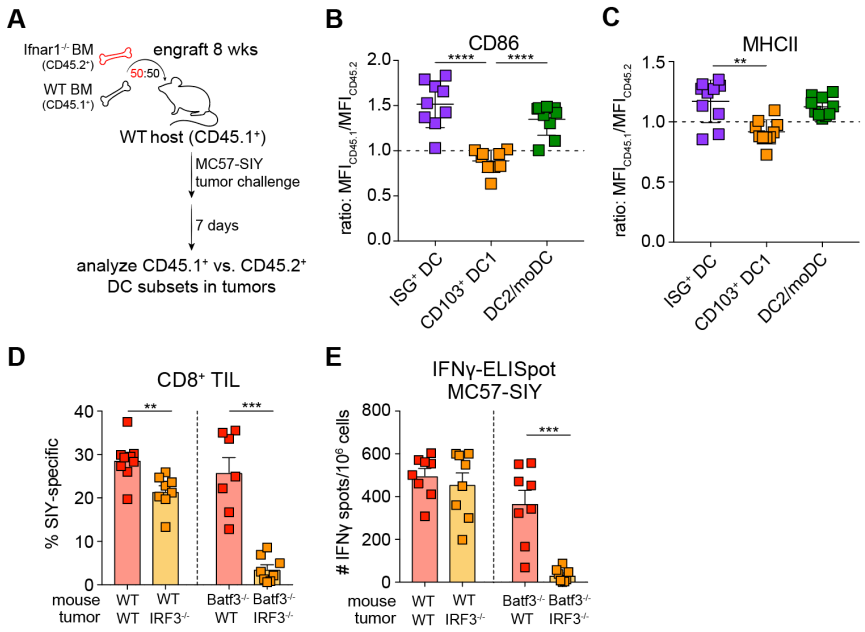

Figure 3.7. IFNAR signaling in the tumor microenvironment drives the enhanced maturation state of ISG$^+$ DC mediates anti-tumor CD8$^+$ T cell responses in Batf3$^{-/-}$ mice lacking DC1.
(A) Experimental design for **(B, C)**.
(B, C) Ratio of the geometric MFI values of CD86 **(B)** or MHCII **(C)** expressed by CD45.1$^+$ DC subsets to CD45.2$^+$ DC subsets. Data shown are pooled from two independent experiments (n = 5 mice per group per experiment).
(D) Quantification of SIY-specific CD8$^+$ T cells in MC57-SIY or MC57-SIY-IRF3$^{-/-}$ tumors from WT or Batf3$^{-/-}$ mice at day 7 post-implantation.
(E) ELISpot quantification of IFNγ-producing splenocytes from WT and Batf3$^{-/-}$ mice bearing MC57-SIY or MC57-SIY-IRF3$^{-/-}$ tumors at day 5 post-tumor inoculation.
(D, E) Data shown are pooled from three independent experiments (n = 3 mice per group per experiment). **p<0.01, ***p<0.001, ****p<0.0001; MWU test (B-E). Data are shown as mean ± s.e.m.

Exogenous addition of IFNβ to progressor tumors restores anti-tumor CD8$^+$ T cell responses in Batf3$^{-/-}$ mice via activation of cross-dressed ISG$^+$ DC.

While immune cells may also be a source of IFN-I in the TME, our data suggests that constitutive IFN-I production by tumor cells is a major driving factor for the induction of the functional ISG$^+$ DC state. To determine whether this observation was generalizable, we screened a panel of murine and human tumor lines representing different tumor types for constitutive IFNAR signaling at baseline. While the vast majority of tumor lines did not exhibit spontaneous IFNAR signaling, a handful did express IFN-I and ISG transcripts at

steady-state, similar to MC57-SIY cells **(Figures 3.8A-3.8B)**. We sorted DC subsets from one such IFN-I/ISG-expressing tumor, the fibrosarcoma 1969-SIY, and evaluated them in our *ex vivo* co-culture assay with 2C T cells **(Figure 3.9A)**. Similar to our observations from MC57-SIY tumors, both DC1 and ISG^{+} DC from 1969-SIY tumors could activate robust 2C CD8^{+} T cell proliferation as measured by replication indices **(Figure 3.9B)**. Systemic anti-tumor T cell responses against 1969-SIY were also preserved in the absence of DC1 as assayed by IFNγ-ELISpot on splenocytes from tumor-bearing Batf3$^{-/-}$ mice **(Figure 3.9C)**. Thus, these data indicate that ISG^{+} DC are contributors to anti-tumor T cell responses in tumors that constitutively produce IFN-I.

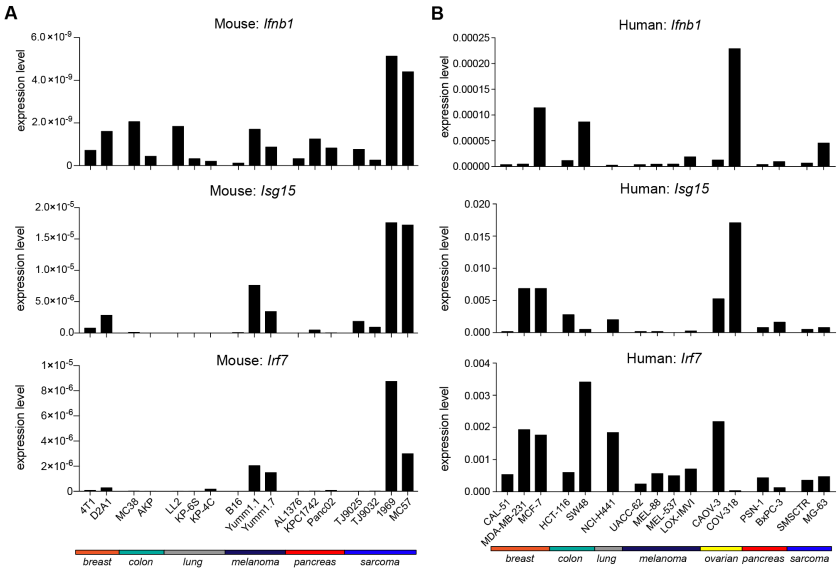

Figure 3.8. The majority of murine and human tumor cell lines do not exhibit constitutive IFNAR signaling at baseline.
(A, B) Expression level of *Ifnb1*, *Isg15*, and *Irf7* across a panel of murine **(A)** and human **(B)** tumor cell lines. Representative data from one of two independent experiments are shown.

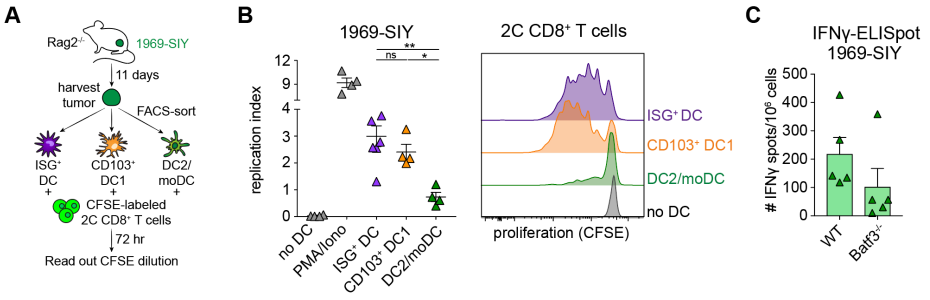

Figure 3.9. Stimulatory ISG$^+$ DC are present in 1969-SIY fibrosarcoma tumors that exhibit constitutive IFNAR signaling at baseline.
(A) Experimental design for **(B)**.
(B) *Left*, replication index of 2C T cells following 72 hr co-culture with ISG$^+$ DC, CD103$^+$ DC1, and DC2/moDC. *Right,* representative example of 2C T cell proliferation peaks following co-culture with DC. DC were sorted from combined 1969-SIY fibrosarcoma tumors from Rag2$^{-/-}$ mice at day 11 post-implantation. Data shown are pooled from two independent experiments (n = 5 mice per experiment).
(C) ELISpot quantification of IFNγ-producing splenocytes from WT and Batf3$^{-/-}$ bearing 1969-SIY tumors at day 5 post-tumor inoculation. Data shown are pooled from two independent experiments (n = 2-3 mice per group per experiment). *p<0.05, **p<0.01, ns = not significant; MWU test (B, C). Data are shown as mean ± s.e.m.

As evident from our IFN-I and ISG screens, however, the majority of tumor cell lines do not exhibit constitutive IFNAR signaling at steady-state **(Figure 3.8)**. Therefore, they are unlikely to harbor stimulatory ISG$^+$ DC. This notion prompted us to ask whether we could induce functional ISG$^+$ DC by exogenously supplying IFN-I to tumor cell lines that otherwise do not secrete IFN-I at baseline. To this end, we co-injected progressor MC38-SIY cells with or without recombinant murine IFNβ into the flanks of Batf3$^{-/-}$ mice and assessed for the rescue of systemic anti-tumor T cell responses as a readout for ISG$^+$ DC activation **(Figure 3.10A #1-3)**. Whereas implantation of MC38-SIY tumor cells alone failed to mount a T cell response in the absence of DC1, co-injection with IFNβ resulted in rescue of the systemic anti-tumor T cell response to 84% of what is observed in WT mice **(Figure 3.10A-3.10B #1-3)**. Furthermore, MC38-SIY tumors co-injected with IFNβ in Batf3$^{-/-}$ mice were more infiltrated with functional SIY-specific CD8$^+$ T cells **(Figure 3.10C)** as well as ISG$^+$ DC that exhibited higher expression of CD86 and CD80

costimulatory molecules **(Figure 3.10D-3.10F)**. To further confirm the role of ISG$^+$ DC, we assayed whether the restored T cell response was dependent on cross-dressing as a functional readout for ISG$^+$ DC. To this end, we co-injected MC38-SIY-β2M$^{-/-}$ tumor cells, lacking MHC-I for cross-dressing, with IFNβ and implanted them into Batf3$^{-/-}$ mice **(Figure 3.10C #4)**. Strikingly, we observed that when ISG$^+$ DC were precluded from acquiring tumor-derived pMHC (via β2M$^{-/-}$ tumor), there was no rescue of T cell responses in Batf3$^{-/-}$ mice despite the presence of IFNβ **(Figures 3.10A-3.10B #4 and 3.10C)**. These data indicate that exogenously provided IFNβ can activate ISG$^+$ DC which rely on cross-dressing to drive CD8$^+$ T cell responses. Importantly, IFNβ-mediated activation of ISG$^+$ DC in Batf3$^{-/-}$ mice could also rescue functional anti-tumor T cell responses against parental MC38 tumors lacking a model antigen both systemically by IFNγ-ELISpot and locally by flow immunophenotyping tumor-infiltrating CD8$^+$ T cells **(Figures 3.10G-3.10J)**. We further extended these observations to the poorly immunogenic B16-SIY model, another progressor tumor that did not exhibit constitutive IFN-I production at baseline **(Figure 3.8A)**. B16-SIY tumors also exhibited rescued T cell responses in Batf3$^{-/-}$ mice when co-injected with exogenous IFNβ **(Figures 3.10K-3.10L)**.

<u>Exogenous addition of Poly(I:C) can also rescue anti-tumor CD8$^+$ T cell responses against progressor tumors in the absence of DC1.</u>

As IFN-I are produced downstream of PRR engagement (Cheon et al., 2014; Musella et al., 2017), we next sought to determine whether exogenous addition of various PRR agonists could similarly rescue systemic CD8$^+$ T cell responses against progressor MC38-SIY tumors in Batf3$^{-/-}$ mice. The cytosolic nucleic acid-sensing pathways,

cGAS/STING and RIG-I/MAVS, which detects cytosolic DNA and RNA, respectively, comprise a critical component of the innate immune sensing of tumors that drives IFN-I production (Iurescia, Fioretti, & Rinaldi, 2018). We thus focused our analysis on comparing whether the IFN-I response induced by Poly(I:C) (RIG-I ligand) or DMXAA (murine STING agonist) was sufficient to drive activation of ISG^+ DC and restore anti-tumor T cell responses in $Batf3^{-/-}$ mice. To this end, we co-injected MC38-SIY tumor cells with naked Poly(I:C) or DMXAA and repeated the IFNγ-ELISpot experiments **(Figure 3.11A)**. Interestingly, only the addition of Poly(I:C) could significantly rescue systemic $CD8^+$ T cell responses against MC38-SIY tumors in $Batf3^{-/-}$ mice, whereas the effect of DMXAA was considerably weaker **(Figure 3.11B)**. To ensure that this effect was specific to PRR agonists that drive a strong IFN-I response, we also co-injected MC38-SIY tumor cells with a TLR2 agonist (Pam2CSK4), which has been shown to dampen IFN-I transcription and favor T_H2 responses (Wenink et al., 2009). In agreement with our data, Pam2CSK4 failed to rescue anti-tumor $CD8^+$ T cell responses in $Batf3^{-/-}$ mice **(Figure 3.11B)**, thus supporting the notion that IFN-I induction is required for restored priming in the absence of DC1.

We were intrigued by the muted effect of DMXAA in restoring anti-tumor priming in $Batf3^{-/-}$ mice, particularly given the reports that STING-mediated IFN-I production is critical for anti-tumor immunity. In an attempt to understand this discrepancy, we tested the ability of naked Poly(I:C) or DMXAA to induce IFN-I production in Flt3-L/GM-CSF-differentiated BM-DC or MC38-SIY tumor cells *in vitro*. Interestingly, naked Poly(I:C) was considerably more potent than DMXAA in driving *Ifnb1* expression in Flt3-L/GM-CSF-differentiated BM-DC **(Figure 3.11C)** but failed to induce *Ifnb1* expression in MC38-SIY

tumor cells **(Figure 3.11D)**, presumably because of inefficient uptake by tumor cells. We thus opted to also use Poly(I:C) complexed with the LyoVec transfection reagent (Poly(I:C)-LyoVec) to stimulate MC38-SIY tumor cells. Similar to our observations using BM-DC, Poly(I:C)-LyoVec but not DMXAA robustly drove *Ifnb1* expression in tumor cells **(Figure 3.11D)**. These data suggest that perhaps there is a threshold level of IFN-I required for successful activation of ISG^+ DC that is not achieved by DMXAA-mediated STING agonism; however, this notion requires further evaluation.

To determine whether the rescued T cell responses induced by Poly(I:C) in $Batf3^{-/-}$ mice were mediated by ISG^+ DC, we assessed for cross-dressing as a functional proxy for these cells. To this end, we co-injected MC38-SIY-$\beta2M^{-/-}$ cells (unable to transfer peptide-MHC) with Poly(I:C) and implanted them in $Batf3^{-/-}$ mice **(Figure 3.11E)**. We observed failed induction of any systemic anti-tumor $CD8^+$ T cell responses **(Figure 3.11F)**, thereby indicating that cross-dressing ISG^+ DC are indeed driving systemic T cell responses in Poly(I:C)-coated MC38-SIY tumors in $Batf3^{-/-}$ mice. Collectively, these data provide evidence that the activation of cross-dressing ISG^+ DC is mediated by IFN-I-induced signaling. Exogenous administration of IFN-I or PRR agonists that drive IFN-I production can induce stimulatory DC states that are distinct from DC1 to enhance anti-tumor $CD8^+$ T cell immunity.

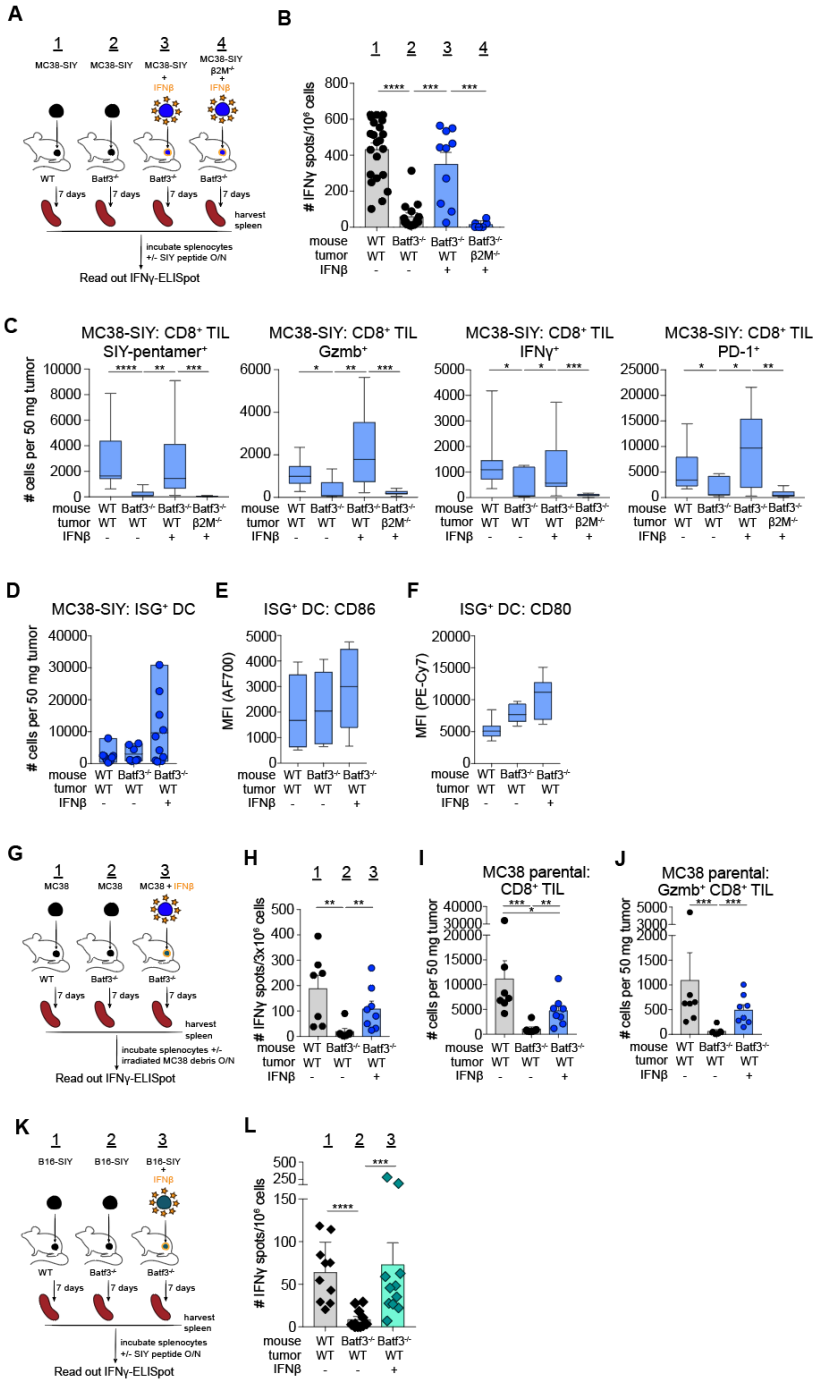

Figure 3.10. Exogenous IFNβ addition activates ISG$^+$ DC and rescues anti-tumor CD8$^+$ T cell responses against progressor tumors in Batf3$^{-/-}$ mice.
(A) Experimental design for **(B)**.
(B) ELISpot quantification of IFNγ-producing splenocytes from WT or Batf3$^{-/-}$ mice implanted with MC38-SIY or MC38-SIY-β2M$^{-/-}$ with or without 50 μg IFNβ at day 7 post-implantation. Data shown are pooled from seven independent experiments (n = 3-5 mice per group per experiment).
(C) Number of SIY-pentamer$^+$, Gzmb$^+$, IFNγ$^+$, and PD-1$^+$ CD8$^+$ T cells in 50 mg of MC38-SIY or MC38-SIY-β2M$^{-/-}$ tumors (+/- 50 μg IFNβ) at day 7 post-tumor implantation in WT or Batf3$^{-/-}$ mice. Whiskers indicate min and max. Line indicates median.

(D) Number of ISG^{+} DC in 50 mg of MC38-SIY tumors (+/- 50 µg IFNβ) at day 7 following tumor implantation in WT or Batf3$^{-/-}$ mice.
(E, F) Geometric MFI of CD86 **(E)** and CD80 **(F)** expression on ISG^{+} DC from MC38-SIY tumors (+/- 50 µg IFNβ) at day 7 following tumor implantation in WT or Batf3$^{-/-}$ mice. Whiskers indicate min and max. Line indicates median.
(B-F) Data shown are pooled from three independent experiments (n = 3-4 mice per group per experiment).
(G) Experimental design for **(H-J)**.
(H) ELISpot quantification of IFNγ-producing splenocytes from WT or Batf3$^{-/-}$ mice implanted with MC38 parental cells with or without 50 µg IFNβ at day 7 post-implantation.
(I, J) Number of CD8^{+} T cells **(I)** or Gzmb^{+} CD8^{+} T cells **(J)** in 50 mg of MC38 parental tumors (+/- 50 µg IFNβ) at day 7 following tumor implantation in WT or Batf3$^{-/-}$ mice.
(H-J) Data shown are pooled from two independent experiments (n = 3-4 mice per group per experiment).
(K) Experimental design for **(L)**.
(L) ELISpot quantification of IFNγ-producing splenocytes from WT or Batf3$^{-/-}$ mice implanted with B16-SIY with or without 50 µg IFNβ at day 7 post-implantation. Data shown are pooled from three independent experiments (n = 3-5 mice per group per experiment). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; MWU test (B-F, H-J, L). Data are shown as mean ± s.e.m.

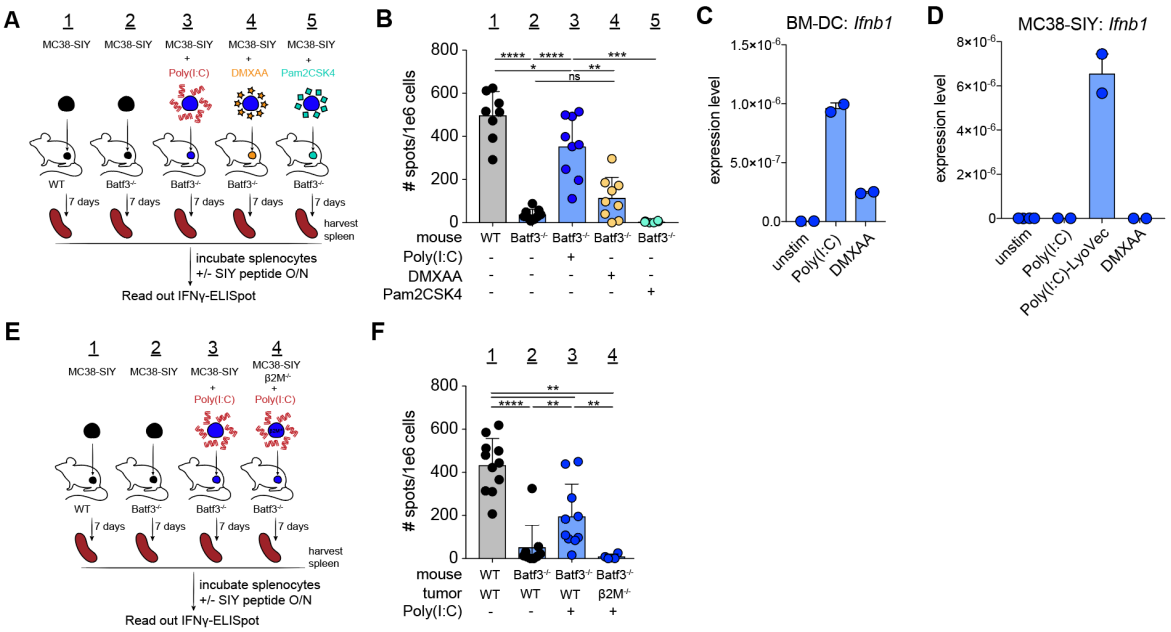

Figure 3.11. Co-injection of Poly(I:C) but not DMXAA or Pam2CSK4 during tumor implantation activates ISG^{+} DC and rescues anti-tumor CD8^{+} T cell responses in Batf3$^{-/-}$ mice lacking DC1.
(A) Experimental design for **(B)**.
(B) ELISpot quantification of IFNγ-producing splenocytes from WT or Batf3$^{-/-}$ mice implanted with MC38-SIY tumor cells (+/- 50 µg PRR agonists) at day 7 post-tumor inoculation. Data shown are pooled from three independent experiments (n = 3 mice per group per experiment).
(C, D) Expression level of *Ifnb1* in Flt3-L/GM-CSF-differentiated BM-DC **(C)** MC38-SIY tumor cells **(D)** following 24 hr stimulation with the indicated PRR agonist. Data shown are from one of at least two independent experiments.
(E) IFNγ-ELISpot experimental design for **(F)**.
(F) ELISpot quantification of IFNγ-producing splenocytes from WT or Batf3$^{-/-}$ mice implanted with MC38-SIY WT or β2M$^{-/-}$ tumor cells (+/- 50 µg Poly(I:C)) at day 7 post-tumor inoculation. Data shown are pooled from four independent experiments (n = 2-3 mice per group per experiment). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant; MWU test (B, E). Data are shown as mean ± s.e.m.

DISCUSSION

In this study, we investigated the signals driving the stimulatory ISG$^+$ DC state. We demonstrated that ISG$^+$ DC in regressor MC57-SIY tumors were activated by tumor-derived IFN-I. IFNAR signaling drove an enhanced maturation state in ISG$^+$ DC, which expressed the highest levels of costimulatory molecules. Given their dependency on IFN-I for activation, we speculate that ISG$^+$ DC are likely most relevant to the immune response in disease settings that trigger a strong IFN-I response, such as in viral infections. Importantly, ISG$^+$ DC could be activated by addition of exogenous IFNβ or Poly(I:C), which stimulates IFN-I production, to drive protective anti-tumor CD8$^+$ T cell responses even in poorly immunogenic tumors lacking DC1.

Constitutive IFN-I production by regressor MC57-SIY tumor cells drives the activation of ISG$^+$ DC

Numerous studies have delved into elucidating tumor-intrinsic signaling pathways that suppress anti-tumor immune responses (Nguyen & Spranger, 2019; Spranger & Gajewski, 2018; Yang, Li, Lei, & Zhang, 2019). We demonstrate here that it is also critical to dissect tumor-intrinsic pathways that are immunostimulatory, as they may yield insights towards modulating the TME to promote productive anti-tumor immune responses. The study of natural spontaneously regressing tumors proves particularly useful in this context. The stark contrast in the anti-tumor immune response between progressor MC38-SIY and regressor MC57-SIY tumors in Batf3$^{-/-}$ mice was mediated by differential tumor cell-intrinsic IFNAR signaling at baseline. MC57-SIY tumor cells constitutively produced IFN-I, whereas MC38-SIY tumor cells did not. The increased presence of IFN-I in the

regressor MC57-SIY TME was then sufficient to drive the maturation and activation of stimulatory ISG^{+} DC, which enabled for anti-tumor CD8^{+} T cell immunity even in Batf3$^{-/-}$ mice lacking DC1.

Our screen of a panel of murine and human tumor lines revealed that while constitutive IFNAR signaling can be observed in other tumor cell lines at steady-state, this is a rather rare phenotype. This is an expected outcome given the immunostimulatory and anti-cancer effects of IFN-I. IFN-I has been shown to promote cross-presentation and enhance cross-priming by DC and is important for the acquisition of effector and memory function by T cells (Hervas-Stubbs et al., 2011; Huber & Farrar, 2011; Schiavoni, Mattei, & Gabriele, 2013). IFN-I also exerts anti-proliferative and anti-angiogenic effects on tumor cells, which promotes tumor cell death (Medrano, Hunger, Mendonça, Barbuto, & Strauss, 2017; Musella et al., 2017). Accordingly, tumor cell-intrinsic suppression of IFNAR signaling via the acquisition of mutations in IFN-I pathway-associated genes (i.e. JAK1/2, STAT1/2, IRF3/7) has been frequently reported as a means of tumor immune evasion (Albacker et al., 2017; Bidwell et al., 2012; Katlinskaya et al., 2016; Katlinski et al., 2017; Linsley et al., 2014). The lack of baseline IFN-I production by most tumors, as we have observed in our screen of tumor cell lines, may thus explain why the ISG^{+} DC state has not been widely described in the tumor context.

Engaging ISG^{+} DC using exogenous IFNβ or Poly(I:C) addition

While we have yet to determine the upstream pathway(s) triggering IFNAR signaling in regressor MC57-SIY tumors, several reports indicate that tumor cell-intrinsic IFN-I can be induced by the aberrant accumulation of intracellular dsRNA or cytosolic

DNA in tumor cells (Ishizuka et al., 2019; H. Liu et al., 2019; Schadt et al., 2019; Takahashi et al., 2021), which signals through the RIG-I/MDA5/MAVS (and other dsRNA sensors) and cGAS/STING pathways, respectively. In line with the immunostimulatory effects of IFN-I, these tumors generated more inflamed microenvironments and were more sensitive to immunotherapy (Ishizuka et al., 2019; H. Liu et al., 2019; Schadt et al., 2019; Takahashi et al., 2021).

In our work, we observed that exogenous addition of IFNβ or the dsRNA mimetic naked Poly(I:C), which drives robust IFN-I production, was able to activate ISG^+ DC and rescue anti-tumor $CD8^+$ T cell responses against progressor tumors in $Batf3^{-/-}$ mice lacking DC1. Interestingly, however, the STING agonist DMXAA was not able to significantly restore anti-tumor $CD8^+$ T cell responses, which was surprising given that activation of the cGAS/STING pathway has been reported to be a major driver of IFN-I production and drives anti-tumor immunity (Corrales et al., 2015; Woo et al., 2014).

A caveat of our assay is that we cannot discern the cell type(s) that sense the PRR agonist and drive the first wave of IFN-I (i.e. tumor, immune, or stromal cells). DMXAA, which is dissolved in DMSO, should be cell membrane permeable and thus likely drives STING-mediated IFN-I production in many cell types. Naked Poly(I:C), on the other hand, requires cellular uptake in order to activate RIG-I/MDA5 and therefore likely only triggers IFN-I production by antigen-presenting cells that have phagocytic ability. In our efforts to understand the blunted ability of DMXAA to induce ISG^+ DC, we pursued a reductionist approach and sought to understand the scale of the IFN-I response induced by Poly(I:C) or DMXAA when used to stimulate immune cells (BM-DC) or tumor cells. Although both naked Poly(I:C) and DMXAA drove robust expression of *Ifnb1* transcripts in BM-DC

compared to unstimulated counterparts, the IFN-I response induced by DMXAA was considerably weaker compared to the response driven by Poly(I:C) stimulation. Intriguingly, we observed that DMXAA completely failed to induce *Ifnb1* transcripts in MC38-SIY tumor cells, while transfected Poly(I:C) (complexed with LyoVec) was able to drive a robust IFN-I response. These data suggest that the failure of DMXAA to activate ISG$^+$ DC likely derives from its inability to drive sufficient IFN-I production. While more work is needed to confirm this notion, our findings fit with data from studies suggesting that loss-of-function mutations or epigenetic silencing of the cGAS/STING promoter regions are frequently observed in a variety of tumor types, thus rendering them non-responsive to cytosolic DNA or DNA damage events (Konno et al., 2018; Sokolowska & Nowis, 2018; Suter et al., 2021; Xia, Konno, Ahn, & Barber, 2016). Interestingly, the RIG-I/MDA5/MAVS cytosolic RNA-sensing pathways were less affected by epigenetic silencing and retained the ability to respond to cytosolic RNA stimulation (Konno et al., 2018). This observation in conjunction with our data carries significant implications for STING-targeted therapies and other PRR agonists that are in clinical development (Amouzegar, Chelvanambi, Filderman, Storkus, & Luke, 2021; Iurescia et al., 2018; Iurescia, Fioretti, & Rinaldi, 2020; Le Naour, Zitvogel, Galluzzi, Vacchelli, & Kroemer, 2020). Specifically, RIG-I/MDA5 agonists may more reliably elicit strong IFN-I responses compared to STING agonists and thus might be the more promising agents to treat cancer.

Outlook and Future Directions

The contribution of ISG$^+$ DC in the context of cancer and cancer therapy warrants further investigation. There is substantial evidence that the success of radiation therapy,

chemotherapy, and immunotherapy is dependent on induction of IFNAR signaling (Burnette et al., 2011; Sistigu et al., 2014; Zaretsky et al., 2016). As ISG$^+$ DC are activated by IFN-I, they are thus likely relevant to the anti-tumor immune response induced by these therapies. Importantly, our data suggests that IFN-I does not have to derive from tumor cells per se. The ability of naked Poly(I:C) addition to activate ISG$^+$ DC and rescue anti-tumor CD8$^+$ T cell responses supports this notion, as it is most likely taken up by phagocytic immune cells. Rather, it seems that the total intratumoral IFN-I concentration appears to be the critical factor for driving ISG$^+$ DC activation. When we exogenously supplied recombinant IFNβ to progressor tumors that lacked constitutive IFNAR signaling at baseline, we could rescue anti-tumor CD8$^+$ T cell responses in Batf3$^{-/-}$ mice in a manner dependent on cross-dressing ISG$^+$ DC. This observation presents an opportunity for therapeutic intervention using tumor-localized IFNβ as well as PRR agonists that drive strong IFN-I responses. Our work suggests that these IFN-I-related therapies might be most beneficial in patients with DC1-excluded tumors (Barry et al., 2018; Böttcher et al., 2018) or poorly immunogenic tumors with defective tumor cell-intrinsic IFN-I signaling (Kalbasi & Ribas, 2020; Zaretsky et al., 2016; Zitvogel et al., 2015).

SUPPLEMENTAL TABLES

The following supplemental tables can be found at the link:

https://www.dropbox.com/sh/6km0bmlt16dlzap/AACiRFh5DeaJjaXcxHR7G66ha?dl=0

Table S5. Antibodies and primer sequences.

Table S6. Mouse and human tumor cell lines for the IFN-I and ISG screen.

MATERIALS AND METHODS

Please refer to Chapter 2 for Materials and Methods related to: *Mice* / *Generation of CRISPR knockout constructs* / *Tumor cell lines and tumor outgrowth studies* / *IFNγ-ELISpot* / *Generation of bone marrow chimeric mice* / *DT-mediated depletion* / *Tumor dissociation* / *Flow cytometry and cell sorting* / *SIY-pentamer staining* / *Ex vivo APC-T cell coculture assay* / *Statistical analysis*.

Generation of recombinant IFNβ. Murine IFNβ1 was cloned with C-terminal His-tags into the gWiz expression vector (Gelantis) using the In-Fusion HD cloning kit (Takara Bio). HEK293 cells were transfected with endotoxin free plasmid DNA (Macherey-Nagel) using OptiPRO serum-free media (Gibco) and polyethylenimine 25K (Polysciences). Six days later, proteins were purified from filtered supernatant using TALON metal affinity resin (Takara Bio), eluted with PBS 200 mM imidazole, buffer exchanged into PBS, and sterile-filtered. IFNβ had the correct molecular weight as determined by SDS-PAGE, and <0.001 endotoxin units per μg as measured by a LAL chromogenic kit (Pierce). IFNβ activity was confirmed using RAW-Lucia ISG Cells (InvivoGen).

In vivo IFNβ or PRR agonist co-injection. For *in vivo* experiments involving IFNβ or PRR co-injection, $2x10^6$ tumor cells were resuspended with 50 μg IFNβ (generated as described above by the Wittrup Lab) in PBS and injected subcutaneously into the flanks of mice.

Collection of tumor-conditioned media. Tumor-conditioned media was collected when flasks containing MC38-SIY, MC57-SIY, or MC57-SIY-IRF3$^{-/-}$ tumor cells reached 100% confluency. Tumor supernatant was centrifuged at 500 *g* for 3 min to pellet cell debris and subsequently filtered through a 0.45 µm PVDF syringe filter (EMD Millipore). The resultant cell-free tumor-conditioned media was aliquoted and stored at -20°C.

Generation of BM-DC. Adapted from (Mayer et al., 2014). BM was harvested from the femur and tibia of mice by flushing the bones with RPMI using a 1 mL syringe. Cells were passed through a 70 µm filter, washed twice with PBS, and cultured at a density of 1.5x10^6 cells/mL in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, 1X HEPES, 1X MEM Non-Essential Amino Acids, 1X β-mercaptoethanol, 100 ng/mL recombinant human FLT3-L (Bio X Cell), and 5 ng/mL recombinant mouse GM-CSF (BioLegend) for 7 days at 37°C and 5% CO_2. BM-DCs at day 7 of culture were either used directly in assays or frozen in 10% DMSO in FBS and stored in liquid nitrogen.

BM-DC IFN-I and ISG induction assay. BM-DCs at day 7 of culture were plated at 3x10^6 cells per well of a 6-well tissue culture-treated plate and cultured with 2 mL of tumor-conditioned media. BM-DC were cultured with 2 mL of fresh complete DMEM media as a negative control or with complete DMEM media containing 20 µg/mL DMXAA (InvivoGen) as a positive control. Following 24 hr incubation at 37°C and 5% CO_2, BM-DC were washed, lysed with RLT Buffer (Qiagen), and frozen at -80°C for subsequent RNA extraction.

Baseline IFN-I and ISG screen with mouse and human tumor cell lines. Murine and human tumor cell lines used for the IFN-I and ISG qPCR screen were either available in-house, gifts, or purchased from ATCC or the High Throughput Sciences Core at the Koch Institute Swanson Biotechnology Center and cultured at the indicated conditions (Table S6). For the experiment, $3x10^5$ cells were washed with PBS, lysed with RLT Buffer, and frozen at -80°C for subsequent RNA extraction.

RNA isolation, cDNA reaction, and qRT-PCR. RNA was isolated using the Qiagen RNeasy Kit (Qiagen) following manufacturer's instructions. Extracted RNA was quantified by NanoDrop and 500 ng of RNA was reverse transcribed into cDNA using the Applied Biosystems Reverse Transcriptase Kit, following manufacturer's instructions. For each qRT-PCR reaction, 1 µL of the cDNA was assayed using the Applied Biosystems SYBR Green PCR Master Mix with defined primer sets for each target gene (Table S5). Reactions were run on the StepOne Real-Time PCR System (Applied Biosystems) and the expression level was calculated as $2^{-\Delta CT}$, where ΔCT is the difference between the CT values of the target gene and 18S.

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CHAPTER 4

DISCUSSION

In this work, we characterized the intratumoral DC compartment of a spontaneously regressing tumor (MC57-SIY fibrosarcoma) and a progressing tumor (MC38-SIY colon carcinoma) with the aim of identifying DC states associated with productive or dysfunctional anti-tumor immunity. In contrast to MC38-SIY tumors, MC57-SIY constitutively produced IFN-I at steady-state, and this significantly enhanced the functionality of the DC compartment. While anti-tumor T cell responses against MC38-SIY tumors were fully dependent on cross-presenting DC1, those against MC57-SIY tumors were driven by both cross-presenting DC1 and cross-dressing ISG$^+$ DC. These nuances in intratumoral DC compartment were reflected in the quality of the induced T cell response, where MC57-SIY tumors harbored a more functional CD8$^+$ T cell infiltrate compared to MC38-SIY tumors. Interestingly, we determined that stimulatory ISG$^+$ DC could be induced by exogenous IFNβ addition to drive anti-tumor CD8$^+$ T cell responses against MC38-SIY and poorly immunogenic tumors even in the absence of DC1, which carries therapeutic implications. In this chapter, I will discuss the implications of this work on our current understanding of productive anti-tumor immunity and for the development of novel strategies to enhance anti-tumor T cell responses and immunotherapy responses.

4.1. Activation of the intratumoral DC compartment is critical for productive anti-tumor immunity

The notion that DC activation is critical for successful T cell priming in draining LN has been well-established. As reviewed in **Section 2.4.2 of Chapter 1**, mature DC upregulate costimulatory molecules, such as CD80 and CD86, which are required for signal 2 of T cell activation. Recent reports suggest that DC activation in tumors is also critical for anti-tumor immunity. IFNγ signaling in the TME induces the expression of the coinhibitory ligand PD-L1 on many cell types as a mechanism to negatively regulate activity of anti-tumor T cells that express the receptor PD-1 (Garcia-Diaz et al., 2017). A recent study found that DC but not macrophages or tumor cells provides the critical source of PD-L1 that binds to PD-1 on T cells, thereby dampening T cell function (Oh et al., 2020). Interestingly, activated DC can overcome this PD-L1-PD-1 regulatory axis. High levels of CD80 on activated DC has been shown to bind PD-L1 in *cis*, and thus, limits the availability of PD-L1 on DC to bind to PD-1 on T cells (Sugiura et al., 2019). As a result of this *cis*-PD-L1/CD80 interaction, T cells remain in an activated state to sustain the anti-tumor immune response (Sugiura et al., 2019). Interestingly, CD28 ligation on T cells has been reported to be critical for the efficacy of anti-PD-1 CBT (Kamphorst et al., 2017), which again points to the requirement for DC activation in tumors for successful response to CBT.

In line with these findings, the work presented in this dissertation also demonstrates that activation of the intratumoral DC compartment is critical for productive anti-tumor immunity. Although MC38-SIY and MC57-SIY tumors induced a comparable tumor-reactive $CD8^+$ T cell response as measured by pentamer staining for SIY-reactive T cells, there was a striking qualitative difference between the T cell infiltrates observed as early as day 7 post-tumor implantation. MC57-SIY tumors harbored a more functional

effector T cell response characterized by robust expression of granzyme B and IFNγ. These effector molecules were lowly expressed by the T cell infiltrate of MC38-SIY tumors, which exhibited upregulated expression the exhaustion markers PD-1, 4-1BB, and Lag-3, signifying the early stages of dysfunction **(data not shown)**. As DC are the critical mediators of T cell priming, we profiled the DC compartment of MC38-SIY and MC57-SIY tumors. While there were differences in the intratumoral DC composition between the two tumor types, a more striking observation was that the DC subsets derived from MC38-SIY tumors were poorly stimulatory in our *ex vivo* DC:T cell co-culture assay compared to those from MC57-SIY tumors where we normalized cell numbers. Although we cannot rule out the contribution of tumor type-specific or environmental factors, we determined that differential tumor cell-intrinsic IFN-I production at baseline was a major factor impacting the functionality of the intratumoral DC compartment. IFNAR signaling was critical for enabling ISG^+ DC to activate $CD8^+$ T cells, and although we did not specifically evaluate this point, we speculate that IFN-I sensing also enhanced the cross-presentation ability of DC1. In the following sections, I will discuss the connection between IFNAR signaling and productive anti-tumor immunity.

4.1.a. *IFN-I as a potent DC maturation agent*

As critical mediators of innate and adaptive immunity, DC govern the outcome of T cell priming responses. Whether a tolerogenic or immunogenic T cell response is induced against an antigen is dependent on the activation state of the presenting DC. Canonically, it is believed that mature DC expressing costimulatory molecules drive immunogenic responses, whereas immature DC induce tolerogenic responses. In turn,

DC maturation state is dictated by the danger signals sensed during antigen uptake. Danger signals activating various PRR pathways, such as Toll-like receptors (TLR) and RIG-I-like receptors (RLR), are the best characterized inducers of DC maturation, and many synthetic PRR agonists have been made for the purpose of activating DC. However, evidence exists that IFN-I are also potent mediators of DC maturation. A study reported that bone marrow-derived DC lacking the ability to sense IFN-I (Ifnar1$^{-/-}$) exhibited lower levels of costimulatory molecules CD40, CD80, and CD86, as well as MHC-II, which blunted their ability to activate naïve T cells (Montoya et al., 2002). Studies to unravel the nuances between PRR and IFN-I-induced DC maturation have yielded interesting results. One study found significant differences in the maturation program induced by TLR agonism versus IFN-I stimulation with regards to antigen-processing activities (Simmons et al., 2012). During the process of DC maturation, it is generally believed that DC downregulate their antigen uptake and processing activities in order to favor antigen-presentation. As such, antigen-presentation is only restricted to the antigens that were encountered during exposure to the stimulating agent or danger signal. The authors were able to largely recapitulate these canonical notions using TLR agonists (i.e. LPS and Pam3CSK4) to mature Flt3-L-derived DC, noting decreased antigen-processing activities and enhanced costimulatory molecules (Simmons et al., 2012). However, interestingly, the authors found that IFN-I effects on maturation deviated significantly from those induced by TLR agonism. There was no inhibition of antigen-processing activities, and thus IFN-I-matured DC could continue to sample antigens, while concurrently expressing high levels of MHC-II and costimulatory molecules (Simmons et al., 2012). It is interesting to consider the implications of these findings for anti-tumor immunity. If antigen-

processing is not downregulated, perhaps IFN-I-induced maturation of intratumoral DC enables for more robust T cell priming against multiple tumor antigens leading to a more productive anti-tumor immune response. As IFN-I are held to be the downstream mediators of nucleic acid sensing, it may be assumed that nucleic acid sensor agonists and IFN-I induce overlapping effects. Indeed, my data in Chapter 2 comparing the RIG-I/MDA5 agonist Poly(I:C) and IFN-I suggests that this might be the case, as both were able to activate ISG^+ DC to rescue T cell priming in the absence of DC1. In support of this notion, a study on TLR3 and MDA5 agonists demonstrated that intact IFNAR signaling was required for the maturation effects induced by Poly(I:C) (Pantel et al., 2014). In fact, direct IFN-I stimulation supplanted the requirement for TLR3 or MDA5 for DC maturation (Pantel et al., 2014). The authors also showed that IFN-I mediated the metabolic reprogramming of DC from oxidative phosphorylation to glycolysis to better support DC survival and thus sustain the anti-viral immunity (Pantel et al., 2014). These observations suggest that IFN-I and PRR agonists may indeed be redundant in mediating DC maturation. However, this may be specific only to nucleic acid PRR agonists as another study found that combining TLR ligands with IFN-I and IFN-II induced highly functional DC that exhibited enhanced migratory ability and high levels of IL-12 production (Nguyen-Pham et al., 2011). The nuances of PRR agonist versus direct IFN-I stimulation on DC maturation and the impact on anti-tumor immunity warrant further investigation.

Another interesting facet of IFN-I biology is the differential impact on DC subsets. This notion was highlighted by our study and indicates that IFNAR signaling may be more complex than initially perceived. In contrast to some reports indicating a requirement for IFN-I sensing by DC1, we observed that DC1 could still infiltrate tumors in $Ifnar1^{-/-}$ mice

and their cross-priming function in the absence of MC57-SIY-derived IFN-I was seemingly unaffected. These data suggest that these basic functions can occur independently of IFN-I, and that perhaps IFN-I primarily acts to enhance these functions in line with its function as a maturation agent. While ISG^+ DC and DC2/moDC development and recruitment to the tumor were similarly not affected by a deficiency in IFN-I sensing, we observed that the maturation of these $CD11b^+$ DC subsets, particularly ISG^+ DC, were more sensitive to IFNAR signaling compared to DC1. Compared to their WT counterparts, $Ifnar1^{-/-}$ ISG^+ DC displayed a blunted maturation profile characterized by reduced levels of costimulatory molecules and decreased MHC-II levels. Ablation of the strong IFN-I signals needed for ISG^+ DC maturation and stimulatory function rendered them unable to drive anti-tumor T cell responses leading to outgrowth of MC57-SIY tumors in $Batf3^{-/-}$ mice. It will be interesting to investigate the differential sensitivity of distinct DC subsets to IFNAR signaling further and how this impacts the anti-tumor immune response. It is believed that IFNAR is constitutively expressed by all nucleated cell types and that IFN-I can be induced by virtually all cells downstream of PRR activation or IFNAR signaling. However, are there variations in the level of IFNAR or PRR expression on different DC subsets? Are there differences in the epigenetic regulation of the response to PRR or IFNAR activation in distinct DC subsets? Does that imply that some DC subsets are better suited to respond to certain immunological insults than others? All of these questions merit further investigation.

There are many other unknowns on the specifics of IFNAR signaling in the TME and what is needed for a robust anti-tumor immune response. One unknown relates to the source of IFN-I. Canonically, it is believed that a strong IFN-I in the tumor setting is

induced downstream of STING activation in DC and other innate immune cells. However, in our work, we demonstrated that tumor cells can be a significant source of IFN-I that can enhance anti-tumor immune responses. This notion is a bit paradoxical. Given the anti-tumorigenic and immunostimulatory effects of IFN-I, it is clearly not beneficial for tumor cells to maintain the IFNAR pathway. Supporting this notion, tumor cell-intrinsic downregulation of IFNAR or downstream components of the IFNAR pathway, such as JAK/STAT mutations, have been frequently reported to enable tumor immune escape and resistance to various therapies (Kalbasi et al., 2020; Sistigu et al., 2014; Zitvogel, Galluzzi, Kepp, Smyth, & Kroemer, 2015). Our screen of a panel of murine and human cell lines further confirmed that constitutive tumor cell-intrinsic IFN-I production is a rather rare phenotype. However, our data also suggests that IFN-I does not need to derive from tumor cells in order to activate ISG$^+$ DC. A high dose of exogenously added IFN-I was sufficient to rescue the anti-tumor T cell response by ISG$^+$ DC against progressor tumors lacking baseline IFN-I production in Batf3$^{-/-}$ mice. While we did not assess the impact of varying the amount of IFN-I on ISG$^+$ DC activation, this will be of interest for further study to determine whether there is a minimum threshold level of IFN-I needed in the TME for the successful activation of ISG$^+$ DC, and more broadly, how different levels of IFN-I impact the DC compartment and anti-tumor T cell priming. Our preliminary data, however, does suggest that IFN-I signaling strength may play a role. At equivalent dosage, we observed that only IFNβ but not IFNα (specifically IFNα3) could activate ISG$^+$ DC and rescue anti-tumor T cell responses in Batf3$^{-/-}$ mice **(data not shown)**. Although it is possible that the biology can be more complex than we realize, an obvious factor is the differential binding affinities of IFNβ and IFNα to IFNAR, with the former being 10-100 fold

higher than most IFNα subtypes which thus correlates with stronger signal strength (van Pesch, Lanaya, Renauld, & Michiels, 2004). More refined studies are required to determine whether this is true and if a higher dosage of IFNα can compensate for lower signaling strength to activate ISG^+ DC. In broader terms, it will also be interesting to investigate whether there are differences in IFNβ versus IFNα-induced signaling on different immune cell types, or whether they are redundant for each other.

The kinetics of IFNAR signaling is another variable that can impact anti-tumor immunity. One study investigated this question by administering an IFNAR blocking antibody into mice at various timepoints during growth of the immunogenic H31m1 sarcoma cell line, which is spontaneously rejected in immunocompetent mice (Diamond et al., 2011). They observed that early blockade of IFNAR signaling enabled tumor immune evasion and uncontrolled growth, whereas late IFNAR blockade had no impact on the rejection of the tumors (Diamond et al., 2011). This suggests that IFN-I-induced signaling is likely required at the priming stage of the anti-tumor immune response, and our data is largely consistent with this notion. We demonstrated that a single high dose administration of IFNβ at the time of tumor implantation was sufficient to rescue anti-tumor $CD8^+$ T cell responses against progressor tumors in $Batf3^{-/-}$ mice lacking DC1. It will also be of interest to determine whether IFNβ remains efficacious when administered at later timepoints when the tumors are established, as this carries clinical relevance. Historical clinical studies using IFN-I have primarily focused on repeated high doses of IFNα administered to patients at advanced stages of disease. Given the significant toxicities associated with high dose IFNα and in light of the findings that chronic IFN-I exposure

can actually induce immunosuppression, it may be worth revisiting the dosing regimen for further IFN-I-related therapies to drive productive anti-tumor immune responses.

4.1b. *Drivers of IFN-I production in the TME*

In our work, we identified that MC57-SIY tumors constitutively produce IFN-I at baseline. The drivers of constitutive IFNAR signaling in MC57-SIY tumors remain unknown but is an area for future investigation. As the PRR pathways driving IFN-I were reviewed in detail in Chapters 1 and 4, suffice it to say that we speculate that aberrant amounts or localization of tumor-derived nucleic acids are the likely culprits driving the IFN-I response in the tumor context. Tumor cells are inherently genetically unstable and sensitive to micronuclei formation, and this has been linked to innate immune activation via activation of the cytosolic dsDNA sensor cGAS/STING to trigger an IFN-I response (Gekara, 2017; Harding et al., 2017; Mackenzie et al., 2017). Relatively recent work has also shown that cytosolic dsRNA sensors also contribute to IFN-I induction. Loss of the RNA-editing enzyme ADAR in tumor cells induced aberrant accumulation of dsRNA, which triggered activation of tumor cell-intrinsic dsRNA sensors and led to IFN-I production and a more inflamed microenvironment (Ishizuka et al., 2019; H. Liu et al., 2019). These studies reveal vulnerabilities of tumor cells that can be therapeutically targeted. The development of PRR agonists, such as RIG-I or STING agonists, to treat cancer is not a novel concept (Amouzegar, Chelvanambi, Filderman, Storkus, & Luke, 2021; Corrales et al., 2015; Elion & Cook, 2018; Iurescia, Fioretti, & Rinaldi, 2018, 2020; Le Naour, Zitvogel, Galluzzi, Vacchelli, & Kroemer, 2020; Wu, Wu, Wu, Wang, & Liu, 2017). Our data suggests that agonism of the RIG-I pathway may elicit a stronger IFN-I

response than the STING pathway in both tumor and immune cells, which has interesting clinical implications. Though STING agonists have shown anti-tumor efficacy in mice, they are less efficacious in the clinic despite attempts to improve their design (Le Naour et al., 2020). One important consideration is that STING has been found to be more frequently epigenetically silenced compared to dsRNA sensors (Konno et al., 2018). Based on these data points, perhaps RIG-I agonists might be the more promising therapeutic agents to induce a strong IFN-I response and anti-tumor immunity, but this notion needs to be further evaluated.

4.1c. *Viral mimicry and anti-tumor immunity*

IFN-I are highly associated with anti-viral immunity. Indeed, they were initially discovered as signaling agents that controlled viral loads (Isaacs & Lindenmann, 1957; Isaacs, Lindenmann, & Valentine, 1957). An interesting notion is whether activation of an anti-viral immune response could help boost anti-tumor immunity and lead to better control. Induction of an anti-viral immunity can be achieved in additional ways besides relying on the use of nucleic acid PRR agonists. Genotoxic therapies like radiation and chemotherapy, for example, have been shown to generate micronuclei as a result of DNA damage, which can activate DNA-specific PRR pathways. Genotoxic insults can also lead to de-repression of endogenous retroviruses (ERVs), which are thought to originate from ancestral germ-line retroviral infections (Löwer, Löwer, & Kurth, 1996; Zhang, Liang, & Zheng, 2019). Priming against retroviral proteins in the tumor context can induce potent anti-viral immune responses, referred to as a state of ‘viral mimicry,’ which can be beneficial for tumor control (Chiaro et al., 2020). In fact, DNA-demethylating agents have

been shown to be effective against colorectal cancer cells by reawakening endogenous retroviral elements and inducing viral mimicry (Roulois et al., 2015). Another study found that high expression of a viral defense signature in tumors was associated with response to CBT (Chiappinelli et al., 2015). In a preclinical model of melanoma, use of a DNA methyltransferase inhibitor reactivated ERVs and drove anti-viral immunity and sensitized tumors to anti-CTLA-4 therapy (Chiappinelli et al., 2015). However, studies have shown that in some cases, the induction of viral mimicry can actually be detrimental for anti-immunity and promote tumor growth (Gao, Yu, & Chen, 2021). Further investigation is thus required to delineate the specific contexts where viral mimicry would be beneficial and detrimental to anti-tumor immunity.

Another interesting therapeutic strategy to induce viral mimicry for the treatment of cancer is the use of oncolytic viruses. This therapy uses engineered viruses that specifically target and kill tumor cells (Cao et al., 2020). Though the notion of using viruses to treat cancer has been studied for over a century, the first oncolytic virus, talimogene laherparepvec (T-VEC), received FDA approval only a few years ago in 2015 (Cao et al., 2020; Pyeon, Vu, Giacobbi, & Westrich, 2020). Besides inducing direct lysis of tumor cells, oncolytic viruses can enhance the immune response via the release of viral antigens and viral nucleic acids that can robustly activate DC and other innate immune cells to drive concomitant anti-viral immunity and thus strengthen tumor control (Cao et al., 2020).

4.2. Functional plasticity of DC subsets

The myeloid APC compartment is heterogeneous and comprises DC, macrophages, and monocytes. Initial efforts to define APC cell types focused on

phenotypic markers. Cells expressing CD45, MHC-II, and CD11c but lacking markers of other immune cell lineages were loosely labeled as DC, though more than likely, this definition also captured macrophages and monocytes as well. Advances in multicolor flow cytometry enabled for more descriptive profiling of DC subsets as more markers could be assayed, leading to a greater appreciation of the heterogeneity of the DC compartment (Merad, Sathe, Helft, Miller, & Mortha, 2013; Mildner & Jung, 2014). This led to the phenotypic characterization of DC subsets based on expression of certain surface markers such as $CD103^+/CD8\alpha^+$ DC1, $CD11b^+Sirp\alpha^+$ DC2, and Siglec-H^+ pDC. However, it was quickly realized that sole reliance on surface markers had significant limitations, especially under inflammatory contexts, where cellular phenotypes of DC and APC changed. To overcome the shortcomings of reliance on phenotypic descriptions, a greater emphasis has been placed on using ontogeny and functional specialization to define DC, as well as using DC subset-specific gene signatures, which have been made possible by recent advances in genomics and transcriptomics profiling technologies. Thus, our current definitions of the major DC subsets include: Zbtb46-dependent conventional DC that comprise (1) IRF8 and Batf3-driven cross-presenting DC1 that primarily activate $CD8^+$ T cell responses and (2) IRF4-dependent DC2 that predominately drive $CD4^+$ T cell responses, as well as TCF4-driven pDC that produce copious amounts of IFN-I. However, as my work and that of other groups have recently shown, these descriptions still have limitations as the DC compartment exhibits far greater functional plasticity than initially perceived, particularly under inflammation.

In contrast to the prevailing notion that DC1 are the critical subset for priming $CD8^+$ T cell responses, we demonstrated that in MC57-SIY tumors, DC2 can also acquire the

ability to activate $CD8^+$ T cells and mediate tumor rejection in $Batf3^{-/-}$ mice lacking DC1. This stimulatory DC2 state, which we termed ISG^+ DC, was dependent on strong IFNAR signaling in the TME, as well as the acquisition of tumor-derived pMHC-I via cross-dressing. In our system, we determined that IFN-I enhanced the maturation of ISG^+ DC, which in turn enabled $CD8^+$ T cell activation. It will be interesting to compare ISG^+ DC to the recently identified Inf-cDC2 subset, as both are induced by IFN-I, though there are some notable differences (Bosteels et al., 2020). We demonstrated that the bulk of ISG^+ DC are dependent on the transcription factor IRF4, but the authors found no defects in Inf-cDC2 development in the absence of IRF4; rather, loss of IRF4 seemed to impact the ability of Inf-cDC2 to migrate to the draining LN (Bosteels et al., 2020). Furthermore, though Inf-cDC2 also developed in the absence of IRF8, the authors found that IFNAR signaling upregulated an IRF8-dependent maturation module in Inf-cDC2 (Bosteels et al., 2020). This is an avenue we could follow-up on to determine whether the enhanced maturation state of ISG^+ DC is similarly dependent on IRF8 upregulation. Additionally, the authors determined that Fc receptor-mediated endocytosis by Inf-cDC2 was the route of antigen internalization for priming T cells (Bosteels et al., 2020). In our system, we found that ISG^+ DC presented tumor antigens via cross-dressing with tumor-derived pMHC-I. It will be interesting to determine whether this is also a possible pathway of antigen uptake by ISG^+ DC. These additional studies will yield better insights into the similarity of these two IFN-I-induced DC2 activation states.

Another example of DC functional plasticity was recently described in the context of lung tumors. The authors described a paradoxical situation wherein tumors were refractory to CBT despite harboring a DC1 infiltrate (Maier et al., 2020). This suggested

that DC1 function might be altered in the lung TME. Indeed, using scRNA-seq and CITE-seq, the authors found that DC1 expressed an immunoregulatory program upon antigen uptake that was characterized by both maturation (CD40, CCR7, IL-12) as well as regulatory markers (PD-L1, PD-L2, IL4Rα) (Maier et al., 2020). While these 'mature DC1 enriched in immunoregulatory molecules' (mregDC1) could prime $CD8^+$ T cells, they also promoted T_{reg} expansion (Maier et al., 2020). Thus, similar to DC2, the phenotype and function of DC1 is not static and can change under different environmental contexts.

Understanding the range of DC functional states in different tumor contexts can facilitate the development of novel therapeutic strategies to modulate the DC compartment to strengthen anti-tumor immunity. In this work, we identified that a single high dose of IFNβ at the time of tumor implantation could induce stimulatory ISG^+ DC and thus drive an anti-tumor $CD8^+$ T cell response in $Batf3^{-/-}$ mice lacking DC1. Though we need to evaluate whether this holds true in the context of established tumors, this presents a therapeutic rationale to use IFNβ to promote a more stimulatory DC compartment. Similarly, regarding mregDC1, the authors demonstrated that IL-4 blockade could be used to inhibit the immunoregulatory functions of mregDC1 and thus enhance their immunostimulatory functions to enable better tumor control. A greater awareness of different DC activation states and the signals that induce or suppress them will significantly expand our therapeutic toolkit to fight cancer.

4.3. Implications of T cell activation via cross-dressing for anti-tumor immunity

Our finding that cross-dressed ISG^+ DC can induce protective systemic anti-tumor immunity in DC1-deficient mice has several implications. First, cytotoxic anti-tumor T cells

need to be able to recognize peptides presented by tumor cells for successful tumor eradication. For this to happen, naïve T cells must be primed by DC presenting the same peptide that is presented by tumor cells. As cross-presentation entails antigen uptake and further internal processing by DC, the peptide that is ultimately presented on the surface of DC might not match what is presented by the tumor cell. In contrast, cross-dressing utilizes tumor-derived pMHC-I complexes for presentation and thus guarantees that naive T cells are primed with the specific peptides that are presented by the tumor cells. This ensures that the induced $CD8^+$ T cell response can recognize and kill tumor cells. Second, antigen-presentation by cross-dressing can bypass the requirement for cross-presentation to mount anti-tumor T cell responses. We demonstrate that in the absence of conventional cross-presentation, T cell responses induced by cross-dressing can drive protective systemic immunity. This is significant given that the exclusion or functional impairment of cross-presenting Batf3-driven DC1 is a frequent mechanism of immune evasion by the tumor and contributes to immunotherapy resistance (Broz et al., 2014; Hildner et al., 2008; Salmon et al., 2016; Sánchez-Paulete et al., 2016; Spranger, Bao, & Gajewski, 2015; Spranger, Dai, Horton, & Gajewski, 2017). Thus, identifying ways to induce or harness cross-dressing as a means of antigen-presentation might be highly beneficial for mounting tumor-reactive T cell responses.

4.3a. *Speculations on the mechanism of cross-dressing*

We demonstrate that ISG^+ DC are highly efficient at cross-dressing compared to other DC subsets. The notion that distinct DC subsets have differing propensities to cross-dress with tumor-derived peptide-MHC complexes suggests that there might be some

specificity in the membrane exchange process. Specifically, the enhanced ability of ISG$^+$ DC to cross-dress could be conferred by their high expression of AXL, which has been reported to be an endocytic receptor involved in antigen uptake (Schmid et al., 2016; Subramanian et al., 2014). Moreover, AXL is an ISG and if it is involved in membrane exchange, this would imply that the ability to cross-dress might be induced downstream of the initial PRR- or IFN-I-sensing event. It will be important to address this point in future studies in order to harness this unique mode of antigen-presentation to boost anti-tumor immune responses.

Though preliminary, our immunofluorescence images from the *ex vivo* cross-dressing assay provided some insights on how tumor-derived pMHC-I might be transferred to ISG$^+$ DC. After an overnight co-culture of sorted Balb/c ISG$^+$ DC (H-2^d) and MC57-SIIN-SIY (H-2^b) tumor cells, we observed the acquisition of H-2K^b:SIIN complexes on Balb/c ISG$^+$ DC. In accordance with our data from surface staining via flow cytometry, these H-2K^b:SIIN complexes were detectable on the surface of cells. Intriguingly, however, we also were able to detect these complexes intracellularly in what appeared to be vesicle-like structures. These data suggest that perhaps ISG$^+$ DC acquire tumor-derived pMHC-I via the transfer of intracellular vesicles, which has been described to occur between DC subsets in the tumor context (Ruhland et al., 2020). Similar to our findings, the authors determined that intracellular vesicle transfer is frequently accompanied by pMHC-I cross-dressing, though they did not report a direct correlation between the degree of pMHC-I acquisition and T cell stimulatory capacity (Ruhland et al., 2020). However, the authors were studying the dissemination of tumor antigens among DC subsets in the draining LN, whereas our studies have been focused on the direct interactions between

ISG$^+$ DC and tumor cells at the tumor site. It is possible that the authors did not observe a direct correlation between pMHC-I levels and T cell stimulatory capacity because of antigen decay over time or pMHC-I turnover, as it is unknown how stable the acquired pMHC-I complexes are on the surface of cross-dressing DC. More work is needed to determine whether intracellular (or extracellular) vesicles are involved in the mechanism of cross-dressing by ISG$^+$ DC.

Assuming vesicle-like structures are involved in the transfer of pMHC-I to ISG$^+$ DC, it is interesting to consider the impact of vesicle contents on DC activation and function. Our immunofluorescence images showed that some fraction of the intracellular vesicles stain positive for both tumor-derived H-2K^b:SIIN and DAPI. This suggests that the sensing of danger signals (i.e. tumor-derived nucleic acids) might occur concurrently with the acquisition of tumor-derived pMHC-I. If true, this would be a unique mechanism for efficiently and specifically activating DC against a given antigen, enabling them to mount an immunogenic antigen-specific cell response. We have not yet identified the driver of constitutive IFN-I production in MC57-SIY tumor cells, but based on these data, we could speculate that activation of nucleic acid PRR are involved. Targeting the co-delivery of antigens and danger signals to DC could be a unique way to induce anti-tumor immunity. The advances in mRNA vaccine platforms for *in vivo* delivery (i.e. mRNA-complexed lipid nanoparticles) make this a promising and feasible strategy to treat cancer (Pardi, Hogan, Porter, & Weissman, 2018).

Another interesting application for the co-delivery of antigens and danger signals in particulate structures to activate DC is through the use of fusogenic viruses for oncolytic immunotherapy (Krabbe & Altomonte, 2018). Fusogenic viruses rely on membrane

glycoproteins to mediate infection, wherein the viral envelope fuses with the target cell membrane (Krabbe & Altomonte, 2018). As these glycoproteins are synthesized and expressed on the surface of infected tumor cells, this can cause cell-cell fusion of infected and non-infected cells, leading to the formation of large multinucleated cells called syncytia (Krabbe & Altomonte, 2018). Syncytia are viable for a few days before dying as a result of nuclear fusion, ATP depletion, and autophagy (Bateman et al., 2002; Krabbe & Altomonte, 2018). Dying syncytia release large amounts of vesicle-like structures called syncytiosomes that have been shown to impact DC function (Bateman et al., 2002). Syncytiosomes contain tumor antigens and danger signals (i.e. viral nucleic acids), and though more work is needed to confirm, they may also harbor tumor-derived pMHC-I complexes (Bateman et al., 2002). DC that have taken up syncytiosomes are able to induce potent anti-tumor T cell responses as a result of enhanced activation and relevant antigen-presentation (Bateman et al., 2002). Thus, the combination of direct lysis of tumor cells via fusogenic viruses in addition to priming by syncytiosome-loaded DC can induce robust anti-tumor immunity. It will be of interest to determine whether cross-dressing is involved in the ability of syncytiosome-loaded DC to drive $CD8^+$ T cell responses, and specifically, to assess the involvement of ISG^+ DC as it is well-established that anti-viral immunity induces a strong IFN-I response.

4.3b. *Impact of cross-presentation versus cross-dressing on the induced $CD8^+$ T cell response*

Our work underscores the importance of delineating the contributions of specific DC subsets in the TME. DC1 and ISG^+ DC can robustly stimulate cytotoxic T cell

responses. However, they do so through fundamentally distinct mechanisms, which can conceivably impact the resultant T cell response. Whereas DC1 primarily cross-present tumor antigens, we demonstrate that ISG$^+$ DC activate T cells by cross-dressing with tumor-derived pMHC-I complexes. Furthermore, ISG$^+$ DC express higher levels of CD80, CD86, and CD40 costimulatory molecules compared to DC1. These differences in signal one and two of T cell activation, though subtle, can drive functionally distinct T cell responses. In support of this notion, we demonstrate that while cross-presenting DC1 predominantly activates an effector or effector memory T cell (T_{EM}; CD44$^+$ CD62L$^-$) response, cross-dressing ISG$^+$ DC promotes a central memory T cell (T_{CM}; CD44$^+$ CD62L$^+$) response **(data not shown)**. This observation is consistent with the prevailing thought on priming thresholds for activation of different T cell states established from viral immunology studies (Henrickson et al., 2013). The generation of effector or T_{EM} responses is believed to require strong and persistent antigen stimulation, and cross-presentation by Batf3-driven DC1 has been shown to prolong antigen-presentation to CD8$^+$ T cells (Henrickson et al., 2013; Iborra et al., 2016). Conversely, it is believed that circulating T_{CM} require a lower threshold of antigen priming for their induction (D'Souza & Hedrick, 2006; Iborra et al., 2016; van Faassen et al., 2005). In the viral context, Wakim and Bevan demonstrated that cross-dressing was less efficient in driving T cell simulation compared to direct or cross-presentation (Wakim & Bevan, 2011), which can be attributed to a lower density of pMHC-I complexes acquired through membrane exchange. Their study showed that cross-dressed DC primarily activated memory CD8$^+$ T cells, which is consistent with our results (Wakim & Bevan, 2011). Furthermore, several studies have demonstrated that Batf3-driven DC1 are not required for the generation of T_{CM}, while they

are required for the reactivation and further differentiation of T_{CM} into more effector-like cells, such as T_{EM} and resident memory T cells (T_{RM}) (Enamorado et al., 2017; Iborra et al., 2016). The functional implications of distinct T cell subsets in anti-tumor immunity are currently an active area of investigation (Q. Liu, Sun, & Chen, 2020; Reading et al., 2018; St. Paul & Ohashi). Nonetheless, our results suggest that ISG$^+$ DC and DC1 may work in concert to maintain a pool of T_{CM} and induce their differentiation into effector cells, such as T_{EM} or T_{RM}.

4.4. Role of ISG$^+$ DC in the Cancer-Immunity Cycle, the broader context, and beyond

In our work, we primarily studied the functions of ISG$^+$ DC in the tumor site. ISG$^+$ DC isolated from MC57-SIY tumors were able to stimulate naïve CD8$^+$ T cells in our *ex vivo* co-culture assay. Specifically, we determined that they acquired pMHC-I from tumors via cross-dressing to mediate T cell activation, and that their stimulatory function was dependent on IFNAR signaling in the TME. As described in **Section 2.1 of Chapter 1**, the generation of productive anti-tumor immunity occurs in a multi-step and iterative process termed the Cancer-Immunity Cycle. Thus, it will be important to identify the role of ISG$^+$ DC within the framework of our current understanding on how anti-tumor T cell responses are mounted. One immediate question is whether ISG$^+$ DC are involved in priming of anti-tumor T cell responses in the draining LN. Interestingly, we were unable to detect high numbers of ISG$^+$ DC in the draining LN of mice bearing MC57-SIY tumors, which might lead us to postulate that their primary role is either *in situ* naive T cell priming or local restimulation of effector T cells. In support of this notion, preliminary data using FTY-720, which blocks T cell egress from LN, had no impact on the clearance of MC57-

SIY tumors in WT mice even though it was administered two days prior to tumor implantation **(data not shown)**. To determine whether this immune response is mediated specifically by ISG^{+} DC, this FTY-720 experiment needs to be repeated in Batf3$^{-/-}$ mice Nonetheless, this data suggests that *in situ* T cell priming does occur and is sufficient to mediate MC57-SIY tumor rejection. If this is the case, it will be interesting to assess whether ISG^{+} DC reside in or are involved in the formation of tertiary lymphoid structures (TLS), as these structures have been shown to correlate with CBT response (Cabrita et al., 2020; Engelhard et al., 2018; Helmink et al., 2020; Sautès-Fridman, Petitprez, Calderaro, & Fridman, 2019). It must be noted, however, that our inability to detect ISG^{+} DC in the draining LN does not mean they are not actually there. Instead, this could be due to a limitation of our flow cytometry panel to identify them, which heavily relies on the use of AXL, an IFN-I-induced marker. It is possible that AXL is downregulated upon migration to the draining LN. Thus, it will be important to use other less biased techniques, such as scRNA-seq, to address whether ISG^{+} DC can be found in draining LN. As some proportion of ISG^{+} DC do express the chemokine receptor CCR7, it is likely that they can enter a migratory state and traffic to the draining LN. Thus, further studies are needed to truly understand where the contributions of ISG^{+} DC fit within the framework of the Cancer-Immunity Cycle.

While our comparative model of regressor MC57-SIY and progressor MC38-SIY tumors facilitated the study of stimulatory DC states, it also has limitations. Specifically, it will be important to broaden our findings and establish human relevance. In pursuit of these objectives, we screened additional murine tumor cell lines and found that our observations could indeed be extended beyond our model system. Moving forward, it will

be critical to assess for ISG$^+$ DC in additional murine tumor models, and more importantly, to establish the relevance of ISG$^+$ DC in human tumors. As we identified a handful of human tumor cell lines exhibiting constitutive IFNAR signaling at baseline, it is conceivable that these tumors, though rare, may harbor the ISG$^+$ DC state. Indeed, we observed enrichment of the ISG$^+$ DC signature in a subset of tumor-infiltrating human DC2, suggesting that they exist in the human tumor setting; however, their functional contribution to anti-tumor immunity in human cancer patients requires further study. Nonetheless, our study demonstrates that IFNβ can be used to induce ISG$^+$ DC in poorly immunogenic murine tumors, and it will be interesting to determine whether this therapeutic implication holds true in the human setting.

Given that ISG$^+$ DC are activated by strong IFNAR signaling, we speculate that they might be involved in the immune response against disease settings that trigger a strong IFN-I response. Viral infections are an obvious setting, as described earlier, and it will be of interest to determine the role of ISG$^+$ DC in diseases such as HIV and COVID-19. As the immune response between acute versus chronic viral infection has been shown to be drastically different (Rai et al., 2021; Virgin, Wherry, & Ahmed, 2009; Zuniga, Macal, Lewis, & Harker, 2015), it will be worth investigating how ISG$^+$ DC function is impacted in these two settings. Beyond viral infection, another potentially relevant context for ISG$^+$ DC is in genotoxic therapies (i.e. radiation and chemotherapy) where an IFN-I response is induced and required for efficacy (Budhwani, Mazzieri, & Dolcetti, 2018; Minn, 2015). Our lab is further investigating the anti-tumor immune response against homologous repair (HR)-deficient or HR-sufficient ovarian cancer. Though not absolute, HR-deficient ovarian lines expressing BRCA1/2 mutations are associated with increased

IFN-I production due to DNA damage from defective DNA repair (Bruand et al., 2021; McAlpine et al., 2012). It will be interesting to determine whether ISG$^+$ DC are induced in these settings, and whether our observations from our comparison of MC57-SIY and MC38-SIY tumors are translatable to this more clinically relevant model system.

In summary, therapeutic engagement of stimulatory DC subsets that drive distinct T cell responses, such as cross-presenting DC1 and cross-dressing ISG$^+$ DC, has the potential to strengthen anti-tumor immunity and boost CBT responses. Our work broadens the current knowledge of functional DC states distinct from DC1 that are capable of driving anti-tumor CD8$^+$ T cell responses.

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APPENDIX I

Type-1 conventional dendritic cell dysfunction enables tumor immune evasion and resistance to anti-PD-1 therapy

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ED, MZ, FC, and SS conceptualized and designed the study. ED, MZ, and FC performed experiments and analyzed the data. ED wrote the original draft of the chapter. All authors revised and edited the chapter.

ABSTRACT

Checkpoint blockade therapy (CBT) has demonstrated remarkable clinical efficacy but fails to work for the majority of cancer patients. While the presence of a T cell infiltrate is generally associated with sensitivity to CBT, there are reports of T cell-rich tumors that are CBT-refractory. Thus, a stronger predictor of response is T cell activation in the tumor. Attempts to sustain T cell activation in a T cell-intrinsic manner, however, have proven insufficient. As the critical initiators of T cell activation, it is important to consider the functional status of dendritic cells (DC) in the tumor over time. Indeed, studies have shown that DC exclusion from the tumor impairs priming of anti-tumor $CD8^+$ T cell responses, which enables uncontrolled tumor growth. However, the suppression of DC function can similarly promote tumor immune evasion. In Chapters 2 and 3, we observed that DC1 in progressor MC38-SIY tumors lose stimulatory capacity over time. In this work, we identify a correlation between DC1 dysfunction at late stages of tumor growth with resistance to anti-PD-1 CBT. Through flow cytometric profiling, single-cell RNA-sequencing, and functional studies, we dissect the mechanism(s) mediating DC dysfunction in MC38-SIY tumors overtime with the aim to identify novel strategies to restore DC functionality.

INTRODUCTION

Cytotoxic $CD8^+$ T cell responses are critical for tumor clearance and the induction of durable anti-tumor immunity (Fridman, Pagès, Sautès-Fridman, & Galon, 2012). However, T cells that infiltrate the tumor frequently undergo exhaustion, a state characterized by expression of inhibitory checkpoint receptors and progressive loss of proliferative and cytolytic function, thereby enabling immune evasion (Thommen &

Schumacher, 2018; Xia, Zhang, Xu, Yin, & Lu, 2019; Zhang et al., 2020). Most immunotherapy strategies aim to reactivate dysfunctional T cells to fight cancer (Durgeau, Virk, Corgnac, & Mami-Chouaib, 2018; Waldman, Fritz, & Lenardo, 2020). Notably, checkpoint blockade immunotherapy (CBT) has shown remarkable durable clinical benefit in several advanced cancer types (Ribas & Wolchok, 2018). However, only a subset of patients responds to this therapy (Larkin et al., 2019), highlighting a critical need to identify additional approaches to mount and sustain functional anti-tumor T cell responses.

Dendritic cells (DC) are critical initiators of anti-tumor immunity, given their unique ability to cross-present antigens on major histocompatibility complexes class I (MHC-I) and prime cytotoxic $CD8^+$ T cell responses (Chen & Mellman, 2013). Cross-presentation is predominately attributed to a subset of conventional DC called Batf3-driven $CD8\alpha^+/CD103^+$ DC1 that are robust stimulators of $CD8^+$ T cells (den Haan, Lehar, & Bevan, 2000; Edelson et al., 2010; Hildner et al., 2008; Iyoda et al., 2002; Schulz & Reis e Sousa, 2002). In the tumor microenvironment (TME), DC1 also contribute to effector T cell recruitment and provide local restimulation of T cells to promote a T cell-inflamed TME (Broz et al., 2014; Roberts et al., 2016; Spranger, Dai, Horton, & Gajewski, 2017). Several studies demonstrate that the presence of a DC1 signature in tumors correlates with improved survival (Barry et al., 2018; Böttcher et al., 2018; Broz et al., 2014; Michea et al., 2018).

Given their importance in stimulating $CD8^+$ T cell responses, the exclusion of DC1 from the TME has been shown to enable tumor immune escape and impairs CBT efficacy (Salmon et al., 2016; Sánchez-Paulete et al., 2016; Spranger, Bao, & Gajewski, 2015).

Yet paradoxically, most tumors that harbor a DC1 infiltrate still progress, suggesting that while DC1 can initiate anti-tumor immunity, they are not able to sustain functional tumor-reactive T cell responses. This observation might indicate that DC1 in tumors become dysfunctional over time. Indeed, in our work investigating the role of DC in productive versus failed anti-tumor immunity as detailed in Chapters 2 and 3, we observed that the induction of anti-tumor $CD8^+$ T cell responses against MC38-SIY tumors were fully dependent on DC1. At early timepoints (day 7 post-implantation), DC1 were functional and could drive a systemic anti-tumor T cell response against MC38-SIY tumors; however, at late timepoints (day 11 post-implantation), DC1 isolated from MC38-SIY tumors lost their stimulatory capacity and failed to activate $CD8^+$ T cells *ex vivo.*

In this work, we hypothesize that loss of DC1 functionality over time mediates tumor immune evasion and resistance to anti-PD-1 checkpoint blockade therapy (CBT). We tested this notion by comparing the phenotypic and functional profiles of DC1 from early-stage (day 7) or late-stage (day 14) tumors. As DC1 dysfunction can manifest in different ways, we also aimed to elucidate the specific mechanism(s) impairing the ability of DC1 to prime an anti-tumor $CD8^+$ T cell response. Identifying the drivers of DC1 dysfunction in the TME can open new strategies to strengthen anti-tumor $CD8^+$ T cell immunity and sensitize refractory tumors to CBT.

RESULTS

MC38-SIY tumors become resistant to anti-PD-1 CBT at late stages of tumor growth.

The sensitivity of the syngeneic murine colon adenocarcinoma model, MC38, to CBT with monotherapy or combination anti-PD-1/anti-CTLA-4 is well-established. However, most preclinical studies administer CBT at early stages of tumor growth when

tumor size is relatively small (~70-100 mm^3) (Homet Moreno et al., 2016; Selby et al., 2016). The efficacy of CBT treatment at late stages of tumor growth has not been as well characterized. Thus, we aimed to evaluate how the efficacy of CBT changes during MC38 tumor progression from day 7 (early-stage) to day 14 (late-stage) of tumor growth. We chose to focus on anti-PD-1 monotherapy (clone 332.8H3) to best mirror the clinical data for microsatellite-instability high, mismatch repair-deficient metastatic colorectal cancer, which suggests that pembrolizumab (anti-PD-1) treatment showed a statistically significant improvement in progression-free survival compared to chemotherapy as a first-line therapy (Shiu et al., 2021). While early initiation (day 7) of anti-PD-1 treatment led to robust tumor control, considerable loss of efficacy was observed when anti-PD-1 treatment was administered at late stages (day 11) of tumor growth **(Figure A.1)**. This observation suggests that there must be longitudinal changes in the anti-tumor immune response during the course of tumor progression that mediates resistance to anti-PD-1 therapy.

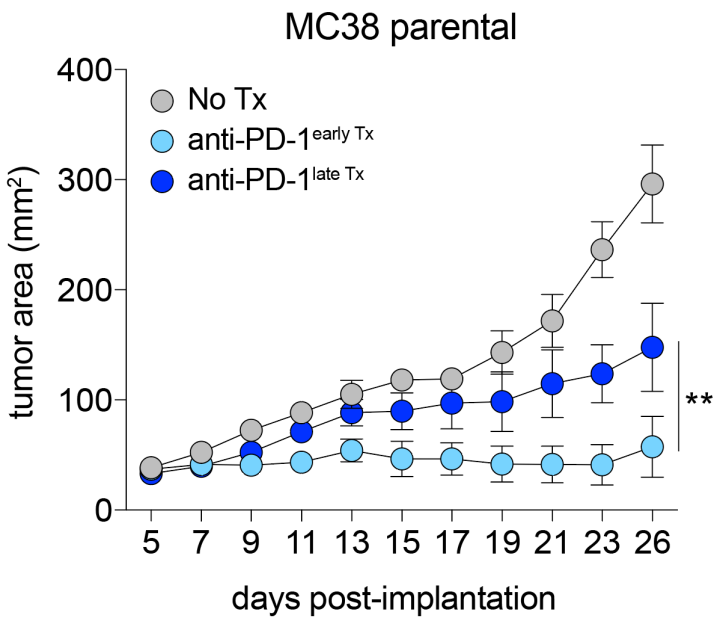

Figure A.1. Loss of anti-PD-1 efficacy is observed when therapy is administered at late-stages of tumor growth. Tumor outgrowth (mm^2) of anti-PD-1 treated or untreated parental MC38-SIY tumors in WT mice. For early treatment (Tx), 200 µg anti-PD-1 is administered *i.p.* on days 7, 10, and 13 post-tumor implantation. For late Tx, 200 µg anti-PD-1 is *i.p.* on days 11, 14, 17 post-tumor implantation. Representative data is shown (n = 4 mice per group per experiment). **p<0.01; two-way ANOVA. Data are shown as mean ± s.e.m. Data generated by Maria Zagorulya.

Endogenous tumor-reactive $CD8^+$ T cell responses in MC38-SIY tumors and tdLN do not expand over time and appear exhausted.

As $CD8^+$ T cells are the key effector cells that mediate tumor destruction, we next aimed to immunophenotype the endogenous T cell infiltrate in MC38 tumors and tumor-draining lymph nodes (tdLN) at day 7 and day 14 of tumor growth. For this experiment, we used the MC38-SIY tumor cell line, which expresses the model T cell antigen SIY (SIYRRGYL) to facilitate analyses of tumor-reactive T cell responses using a commercially available SIY-pentamer staining reagent. From day 7 to day 14 of MC38-SIY tumor growth, there was no expansion of SIY-specific T cells as a proportion of the $CD8^+$ T cell infiltrate **(Figure A.2a)** or in terms of absolute numbers in tumors **(Figure A.2b)**. Furthermore, by day 14 of tumor growth, the SIY-specific T cell infiltrate in tumors

appeared to be canonically exhausted, as indicated by an increased proportion of cells expressing PD-1 and Lag-3, decreased 4-1BB expression, and reduced IFNγ production **(Figure A.3)**. Interestingly, granzyme B expression was unchanged from day 7 to day 14 **(Figure A.3)**. However, as the tumors were growing progressively at day 14 **(Figure A.1)**, this indicates that the cytolytic capacity of T cells must be impaired. In the tdLN, there was also no expansion of SIY-specific T cells over time; in fact, the proportion of SIY-specific T cells decreased from day 7 to day 14 of tumor growth **(Figure A.4a)**. The frequency of SIY-specific T cells expressing any of the activation and effector molecules profiled (PD-1, Lag-3, T-bet, granzyme B) also decreased over time, indicating poor activation **(Figure A.4b)**. These data suggest that T cell priming might become impaired in MC38-SIY tumors over time.

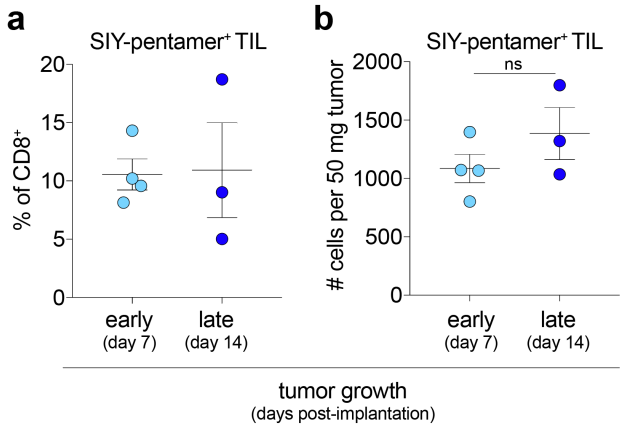

Figure A.2. Anti-tumor SIY-reactive T cells fail to expand over time during progression of MC38-SIY tumors. (a, b) Frequency **(a)** or absolute numbers **(b)** of SIY-reactive tumor-infiltrating lymphocytes (TIL) in MC38-SIY tumors at day 7 and day 14 of tumor growth. Representative data from one of two independent experiments is shown (n = 4 mice per group per experiment). ns, not significant; MWU test (a, b). Data are shown as mean ± s.e.m.

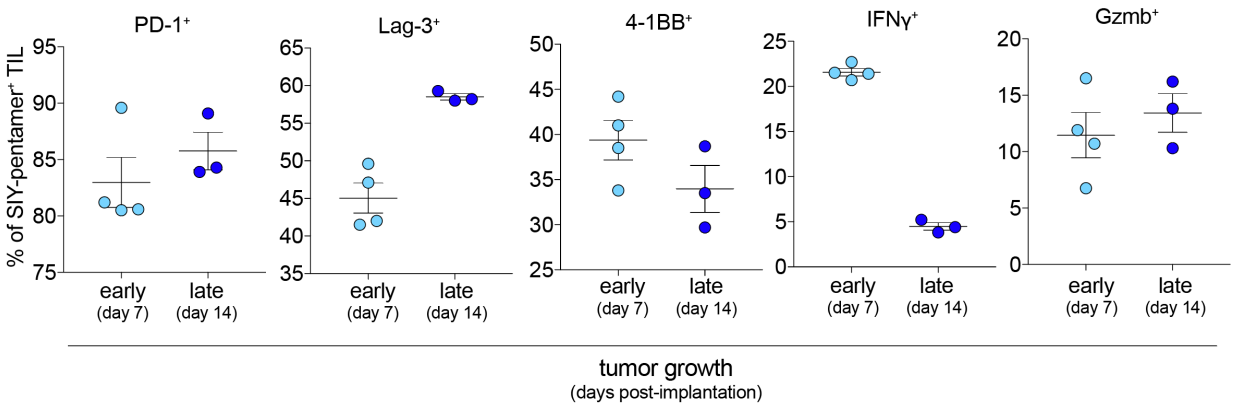

Figure A.3. Anti-tumor SIY-reactive T cell infiltrate becomes exhausted during progression of MC38-SIY tumors. Frequency of SIY-reactive TIL in MC38-SIY tumors expressing the indicated molecules at day 7 and day 14 of tumor growth. Representative data from one experiment is shown (n = 4 mice per group per experiment). Data are shown as mean ± s.e.m.

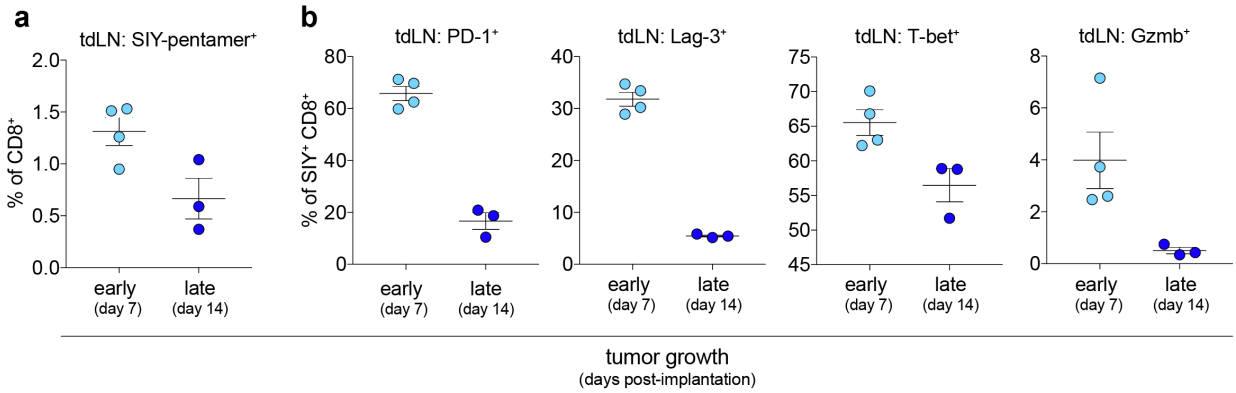

Figure A.4. Anti-tumor SIY-reactive T cells in the tdLN decrease over time and exhibit blunted activation. (a, b) Frequency of SIY-reactive T cells **(a)** and their expression of the indicated molecules **(b)** in the tdLN of mice bearing MC38-SIY tumors at day 7 and day 14 of tumor growth. Representative data from one experiment is shown (n = 4 mice per group per experiment). Data are shown as mean ± s.e.m.

Priming of anti-tumor CD8$^+$ T cell responses is impaired during the course of MC38-SIY tumor progression.

To confirm whether there is a deficiency in T cell priming during the course of tumor progression, we performed an *in vivo* priming assay wherein we intravenously transferred 2C T cell receptor (TCR) transgenic CD8$^+$ T cells that had been labeled with a proliferation dye into mice bearing MC38-SIY tumors at day 7 (early-stage) or day 11 (late-stage) of

tumor growth. Three days later, we analyzed the recovery of the transferred 2C T cells in the tdLN of mice. Indeed, 2C T cells that were transferred at day 11 were less proliferative compared to those transferred at day 7 of tumor growth **(Figure A.5a)**. Consistent with our profiling of endogenous anti-tumor T cells indicating poor activation at late stages of tumor growth, we observed that 2C T cells transferred at day 11 were mostly in a naïve state ($CD62L^+$) **(Figure A.5b)**. In contrast, those transferred at day 7 of tumor growth exhibited more of an activated phenotype, comprising both effector or effector memory T cells (T_{EM}; $CD44^+$ $CD62L^-$) and central memory T cells (T_{CM}; $CD44^+$ $CD62L^+$) **(Figure A.5b)**. Interestingly, there were also striking phenotypic differences among the activated ($CD44^+$) 2C T cells from both time points. 2C T cells transferred at day 7 harbored a greater fraction of T_{EM} and T_{CM} cells expressing PD-1, Lag-3, and T-bet compared to 2C T cells transferred at day 11 **(Figure A.5c)**. Collectively, these data confirm our hypothesis that T cell priming is impaired over the course of MC38-SIY tumor progression.

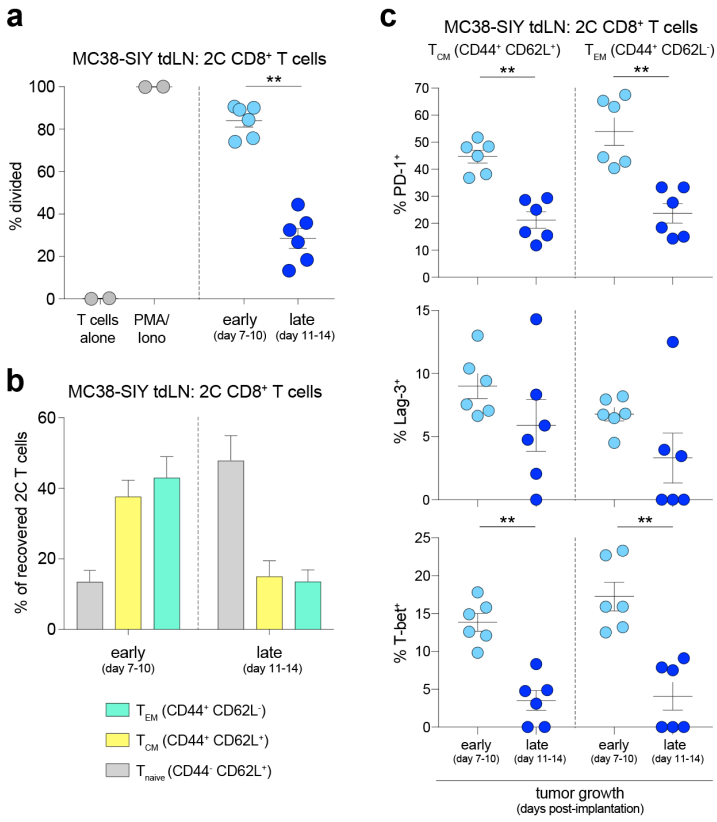

Figure A.5. *In vivo* 2C T cell priming in tdLN of mice bearing MC38-SIY tumors is blunted during the course of tumor progression. (a) Proliferation of 2C T cells in tdLN that were transferred *i.v.* into MC38-SIY tumor-bearing mice at day 7 or day 11 of tumor growth and analyzed three days later. **(b)** Phenotypes of the recovered 2C T cells from tdLN of MC38-SIY tumor-bearing mice at the indicated timepoints. **(c)** Expression of the indicated molecules on T_{CM} or T_{EM} of the recovered 2C T cells in tdLN of MC38-SIY tumor-bearing mice at the specified timepoints. Pooled data from two independent experiments is shown (n = 3 mice per group per experiment). **p<0.01; MWU test (a, c). Data are shown as mean ± s.e.m. Data generated by Maria Zagorulya.

Blunted anti-tumor T cell priming in MC38-SIY tumors over time is mediated by impaired DC1 recruitment and function.

As detailed in Chapter 2, we identified that anti-tumor immunity against MC38-SIY tumors was fully dependent on Batf3-driven DC1. As DC1 are thought to be the critical DC subset required for priming $CD8^+$ T cell responses, we used flow cytometry to profile the DC1 infiltrate in MC38-SIY tumors and tdLN at day 7 and day 14 of tumor growth. Consistent with our earlier data **(Figure 2.2)**, we observed a decrease in DC1 in MC38-

SIY tumors as a proportion of MHC-II$^+$ cells **(Figure A.6a)** and as absolute numbers in the tumor **(Figure A.6b)**. Similarly, a decrease in the frequency of DC1 was also observed in the tdLN **(Figure A.6c)**. These data point towards failed DC1 recruitment to tumors over time as a potential factor mediating defective T cell priming.

Besides impaired DC1 recruitment, it is also possible that defective antigen uptake or migration to the tdLN might render DC1 dysfunctional. To address this possibility, we implanted a MC38 cell line expressing the pH stable fluorophore ZsGreen into WT mice, so we could track the uptake of tumor antigen via ZsGreen expression at early (day 7) and late (day 11) timepoints of tumor growth. We detected similar numbers of ZsGreen$^+$ DC1 in tdLN over time, suggesting that defects in antigen uptake and migration are likely not the causative factors of dysfunction **(Figure A.7)**. Despite carrying antigen, however, DC1 isolated from late-stage (day 11) MC38-SIY tumors were poorly stimulatory in an *ex vivo* co-culture assay with 2C T cells **(Figure 3.1B-3.1C)**. Moving forward, it will be critical to assess whether impaired antigen-processing or presentation is mediating the inability of DC1 to activate 2C T cells.

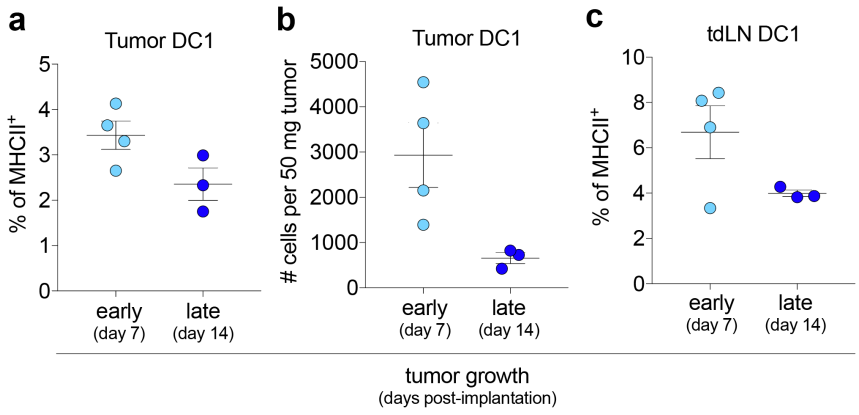

Figure A.6. DC1 decrease over time in MC38-SIY tumors and tdLN. (a, b) Frequency **(a)** and absolute numbers **(b)** of DC1 in MC38-SIY tumors at day 7 and day 14 of tumor growth. **(c)** Frequency of DC1 in tdLN of mice bearing MC38-SIY tumors at day 7 and day 14 of tumor growth. Representative data from one experiment is shown (n = 4 mice per group per experiment). Data are shown as mean ± s.e.m.

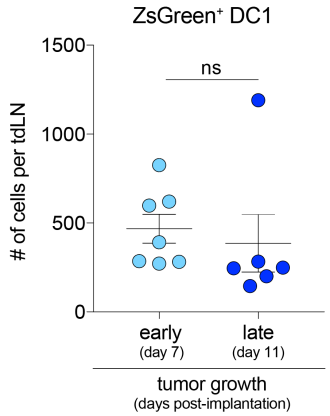

Figure A.7. Antigen uptake by DC1 and trafficking to the tdLN is not impacted by tumor progression. Absolute numbers of ZsGreen$^+$ DC1 in tdLN of mice bearing MC38-SIY tumors at day 7 and day 11 of tumor growth. Pooled data from two independent experiments is shown (n = 3-4 mice per group per experiment). ns, not significant; MWU test. Data are shown as mean ± s.e.m. Data generated by Maria Zagorulya.

Blunted DC maturation is another possible manifestation of dysfunction. As described in Chapter 1, DC need to be able to sense danger signals to mount an immunogenic T cell response. Sensing of danger signals via pattern recognition receptors (PRR) induces a maturation program in DC that is characterized by increased expression of costimulatory molecules, among other functional and phenotypic changes. Interestingly, we observed that DC1 in both MC38-SIY tumors and tdLN appeared to be less mature over time, as indicated by a decreased proportion of CD86$^+$ DC1 from day 7 to day 14 of tumor growth **(Figure A.8)**. Thus, it is likely that a combination of impaired DC recruitment to the tumor, defective antigen-processing and presentation, and blunted DC maturation is mediating failed CD8$^+$ T cell priming in MC38-SIY tumors over time.

To derive additional mechanistic insights, we performed single-cell RNA-sequencing of the DC infiltrate in MC38-SIY tumors at day 7 and day 14 of growth. We observed an increase in the expression of metabolic-related genes suggesting that perhaps dysregulated metabolism might impair DC1 function, maturation, recruitment, or

even possibly survival in MC38-SIY tumors **(Figure A.9)**. All of these factors can induce DC1 dysfunction and are thus open avenues for further evaluation.

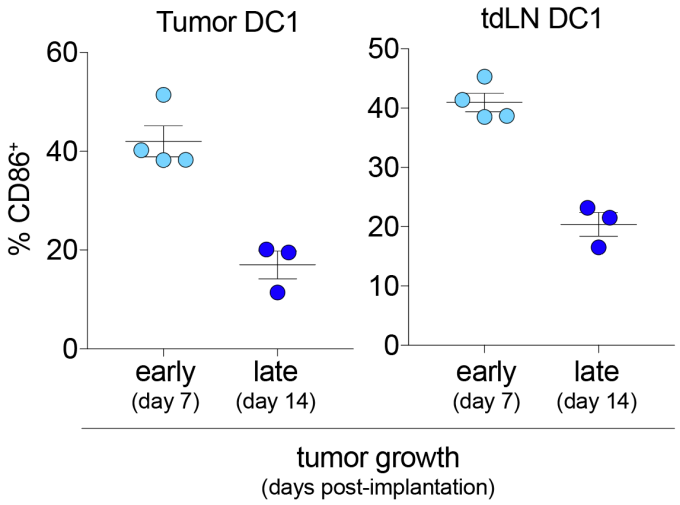

Figure A.8. DC1 from in MC38-SIY tumors and tdLN exhibit decreased maturation profile during tumor progression. Frequency of CD86$^+$ DC1 in tumors *(left)* and tdLN *(right)* of mice bearing MC38-SIY tumors at day 7 and day 14 of tumor growth. Representative data from one experiment is shown (n = 3-4 mice per group per experiment). Data are shown as mean ± s.e.m.

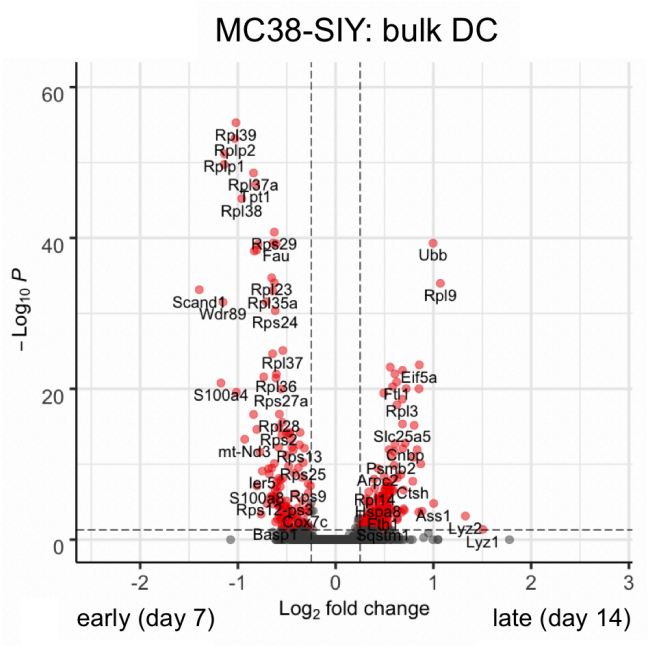

Figure A.9. Volcano plot of differentially expressed genes in bulk DC isolated from MC38-SIY tumors at day 7 and day 14 of tumor growth. Each dot represents a gene. Plot generated by Maria Zagorulya.

DISCUSSION

In this work, we identified a correlation between resistance to anti-PD-1 monotherapy at late stages of tumor growth and DC1 dysfunction. There was a decrease in DC1 presence in the tumor and tdLN from day 7 to day 14 of tumor growth. While antigen uptake and ability of DC1 to traffic to tdLN seemed to be unaffected, DC1 isolated from late-stage tumors failed to stimulate $CD8^+$ T cells *ex vivo.* Suppressed activation or maturation could be a contributing factor because DC1 from day 14 tumors exhibited decreased CD86 expression compared to those from day 7 tumors. Single-cell RNA-seq profiling of the DC compartment in day 7 versus day 14 MC38-SIY tumors indicated that dysregulated metabolism could be another factor mediating DC1 dysfunction over time in tumors. Identifying the drivers of DC1 dysfunction during the course of tumor progression can open new therapeutic avenues to treat cancer by restoring DC1 function.

DC1 contribute several important functions in the induction of an anti-tumor immune response. As the predominant cross-presenting DC subset, they are integral for cross-priming T cells against tumor-derived antigens. To do so, they need to uptake antigens, process and present antigenic peptides on surface MHC-I molecules, and migrate to the tdLN to encounter naïve T cells. Simply presenting antigens to naïve T cells is not sufficient to drive immunogenic T cell activation. DC1 also need to be in an activated or mature state and express costimulatory molecules to successfully induce an effector T cell response against tumors. Tumor-resident DC1 are also critical for recruiting effector T cells into the tumor. Defects in any of these processes would result in DC1 dysfunction and could enable tumor immune evasion.

Our work, though preliminary, indicates that DC1 dysfunction in MC38 and MC38-SIY tumors might be mediated by a failure to maintain DC1 recruitment to the tumors, defective antigen-processing and presentation, impaired DC activation, dysregulated metabolism, or a combination of any of these factors. Studies have shown that exclusion or absence of DC1 prevents the induction of an anti-tumor T cell response (Spranger et al., 2015). Enrichment of a DC1 signature in tumors correlates with better survival and response to CBT (Barry et al., 2018; Broz et al., 2014). By extension, failed DC1 recruitment over time can blunt the anti-tumor T cell response and conceivably mediate acquired resistance to CBT. Studies have shown that Natural Killer (NK) cells are critical for the recruitment of DC1 to tumors (Barry et al., 2018; Böttcher et al., 2018). Thus, it will be interesting to assess for the presence of NK cells in MC38 tumors at day 7 and day 14 to determine whether they also decrease over time. Another means to enable a DC1 excluded TME is via premature death of DC1 in tumors. Indeed, several studies have shown that tumor-produced factors could induce DC apoptosis in tumors in both humans and mice (Esche et al., 1999; Kiertscher, Luo, Dubinett, & Roth, 2000; Ma, Shurin, Peiyuan, & Shurin, 2013; Péguet-Navarro et al., 2003; Yang et al., 2002). Whether tumor-induced DC apoptosis is a contributing factor in MC38 tumors over time requires further evaluation.

Aside from the decreasing presence of DC1 in tumors over time, we also observed that the tumor-infiltrating DC1 from late-stage MC38-SIY tumors failed to activate 2C T cells *ex vivo.* We determined that this functional deficiency was likely not due to defective antigen uptake, but the possibility of defective antigen-processing and/or cross-presentation remains. More work is needed to evaluate whether this might be the case.

Impaired maturation is also detrimental for the induction of an anti-tumor immune response as described in Chapter 1. Indeed, DC1 profiled from day 14 MC38-SIY tumors expressed lower levels of CD86 compared to day 7 tumors, which is suggestive of blunted DC activation. It will be interesting to determine whether *ex vivo* maturation using a PRR agonist like DMXAA or Poly(I:C) would be sufficient to restore the stimulatory capacity of DC1 as this would have therapeutic implications. It is currently unknown whether these DC1 are in a reversible state of dysfunction or if it is permanent.

Lastly, our preliminary scRNA-seq data suggested that dysregulated metabolism might contribute to DC1 dysfunction in late-stage MC38-SIY tumors. Indeed, studies have shown that altered metabolism can influence the immunostimulatory capacity of DC (Bullock & Dong, 2014). One prominent gene upregulated in day 14 DC compared to day 7 DC was *Ftl* encoding ferritin light chain. It is possible that dysregulated iron metabolism is impacting the functional capacity of DC1, and efforts to test this hypothesis are underway. At the same time, it might also be interesting to determine the metabolic profile of day 7 versus day 14 interstitial fluid of MC38 tumors to determine whether there are any significant differences in metabolites that might induce DC1 dysfunction. Once we have potential candidates in hand, we plan to genetically or biochemically perturb them to determine the impact on DC1 in late-stage tumors, as well as to assess whether this perturbation re-sensitizes late-stage MC38 tumors to CBT. Completion of this work has the potential to open new avenues for therapeutic development targeting DC1 dysfunction.

MATERIALS AND METHODS

Please refer to Chapter 2 for Materials and Methods related to: *Tumor dissociation* / *Flow cytometry and cell sorting* / *SIY-pentamer staining* / *Ex vivo APC-T cell coculture assay* / *Statistical analysis*.

Mice. C57BL/6 and Rag2$^{-/-}$ mice were purchased from Taconic Biosciences. T cell receptor transgenic (TCR-tg) 2C Rag2$^{-/-}$ mice were bred and maintained in-house. All mice were housed and bred under specific pathogen free (SPF) conditions at the Koch Institute animal facility. For all experiments, mice were gender-matched and age-matched to be 6-12 weeks old at the time of experimentation. All experimental animal procedures were approved by the Committee on Animal Care (CAC/IACUC) at MIT.

Generation of ZsGreen expression vector. The pLV-EF1α-IRES-puro vector (Addgene #85132) was digested with *BamHI* and *EcoRI* restriction enzymes (NEB) to linearize the vector. The ZsGreen insert was generated from pCAGGS_ZsGreen_minOVA (a gift from Max Krummel at UCSF). The insert was cloned into the linearized pLV-EF1α-IRES-puro vector (final construct referred to as 'pLV-EF1α-ZsGreen-IRES-puro') using the In-Fusion cloning kit (Takara Bio), amplified, and sequenced for accuracy.

Tumor cell lines and tumor outgrowth studies. Parental and SIY-GFP expressing MC38 colon carcinoma tumor cell lines were a gift from the Gajewski laboratory at The University of Chicago. The MC38 tumor line stably expressing ZsGreen was generated by lentiviral transduction of the parental tumor line with the pLV-EF1α-ZsGreen-IRES-puro construct

and puromycin (Gibco) selected. Expression was confirmed using flow cytometry for ZsGreen-expressing cells.

Tumor cell lines were cultured at 37°C and 5% CO_2 in DMEM (Gibco) supplemented with 10% FBS (Atlanta Biologicals), 1% penicillin/streptomycin (Gibco), and 1X HEPES (Gibco). Tumor cells were harvested by trypsinization (Gibco) and washed 3 times with 1X PBS (Gibco). Cells were resuspended in PBS, and $0.5x10^6$ parental MC38 cells, $2x10^6$ MC38-SIY-GFP, or $2 x10^6$ MC38-ZsGreen tumor cells were injected subcutaneously into the flanks of mice. Subcutaneous tumor area measurements (calculated as *length* x *width*) were collected 2-3 times a week using digital calipers until the endpoint of the study.

In vivo anti-PD-1 treatment. For early treatment, 200 µg anti-PD-1 (clone 332.8H3 from Gordon Freeman at Dana-Farber Cancer Institute) in 100 µL PBS was injected intraperitoneally into mice at days 7, 10, and 13 post-tumor implantation. For late treatment, 200 µg anti-PD-1 in 100 µL PBS was injected intraperitoneally into mice at days 11, 14, 17 post-tumor implantation. As a control, 100 µL PBS was injected intraperitoneally into mice at the corresponding time points.

In vivo 2C T cell priming assay. Spleens and inguinal lymph nodes of 2C $Rag2^{-/-}$ $CD45.1^+$ mice were dissected and dissociated into single cell suspensions as previously described. Cells were labeled with CellTrace Violet (Life Technologies) following the manufacturer's instructions. Approximately 1 million labeled cells were transferred to MC38-SIY tumor-bearing C57BL/6 mice at days 7 and 11 post-tumor inoculation. Three days post 2C T

cell transfer, recipient animals were euthanized and the 2C T cells in spleen and tdLN were analyzed by flow cytometry.

Single-cell RNA-sequencing and analysis. Live intratumoral $CD45^+$ cells from WT mice bearing MC38-SIY tumors at day 7 and day 14 post-tumor implantation were FACS-sorted as described. Please refer to Chapter 2 for Materials and Methods related to sample handling and submission, cDNA library preparation using the 10X Genomics platform, and scRNA-seq analysis. Note, the sequencing data from day 7 and day 14 tumors were integrated prior to performing the scRNA-seq analyses.

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