Characterization of Tumor Immune Infiltrate to Elucidate Immune Components Involved in Response to Checkpoint Inhibitor Therapies in Mouse Syngeneic Tumor Models

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Characterization of Tumor Immune Infiltrate to Elucidate Immune Components Involved in Response to Checkpoint Inhibitor Therapies in Mouse Syngeneic Tumor Models

Mark Zielstorff

A thesis in the Field of Biotechnology
For the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Seminal research conducted over the last two decades has confirmed that the immune system plays a crucial role in the processes that regulate tumor growth and development. So-called immune surveillance is an idea that has persisted for decades, and evidence supporting the hypothesis that the immune system detects and destroys neoplastic growth has accumulated over time. More recently the understanding of the role of immune checkpoints in cancer has led to breakthrough immunotherapeutic drugs such as ipilimumab (anti-CTLA-4 antibody), and pembrolizumab and nivolumab (anti-PD-1 antibodies). These drugs, also known as checkpoint inhibitors, act by blocking inhibitory immune receptors on T cells thereby enhancing the ability of the immune system to mount an anti-tumoral response. While immunotherapies can lead to durable, robust responses, the rate of response is quite variable across and within tumor types (Topalian, Taube, Anders, & Pardoll, 2016). There is currently an intense effort to characterize what differentiates responders from non-responders that will lead to the development of predictive criteria capable of identifying these populations prior to the initiation of treatment. For instance, the FDA has approved companion diagnostics that quantify the amount of programmed cell death ligand 1 (PD-L1) there is within a tumor for prediction of response to nivolumab and pembrolizumab (Topalian, Taube, Anders, & Pardoll, 2016). However, a recent analysis found that the response rate to anti-PD-1 antibody in PD-L1+ tumors was 48% while PD-L1- tumors still maintained a 15% response rate.
(Sunshine & Taube, 2015), demonstrating the difficulty that exists in predicting absolute response to these antibodies.

Ongoing work at Merck Research Laboratories (MRL) in Boston is focused on developing next generation immuno-oncology therapeutics. Understanding the molecular mechanisms that regulate response and non-response is central to this work. Traditional mouse tumor models relied on implantation of cells derived from human tumors, called xenografts, that required immunodeficient hosts. These models were suitable for the study of cytotoxic drugs designed to kill tumor cells directly. However, they would not be suitable for the study of tumor-immune interaction. The preclinical research at Merck currently relies heavily on mouse syngeneic tumor models that, crucially, use mice with an intact immune system capable of mounting immune responses directed towards the tumor. Here, tumor bearing mice are generated by exogenous implantation of cultured tumor cell lines derived from primary mouse tumors obtained from either naturally occurring (spontaneous) or carcinogen-induced tumors raised in mice of the same genetic background. Upon subcutaneous inoculation implanted tumor cells grow into solid tumors that can be easily measured for response to therapeutic intervention. The most commonly used murine syngeneic model at MRL uses the MC38 colon adenocarcinoma cell line. Tumors generated from these cells demonstrate robust responsiveness to murine anti-PD-1 antibody (mDX400). Typical response rates in mice bearing 80-120mm³ tumors are between 60-80% complete regressions (with additional partial responses) following 5 milligram/kilogram (mg/kg) dosing of mDX400, administered intraperitoneally (IP) every 5 days (Q5D) for approximately 15-20 days (data not shown). As MC38 tumors grow larger than this 100mm³ average size they have markedly lower
response rates. It is possible that immune cell populations contribute to this observation. Phenotyping of explanted dissociated 80-120mm³ tumors via mass cytometry time of flight (Helios, Fluidigm) revealed that as much as 50% of cells within these tumors are CD45⁺ immune cell infiltrate. Furthermore, the PD-1/PD-L1 axis is abundant: 60+% of T cells express PD-1, ~90% of myeloid cells express PD-L1, and among CD45⁻ cells, which consist of tumor and stromal cells, PD-L1 expression is nearly 50% (data not shown). Moreover, in tumors of this size the CD4⁺ T cell compartment is typically 10-12% CD25⁻Foxp3⁺ regulatory T cells (Treg). Opportunistic phenotyping of large tumors (600mm³ and 1000mm³) revealed drastic increases in Treg cells within the tumor infiltrating lymphocytes (TIL): ~30% and ~60% of total CD4⁺, respectively. Several lines of research have demonstrated the importance of IL-10 in the activation and anti-tumor activity of tumor resident CD8⁺ cytotoxic T cells, which is type I IFN dependent (Emmerich, et al., 2012) (Mumm, et al., 2011) (Stewart, et al., 2013). Additionally, it has been shown that the majority of intratumoral IL-10 is generated by Treg, which plays an important role in restraining tumorigenic Th17 inflammation (Stewart, et al., 2013). Therefore, one possible explanation for the loss of responsiveness to anti-PD-1 treatment as tumors progress is that while Treg numbers increase they become dysfunctional and lose the ability to both suppress Th17 inflammation and activate intratumoral CD8⁺ T cells. Elucidating the role of the type I IFN/IL-10/Treg axis in this phenomenon could provide valuable insight into strategies for treating patients that respond poorly to checkpoint inhibitor therapy.

The current study interrogates whether changes in the type I IFN/IL-10/Treg axis within the tumor microenvironment contributes to poor therapeutic response in syngeneic
tumors across tumor progression through a comprehensive profiling of surface markers and cytokine production.
Table of Contents

List of Tables ................................................................................................................................. ix

List of Figures ................................................................................................................................. x

I. Introduction ................................................................................................................................ 1

The immune system and cancer ................................................................................................. 2

Importance of tumor infiltrating lymphocytes .......................................................................... 4

Immunosuppressive mechanisms in the tumor microenvironment ......................................... 5

Cancer therapeutics ................................................................................................................... 9

Factors limiting efficacy of immunotherapeutics ..................................................................... 11

IL-10 as a pro-inflammatory cytokine ..................................................................................... 12

II. Materials and Methods ............................................................................................................ 14

Mice ................................................................................................................................................. 14

*In vitro* cell culture ..................................................................................................................... 14

*In vivo* studies ............................................................................................................................. 15

Flow cytometry ............................................................................................................................ 16

Mass cytometry ............................................................................................................................... 17

Cytokine analysis .......................................................................................................................... 18

Statistical analyses ....................................................................................................................... 19
III. Results........................................................................................................................................... 21

Mouse CT26 syngeneic model........................................................................................................... 21

Analysis of immune cell infiltrate in small and large tumors.............................................. 23

Changes in immune cell infiltrate and phenotype with anti-PD-1 antibody treatment .......................................................... 25

Innate immune activation................................................................................................................ 31

Combination treatment with STING agonist and anti-PD-1 antibody ...................... 35

Results Summary ..................................................................................................................... 40

IV. Discussion.................................................................................................................................. 43

References........................................................................................................................................ 51
List of Tables

Table 1. Flow cytometry antibody panel. ................................................................. 17
Table 2. Mass cytometry antibody panel. .............................................................. 19
Table 3. Treatment paradigm for CT26 DMXAA mDX400 combination study ........ 35
List of Figures

Figure 1. CT26 efficacy data with mDX400 treatment .................................................... 22
Figure 2. PD-1 and PD-L1 expression in small CT26 tumors .............................................. 26
Figure 3. Flow cytometry data from small CT26 tumors treated with mDX400 .............. 28
Figure 4. Flow cytometry data from large CT26 tumors treated with mDX400 .............. 30
Figure 5. STING pathway schematic ................................................................................ 32
Figure 6. Tumor cytokine data following cGAMP administration ...................................... 33
Figure 7. CT26 efficacy data with cGAMP treatment ........................................................ 34
Figure 8. Tumor volume data from CT26 DMXAA mDX400 combination study .......... 36
Figure 9. Cytokine data from CT26 DMXAA mDX400 combination study ................... 37
Figure 10. Flow cytometry data for CT26 DMXAA mDX400 combination study .......... 39
Figure 11. Type I IFN signaling schematic ...................................................................... 46
Figure 12. IL-12 pathway schematic ................................................................................ 47
Chapter 1

Introduction

Alberts et al. in *Molecular Biology of the Cell* define cancer cells as having two distinct properties: “they and their progeny (1) reproduce in defiance of the normal restraints on cell division and (2) invade and colonize territories normally reserved for other cells” (Alberts, et al., 2002). The mechanisms by which normal, healthy cells acquire these traits are both complex and not fully understood, but broadly involve multiple somatic mutations that result in uncontrolled growth and resistance to apoptotic pathways that normally suppress aberrant behavior. Thus, cancer is a heterogeneous disease with an array of morphologies, clinical manifestations, and treatments, and cancerous growths, called neoplasms, are defined by these varied criteria. The primary, and in many cases most critical distinction is that of malignancy. Non-malignant, or benign, neoplasms remain localized to the tissue or cell type of origin, and in most cases are not dangerous, but if necessary can be effectively treated via surgical resection. However, benign growths can become clinically relevant when they occur in tissues sensitive to compression, such as the brain, or if they occur in tissues that secrete hormone (Boffetta, Boccia, & La Vecchia, 2014). Malignant neoplasms are characterized by growth that results in structural and functional changes compared to the tissue of origin, and in many cases are subject to metastasis, the process by which tumor cells
migrate out of the tissue of origin and colonize separate and distinct tissues at distal sites throughout the body. The presence or absence of metastases often defines a patients’ prognosis and expected success to therapeutic intervention (Boffetta, Boccia, & La Vecchia, 2014). Nomenclature for tumor classification is based on the tissue of origin, and malignancy status. For example, neoplasms that arise from epithelial cells are called carcinomas, while those that arise from muscle or connective tissue are called sarcomas. Additionally, malignancy status can be conveyed; a benign growth of glandular tissue is called an adenoma, while a malignant growth of the same tissue is called an adenocarcinoma. The vast majority of malignant neoplasms in adults are carcinomas. Additionally, the World Health Organization maintains a coding index of neoplasms classified based on a combination of topographical and morphological characteristics called the International Classification of Diseases for Oncology (World Health Organization, 2013).

The immune system and cancer

As early as 1909, the German physician scientist Paul Ehrlich hypothesized that the immune system was responsible for curtailing the growth of neoplasms which he speculated would otherwise occur with greater frequency (Ehrlich, 1909). This concept, which eventually became known as immunosurveillance, was actively debated amongst immunologist and cancer biologists for decades. The Nobel Prize winning physician researcher F. Macfarlane Burnet would pen several articles on the concept of tumor neo-antigens that he believed would elicit an immune response (Burnet, 1957) (Burnet, 1964) (Burnet, 1970). His contemporary, Lewis Thomas also made several important
contributions. Thomas described the evolution of the understanding of cellular immunity, and the natural progression to a theory of immune surveillance as a protective mechanism against neoplasms (Thomas, 1982). Together, Burnet and Thomas are largely credited with developing the modern concept of immunosurveillance. However, it was not until the technological advances in mouse genetics and monoclonal antibody production that scientists would have the tools necessary to sufficiently probe the idea. It was during this time that studies would clearly demonstrate the link between immunity and cancer.

While the contributions of the 20th century clearly implicated the role of the immune system in modulating cancer it became clear that the original concept of immunosurveillance was incomplete. The idea that the immune system is capable of detecting transformed, mutated cells belies the fact that people develop clinically relevant disease at all. In 1998 Robert Schreiber’s lab at the University of Washington in St. Louis showed that insensitivity to interferon gamma (IFNγ), a key pleiotropic cytokine that mediates innate and adaptive immune responses, significantly increased the speed and frequency of both spontaneous and induced tumor formation (Kaplan, et al., 1998). Additional findings out of the Schreiber lab demonstrated that tumors are capable of escaping immune detection by modulating immunogenicity. Shankaran et al. (2001) showed that mice lacking Rag2 (recombination-activation gene 2, which is necessary for recombination of B and T cell receptors, and expressed only in those cells) and STAT1 (signal transducer and activator of transcription 1, necessary for IFNγ signaling) had significantly higher rates of both chemically induced and spontaneous tumor formation compared to wild type mice, and using tumor transplantation approaches demonstrated that tumors that developed in immunodeficient mice are more immunogenic than tumors
raised in the presence of an intact immune system (Shankaran, et al., 2001). These findings demonstrate that the immune system is essential in preventing the development of tumors, but also, paradoxically, contributes to the evolution of less immunogenic tumors. The authors proposed a new concept to provide a fuller understanding of the dynamics between immunity and tumor formation which they termed immunoediting. This concept includes several phases, which begins with immunosurveillance, but if tumors are not initially eradicated they can be edited by the immune system. During the editing process the most immunogenic cells are removed leaving behind the least immunogenic cells, which facilitates the process of tumor escape whereby tumor growth can no longer be controlled by the immune system alone.

Importance of tumor infiltrating lymphocytes

Through the work describing the processes that define the concepts of immunosurveillance and immunoediting, it became clear that infiltration of immune cells into developing malignant neoplasms was a critically important factor in the development of disease. The discussion of tumor immune infiltrate is most commonly limited to lymphocytes, known as tumor infiltrating lymphocytes (TIL), because lymphocytes (predominately CD8+ T cells) are the effector cells that carry out the antitumoral response. It should be noted, however, that immune infiltrate is also comprised of innate immune cells. These cells contribute to both T cell activation and suppression, and are addressed further in the discussion of the immunosuppressive aspects of the tumor microenvironment.
Given the link between the immune system and cancer understanding the composition of TIL and the relevance to clinical outcomes can provide important insights. In fact, investigators have been attempting immunotherapeutic approaches for decades whereby TIL are collected from a patient biopsy, expanded in vitro, and injected back into the patient, long before the mechanistic underpinnings of this approach had been established. Steven Rosenberg’s lab at NIH was among the first to describe methods for expanding patient derived TIL for the use in immunotherapy trials (Topalian, Muul, Solomon, & Rosenberg, 1987) (Beldegrun, Muul, & Rosenberg, 1988). Subsequently it has been shown that the amount of CD8\(^+\) infiltrate in tumors is positively correlated with prognosis, and that the presence of TIL was able to predict increased survival in various types of cancer (Kim, Emi, & Tanabe, 2007) (Whiteside, 2012). Further, in a meta-analysis by Gooden et al. (2011), they revealed that while the presence of TIL moderately influenced prognosis, the effect was most pronounced in studies reporting ratios of CD8\(^+\) to suppressive Foxp3\(^+\) T regulatory (T\(_{reg}\)) cells (Gooden, de Bock, Leffers, Daemen, & Nijman, 2011). These data showed that while the presence of TIL can be an important prognostic indicator, understanding TIL phenotype and composition is critical.

Immunosuppressive mechanisms in the tumor microenvironment

Given that tumor cells have mutated and no longer accurately resemble “self,” and the proinflammatory aspects of tumorigenesis (Landskron, De la Fuente, Thuwajit, Thuwajit, & Hermoso, 2014), one might expect tumors to be highly immunogenic. However, the tumor microenvironment (TME) is highly immunosuppressive (Guan & Chen, 2013) (Topalian, Taube, Anders, & Pardoll, 2016). The factors contributing to the
immunosuppression observed in the TME are complex, but are broadly attributable to regulatory immune cells, mesenchymal stem cells, and upregulation of immune checkpoint inhibitors.

A large percentage of tumor immune infiltrate in most cancer types consist of tumor associated macrophages (TAM) and tumor associated dendritic cells (TADC) (Mantovani, Bottazzi, Colotta, Sozzani, & Ruco, 1992) (Balkwill & Mantovani, 2001). Properly activated macrophages can kill tumor cells and vascular endothelial cells necessary for continued tumor growth, and mature dendritic cells can elicit potent anti-tumor responses by effectively presenting tumor antigens to T cells. However, both TAM and TADC present an immature phenotype, rendering them ineffective (Allavena, et al., 2000) (Mantovani, Bottazzi, Colotta, Sozzani, & Ruco, 1992). In fact, the more recently defined myeloid derived suppressor cells (MDSC) commonly found in tumor immune infiltrate are known to be immature undifferentiated cells of myeloid origin that in the presence of appropriate growth factors can differentiate into mature macrophages and dendritic cells. MDSC actively inhibit T cell responses by upregulating the production of arginase and indoleamine-pyrrole 2,3-dioxygenase (IDO), enzymes that in turn reduce the availability of the amino acids L-arginine and tryptophan, respectively; L-arginine is required by T cells for the expression of the T cell co-receptor CD3ε, while tryptophan is necessary for T cell proliferation (Nagaraj & Gabrilovich, 2007).

The canonical regulatory immune cell is the CD4⁺CD25⁺Foxp3⁺ regulatory T cell (T_{reg}), which has been of interest to tumor biologists for many years, as mentioned previously. Regulatory T cells, which express the transcription factor forkhead box p3
(foxp3), actively engage in maintenance of immunological self-tolerance and immune homeostasis (Sakaguchi, 2004) (Sakaguchi, Wing, Onishi, Prieto-Martin, & Yamaguchi, 2009). T_{reg} cells that are derived in the thymus are referred to as natural T_{reg} (nT_{reg}), and are generated through MHC class II-dependent T cell receptor interactions resulting in high avidity selection (Larkin, et al., 2008), making them potent mediators of immune self-tolerance. Additionally, peripheral CD4^+ naïve T cells can be induced to express foxp3 which confers to them a suppressive phenotype (Apostolou & von Boehmer, 2004). Induced T_{reg} (iT_{reg}) seem to be generated in response to antigen exposure and require, in addition to T cell receptor simulation, exposure to TGFβ and IL-2 (Chen & Konkel, 2010) (Fu, et al., 2004), which is not the case for nT_{reg} (Curotto de Lafaille & Lafaille, 2009). Thus, iT_{reg} can be viewed as an element of adaptive immunity. Curroto de Lafaille et al. speculate that iT_{reg} may have developed through coevolution of the mammalian adaptive immune system and gut commensal bacteria as a result of constant exposure to varied food antigens (Curotto de Lafaille & Lafaille, 2009).Suppressive function of T_{reg} cells seems to be a product of the methylation state of CpG motifs in the foxp3 locus. Demethylation of an evolutionarily conserved region called T_{reg} cell-specific demethylated region (TSDR) is unique to foxp3^+ T cells. Unlike nT_{reg} cells, iT_{reg} cells display only partial demethylation of this region regardless of the level of foxp3 expression, and foxp3 expression in these cells can be lost over time (Huehn, Polansky, & Hamann, 2009). The natural/induced composition of intratumoral T_{reg} has been an area of active research, and has generated conflicting results. For example, Malchow et al. have observed that development of T_{reg} cells found in murine oncogene-driven prostate cancer was dependent on a protein called Aire which is necessary for thymic
development of organ specific nTreg (Malchow, et al., 2013). And Liu et al. have suggested that the ability of tumor cells to generate iTreg in the periphery is one of the mechanisms by which tumor cells escape immune detection (Liu, et al., 2007).

Mesenchymal stem cells (MSC) are pluripotent cells that give rise to a variety of tissues including bone, adipose, and cartilage (Jiang, et al., 2002). Upon migration to the tumor microenvironment they can differentiate into cancer associated fibroblasts (CAF) (Direkze, et al., 2004). MSC contribute to tumorigenesis in a number of ways including the promotion of tumor cell “stemness,” enhancing the ability of tumor cells to migrate, facilitating angiogenesis, increasing resistance to chemotherapies, and suppressing immune responses (Guan & Chen, 2013). Immunosuppressive cytokines secreted by MSC largely overlap with those associated with Treg and MDSC, and MSC may contribute to increased Treg numbers in the TME (Guan & Chen, 2013).

Finally, the chronic inflammatory status of the tumor results in T cells that acquire an exhausted phenotype similar to that seen in chronic viral infection. Exhausted T cells express the inhibitory immunomodulatory receptors (IMRs) programmed cell death 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG3), and T cell immunoglobulin and mucin 3 (TIM3), among others (Blackburn, et al., 2008) (Mahoney, Rennert, & Freeman, 2015). In addition, IFNγ secreted by T cells leads to upregulation of PD-1 ligand 1 (PD-L1) and IDO in the tumor microenvironment, which could potentially lead to a prolonged and dominant immunosuppressive environment.
Cancer therapeutics

Although Paul Ehrlich was the first to document the systematic use of chemicals to treat diseases, including cancers, at the beginning of the 20th century (coining the term chemotherapy), prior to the 1960’s the standard treatment for cancer was surgery and radiation (DeVita & Chu, 2008). It was not until World War II when accidental exposure to nitrogen mustards lead to pioneering research in the 1940’s and 1950’s on alkylating compounds and their effects on cell proliferation that physicians and researchers began in earnest to evaluate chemicals that could be used to treat cancers (DeVita & Chu, 2008). It was during this time that cancer chemotherapy workhorses such as methotrexate and cyclophosphamide, molecules that, like nitrogen mustards, inhibit DNA assembly which ultimately leads to cell death, were discovered. While various cytotoxic agents with various mechanisms of action have been discovered and put to great use as cancer therapeutics, they all suffer the same limitation: by non-specifically killing both healthy and cancerous cells the physicians administering them can only hope that the patient outlives the disease. Ultimately, these agents all have severe, on target, dose limiting toxicities.

The biotechnological advances of the late 20th century lead to new cancer therapeutics with greater specificity and less toxicity. After the discovery of the Philadelphia chromosome in 1959 in people with chronic myelogenous leukemia (CML) (Nowell & Hungerford, 1960) research efforts over the subsequent decades lead to the understanding of the mechanism by which this mutation leads to the development of disease. Briefly, a reciprocal translocation between chromosomes 9 and 22 result in a
fusion gene, BCR-ABL (ABL from chromosome 9 and BCR from chromosome 22). This hybrid protein, which signals through tyrosine kinase activity, is constitutively active resulting in uncontrolled cell division. The detailed understanding of the molecular biology of the mutation responsible for the development of CML lead research teams at Novartis to screen compound libraries against the tyrosine kinase domain of the BCR-ABL fusion protein, which ultimately lead to the discovery and development of imatinib, which gained FDA approval in 2001. Around the same time, the discovery of the over-expression (2-20 fold) of the HER2 (human epidermal growth factor receptor 2) protein on a significant proportion of breast cancers (30%) implicated its role in the pathogenesis of human breast cancer (Slamon, et al., 1987). This discovery led to the development of a monoclonal antibody, trastuzumab, in a joint effort between Genentech and UCLA. These targeted therapies fundamentally changed the way people thought about treating cancer, demonstrating that rational drug design could lead to therapies specific to the disease thereby significantly decreasing overall toxicity.

More recently the understanding of the role of immune modulatory receptors (IMRs; also called immune checkpoints) in cancer has led to breakthrough immunotherapeutic drugs. Inhibitory immune checkpoint pathways are abundant throughout the immune system and consist of receptor ligand pairs designed to maintain self-tolerance and modulate the duration and amplitude of immune responses thereby limiting autoimmunity. It is now clear that tumors co-opt immune checkpoint pathways as a mechanism of immune evasion. Checkpoint inhibitor drugs act by blocking inhibitory immune receptors on T cells, thereby enhancing the ability of the immune system to mount a response to the tumor. While immunotherapies can lead to durable,
robust responses, the rate of response is quite variable across and within tumor types (Topalian, Taube, Anders, & Pardoll, 2016). There is currently an intense effort to characterize what differentiates responders from non-responders, and to develop predictive criteria.

Factors limiting efficacy of immunotherapeutics

Immunotherapies are aimed at increasing the quality of the immune response directed at tumors. Early immunotherapies primarily consisted of adoptive cellular therapy (ACT), where TIL were harvested from patients, expanded in vitro, and injected back. Current approaches have expanded on this idea and include molecular manipulations of the T cell receptor to make the TIL more responsive to tumor antigen. However, tumors that are sculpted by the immunoediting process have been stripped of the most immunogenic cells, leaving behind a tumor that is no longer a potent inducer of immune response. The production of immuno-inhibitory molecules such as IDO, arginase, and TGFβ by tumor cells and well as myeloid cells that have been manipulated by the tumor microenvironment can actively suppress what might otherwise be a capable immune response. And as stated previously, the chronic inflammatory status of the tumor can result in T cells that acquire an exhausted phenotype resulting in the upregulation IMRs, while cancer cells acquire expression of IMR ligands further inhibiting effector cells. It is likely the combination of all of these factors that lead to inconsistent response rates to any one treatment. However, combination treatments targeting multiple mechanisms are in some cases providing responses that are more robust and more durable.
IL-10 as a pro-inflammatory cytokine

The mechanism(s) that contribute to decreased response rates to the checkpoint inhibitor anti-PD-1 antibody across tumor progression in mouse syngeneic models are unknown. The rapid growth of implanted mouse syngeneic tumors compared to that of spontaneous human neoplasm likely results in tumors that undergo rapid changes in tumor intrinsic protein expression and composition of immune infiltrate. The sharp increase in T_{reg} cells within MC38 tumors as they progress (data not shown) is one such example. While the most obvious explanation is that this increase in suppressive cells is contributing to resistance to immunotherapy, evidence for the importance of the T_{reg}-associated cytokine IL-10 for cytotoxic CD^{8+} T cell activation within the TME, as well as T_{reg}-derived IL-10 for the inhibition of tumorigenic T_{h17} inflammation calls this into question.

IL-10 is a cytokine that has long been associated with immune suppression given its broad anti-inflammatory properties (Sanjabi, Zenwicz, Kamanaka, & Flavell, 2009). IL-10 inhibits the canonical T_{h1} cytokine IFNγ by down regulating co-stimulatory molecules on antigen presenting cells (APC) (Fiorentino, et al., 1991) and by suppressing IL-12 production, a potent inducer of IFNγ, in peripheral blood mononuclear cells (D'Andrea, et al., 1993). IL-10 blockade can lead to the development of auto-immune disease-like phenotypes in mice such as spontaneous colitis (Sellon, et al., 1998). And naïve CD^{4+} T cells activated in the presence of IL-10 and TGFβ can be induced to express foxp3, becoming suppressive iT_{reg} cells (Fu, et al., 2004). However, several recent studies have demonstrated the importance of IL-10 for immune cell activation via mechanisms that are specific to the tumor microenvironment. Mumm et al. 2011 showed
that, contrary to what is observed during systemic inflammation, IL-10 in the TME upregulated co-stimulatory molecules, and induced cytotoxic enzyme and IFNγ production in tumor resident CD8+ T cells (Mumm, et al., 2011). The authors suggest the mechanism for this selectivity is a significant upregulation of IL-10 receptor on intratumoral CD8+ T cells as compared to cells from blood or secondary lymphoid organs. In addition, IL-10 has been shown to directly activate and expand CD8+ T cells currently residing in the tumor (Emmerich, et al., 2012). And IL-10 production by T_{reg} has been shown to inhibit tumorigenic T_{h17} inflammation in a manner that is dependent on type I interferon.

The present study seeks to understand whether IL-10 is relevant for effective tumor control, to study the relative abundance of IL-10 within the TME across tumor progression, as well as the sources of its production. Given that restraint of T_{h17} inflammation by IL-10 within the TME is type I interferon dependent, molecules that stimulate type I interferon production will be used alone and in combination with anti-PD-1 antibody which will potentially generate a combination benefit. Further, broad profiling of relevant cytokines will be conduction to understand networks that may contribute to immune inhibition or stimulation. Changes in total TIL composition, and phenotypic changes to specific subsets of immune cells will also be characterized by protein expression.
Chapter II

Materials and Methods

The materials and methods used to conduct the experiments described herein are outlined within this section. All experiments were conducted at Merck Research Laboratories Boston.

Mice

Female C57BL/6 mice were acquired from Jackson Laboratories (Bar Harbor, ME) at eight weeks of age and allowed to acclimate for one week prior to the initiation of in vivo studies. Mice were housed five per cage and allowed free access to food (Teklad Global Rodent Diets) and water in a temperature, humidity, and light cycle (12h on/12h off) controlled vivarium. Cages were maintained with wood chip bedding (Teklad sanichips), changed once every two weeks, and species appropriate enrichment (Bio Serve Mouse Igloo and Ancare nestlet).

In vitro cell culture

All cell culture work was performed aseptically in a biosafety cabinet (BSC) within a biosafety level (BSL) 2 containment facility. CT26 cells were stored
in cryovials in liquid nitrogen until the time of use. Frozen cell aliquots were passaged at least twice prior to injection into animals. Cells were passaged in media containing Dulbecco’s Modified Eagle Medium (Gibco Laboratories, Gaithersburg, MD) with 10% fetal bovine serum (FBS; Gibco) using standard aseptic cell culture technique.

*In vivo* studies

All *in vivo* studies were conducted in accordance with the Guide for Laboratory Animal Care and Use (National Research Council), and were approved by the Merck Boston institutional animal care and use committee. Animals were inoculated with $3 \times 10^5$ CT26 colon adenocarcinoma cells injected subcutaneously into the right flank in a volume of 100 μL phenol red-free DMEM (Gibco). Tumor growth and body weight were recorded twice a week, and animals were routinely assessed for health status by veterinary staff. Animals that lost 20% of their starting body weight, had full dermal lesioning, had tumors that grew longer than 2 cm in either direction or had a volume larger than 2 cm$^3$, or were otherwise moribund were euthanized. Anti-PD-1 antibody (mDX400, Merck Research Laboratories) was dosed intraperitoneally at a concentration of 5 mg/kg in a volume of 5 mL/kg once every 5 days (Q5D) and was formulated in an aqueous buffer containing 2.72 g/L sodium acetate and 90 g/L sucrose titrated to pH 5.5. 5,6-dimethylxanthenone-4-acetic acid (DMXAA) was synthesized by Merck Research Laboratories and dosed intratumorally at 360 μg/mouse (approximately 18 mg/kg) in a volume of 50 μL (approximately 2.5 mL/kg) twice a week and was formulated in an *in situ* basic salt (1.2 equivalents of NaOH) in normal saline, and titrated to pH 8.0. Cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) was synthesized by
Merck Research Laboratories and was dosed intratumorally at 500 μg/mouse (approximately 25 mg/kg) in a volume 50 μL twice a week and was formulated in PBS (Gibco). All animals were euthanized by carbon dioxide (CO₂) inhalation. Following euthanasia tumors were harvested and placed into tubes containing cold DMEM (Gibco), and stored on ice. Single cell suspensions were generated using a mouse tumor dissociation kit (Miltenyi Biotec, Germany) and Miltenyi OctoMACS tissue dissociator per manufacturer’s protocol. Single cell suspensions were cultured in complete culture media (Roswell Park Memorial Institute medium [RPMI] 1640 + Glutamax, 10% FBS, 1% penicillin/streptomycin, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 100 μM β-mercaptoethanol) containing 1x cell stimulation cocktail (eBioscience, San Diego, CA) at 37°C with 5% CO₂ overnight on Costar ultra-low attachment plates (Corning Life Sciences, Tewksbury, MA).

**Flow cytometry**

Cells were stained according to manufacturer’s protocol (BD Biosciences, San Jose, CA). Briefly, single cell suspensions were first stained with 1:500 anti-CD16/32 (FC block; BD Biosciences) in 50 μL cell staining buffer (BD Biosciences) for 10 minutes at room temperature, followed by the addition of 50 μL surface antibody cocktail for 30 minutes at room temperature. Following surface staining, cells were stained with intracellular/intranuclear markers per manufacturer’s protocol (transcription factor staining buffer; Invitrogen, Carlsbad, CA). Briefly, after cells were fixed and permeabilized they were stained with 1:500 anti-CD16/32 in 25 μL permeabilization buffer for 10 minutes at room temperature, followed by the addition of 25 μL intracellular
antibody cocktail for 30 minutes at room temperature. After staining cells were analyzed on a BD LSRFortessa (BD Biosciences) using FacsDiva 8 software (BD Biosciences). Subsequent data analysis was performed using FCS Express 5 (De Novo Software, Glendale, CA). A list of antibodies and dilutions is provided in table 1. Single color stains for compensation were performed using Ultracomp eBeads (Invitrogen). Fluorescence minus one (FMO) controls were used to establish gating with CD69, CD25, IL-10, IL-10R, and Ki67.

### Table 1. Flow cytometry antibody panel.

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<th>Marker</th>
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Mass cytometry

Cells were stained according to previously described protocol (Brodie & Tosevski, 2017). Briefly, single cell suspensions were first stained with 1:500 anti-CD16/32 and 1:4000 Rh<sup>103</sup> in 50 μL cell staining buffer for 10 minutes at room
temperature, followed by the addition of 50 μL surface antibody cocktail for 30 minutes at room temperature. Following surface staining, cells were stained with intracellular/intranuclear markers as previously described (transcription factor staining buffer; Invitrogen). Following intracellular intranuclear staining, cells were placed in Maxpar fix and perm buffer (Fluidigm, South San Francisco, CA) containing 1:4000 Ir\textsuperscript{191/193} and stored at 4°C overnight. Cells were washed and filtered the following morning using MultiScreen plates (Milipore Sigma, Burlington, MA), counted by Countess II automated cell counter (Invitrogen), and appropriate volumes of water were added to the samples to achieve the desired concentration. Cells were analyzed on a Helios mass cytometer (Fluidigm), and data was analyzed using FCS Express 5. A list of antibodies and dilutions is provided in table 2.

Cytokine analysis

Tumors were excised, weighed, and immediately placed in tubes and put on dry ice. To normalize the concentration of protein input lysis buffer was added to each sample equaling three times the sample weight. Samples for which three times the sample weight equaled less than 100 were given 100 μL of lysis buffer, and the cytokine measurement was adjusted using the formula: 
\[
\text{cytokine value [pg/mL] x lysis buffer volume [μL]} / (\text{tumor weigh [mg]} \times 3).
\]
Lysates were analyzed for type I interferon using either IFN\(\alpha\) or IFN\(\beta\) enzyme-linked immunosorbent assay (ELISA) kit (PBL Assay Science, Piscataway, NJ). Lysates were analyzed for IL-10 and IL-17 using 19-plex chemiluminescent immunoassay kit (Meso Scale Discovery, Rockville, MD).
Table 2. Mass cytometry antibody panel.

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Statistical analyses

In life tumor volume data was analyzed by 2-way ANOVA, with Sidak’s multiple comparison test. Cytometry and cytokine data for studies with only two treatment groups were analyzed using two-tailed student’s T test. Cytometry and cytokine data for studies with more than two treatment groups were analyzed using 1-way ANOVA with Tukey’s multiple comparison test. One star represents statistical significance of $p < 0.05$, two stars
represent statistical significance of $p < 0.01$, three stars represent statistical significance of $p < 0.001$, four stars represent statistical significance of $p < 0.0001$. Statistical analyses were performed in GraphPad Prism 7 (GraphPad Software, San Diego, CA).
Chapter III

Results

Experiments were performed to elucidate responses in the CT26 syngeneic tumor model to single agent anti-PD-1 antibody, STING agonist, or the combination of both. *In vivo* tumor growth curves, tumor cytokine production, and cytometric profiling of tumor immune infiltrate are described further in this section.

Mouse CT26 syngeneic model

Mouse syngeneic tumor models use cells isolated from induced or spontaneously forming tumors. The cells are then implanted into wild type mice of the same genetic background which ensures that the tissue will not be rejected. Xenograft models, where foreign tumor cells, typically of human origin, are grown require the use of immunodeficient mice to ensure that the graft is not rejected. The value of syngeneic models is that recipient mice have intact immune systems making them ideal for use in an immuno-oncology setting. However, because tumor cell lines have been passaged repeatedly over time (potentially decades) genetic drift is possible; cells acquire mutations which can result in loss or gain of protein expression. Because of this drift a particular cell line used in one institution may not behave as it does in another setting despite their shared origin (Mosely, et al., 2017).
The CT26 mouse model is routinely used for the evaluation of therapeutic agents in the pre-clinical setting. When CT26 mouse colon adenocarcinoma cells are injected into the right flank solid tumors form within several days and reach an average volume of 100mm³ by around day 8, and endpoint tumor burden by around day 21. Mice treated with 5 mg/kg anti-PD-1 antibody (mDX400) dosed once every 5 days (Q5D) starting on day 8 achieve statistically significant reductions in tumor growth (Two-way ANOVA, time $p < 0.0001$, treatment $p < 0.0001$, interaction $p < 0.0001$), resulting in 89% tumor growth inhibition including 20-40% complete regressions (figure 1).

![Figure 1. CT26 efficacy data with mDX400 treatment. Data represents mean +/- SEM, $n = 7-10$.](image)

However, responses have considerable variability and this model is therefore considered only partially responsive to anti-PD-1 antibody treatment in our experience. Additionally, there is a small tumor volume window in which therapeutic administration of anti-PD-1 antibody is effective. As tumors grow larger they respond less to PD-1
blockade, which is likely due to changes in quantity or phenotype of immune infiltrate. In order to evaluate these changes in immune cells over the course of tumor progression cytometric analysis was performed on both small (~100mm$^3$) and large tumors (~250mm$^3$). Experiments with small and large tumors were each run in duplicate and both produced data sets that were largely consistent across replicates.

Analysis of immune cell infiltrate in small and large tumors

Treatment of small tumors ($n=9$) with IgG1 isotype antibody shows that tumors consisted of 32% ± 16 CD45$^+$ immune cells (figure 3). Of these, 15% ± 6 were CD3$^+$ T cells, 51% ± 8 were CD11b$^+$ myeloid cells. In a separate CyTOF analysis, the remaining cells were shown to be mostly NK cells (data not shown). T cells, being the main anti-tumor effector cells, are of particular interest, and high T lymphocyte infiltration has been associated with good clinical outcome in many cancer types (Fridman, Pagès, Sautès-Fridman, & Galon, 2012). CD4$^+$ helper T cells are heterogeneous as they are composed of T helper 1 (T$_h$1), T$_h$2, T$_h$17, and T$_{reg}$ subsets, among others. While T$_h$1 cells have the ability to activate cytotoxic CD8$^+$ cells, given this heterogeneity, the contribution of total CD4$^+$ infiltrate to clinical outcome is not well characterized. However, increased infiltration of cytotoxic CD8$^+$ T cells is clearly associated with overall survival (Fridman, Pagès, Sautès-Fridman, & Galon, 2012). In the periphery, a normal mouse CD4/CD8 ratio is around 2, and decreased CD4/CD8 ratio in the tumor would indicate a preferential expansion of the CD8$^+$ compartment. In the present study CD4$^+$ T cells represented 36% ± 12 and CD8$^+$ cells represented 20% ± 17 of total T cells, resulting in a 1.8 CD4/CD8 ratio in the tumor. T$_{reg}$ cells have a controversial role in cancer biology and clinical
outcome. In 2004 an influential publication by Curiel et al. showed that high T_{reg} infiltrate was correlated with poor overall survival in breast cancer (Curiel, et al., 2004). However, several publications have shown the opposite is true in other tumor types (Grabenbauer, Lahmer, Distel, & Niedobitek, 2006), (Heimberger, et al., 2008). The impact of T_{reg} cells in the CT26 model is unclear, but given reports of IL-10 secretion activating CD8^{+} cells in the TME (Mumm, et al., 2011) (Emmerich, et al., 2012) and the specific contribution of T_{reg} cells to the generation of IL-10 in the TME (Stewart, et al., 2013) they are of particular interest to the current study. CD25 expression has been shown to identify T_{reg} cells in the TME (Stewart, et al., 2013) (and data not shown), negating the need to stain for the intracellular marker Foxp3. Of the CD4^{+} cells 7\% \pm 8 were CD25^{+} T_{reg}.

In contrast to small CT26 tumors, tumors grown for 12 days to an average size of ~250mm^{3} (n=10-12) have significantly less response to anti-PD-1 antibody. Mice treated with 5 mg/kg mDX400 dosed Q5D achieved 35\% tumor growth inhibition by day 11 with no complete regressions (figure 8). Large tumors consisted of 6.6-fold fewer CD45^{+} immune cells (4.9\% \pm 3.3), with 15\% fewer T cells (13\% \pm 4.9) and a 21\% more myeloid cells (62\% \pm 9.4; figure 4) than small tumors. Within the T cell compartment 48\% \pm 11 were CD4^{+} and 32\% \pm 10 were CD8^{+} resulting in a CD4/CD8 ratio of 1.5 in the tumor. The percent of CD25^{+} T_{reg} in the CD4 compartment was 4.2-fold higher (27\% \pm 9).

Large tumors expressed less IL-10 than small tumors in both the total CD4^{+} population (0.2\% large vs. 0.6\% small) and T_{reg} population (0.4\% vs. 2.1\%). Mumm et al. 2011 suggests that upregulation of IL-10R on tumor infiltrating CD8^{+} cells is a
mechanism by which IL-10 secretion in the TME is immunostimulatory (Mumm, et al., 2011). Here, IL-10R expression on CD8+ cells in large tumors was reduced compared to small tumors (0.5% vs. 17%). Changes in activation markers on CD4+ and CD8+ cells were mixed. Within the CD4+ population CD69 expression was reduced (3.3% vs. 24%) while Ki67 was increased (22% vs. 14%). Within the CD8+ population CD69 expression was unchanged (39% vs. 39%) while Ki67 expression was reduced (25% vs. 43%). Taken together the changes to functional markers on T cells suggests a phenotype consistent with reduced anti-tumor activity in the larger CT26 tumors compared to the smaller tumors.

Changes in immune cell infiltrate and phenotype with anti-PD-1 antibody treatment

Various mechanisms have been identified within the TME that suppress immune response allowing tumor cells to continue to proliferate. The PD-1 pathway has been of particular interest since Merck’s Keytruda, and subsequently Bristol Myers Squibb’s Opdivo, entered the market. These drugs have shown that modulating the interaction of PD-1 and its receptor PD-L1 is a mechanism that enhances the adaptive anti-tumor immune response providing great clinical utility. PD-1 is normally absent on resting T cells, but its expression is upregulated following T cell receptor ligation. Chronic antigenic T cell receptor stimulation results in T cells that become functionally impaired, a state referred to as exhaustion, and exhausted T cells express high levels of PD-1. PD-1 expression is found on tumor infiltrating T cells in many cancers, as is the expression of PD-L1 on many different cell types within the TME including immune cells, stromal cells, and cancer cells (Iwai, et al., 2002) (Zou, Wolchok, & Chen, 2016). In a study of
pooled untreated CT26 tumors (average size ~100mm³) analyzed by mass cytometry 23% of CD4⁺ and 25% of CD8⁺ T cells expressed PD-1, while 75% of CD45⁻ (a pool consisting largely of tumor and stromal cells) and 62% of myeloid cells expressed PD-L1, reflecting the relevance of the PD-1/PD-L1 pathway in this tumor type (figure 2).

Figure 2. PD-1 and PD-L1 expression in small CT26 tumors. Data was generated from multiple tumors that were pooled into a single sample. A) Expression of PD-L1 on CD45⁻ and CD11b⁺ myeloid cells. B) PD-1 expression on CD4⁺ and CD8⁺ T cells.

Given the observation that mDX400 treatment significantly inhibits tumor growth in small CT26 tumors broad changes in tumor infiltrate and phenotype would be expected, and this is indeed reflected in the data. It has been shown that dual blockade of the PD-1 and CTLA-4 pathways in B16F10 melanoma expands tumor infiltrating T cells, a tumor type that is largely devoid of immune infiltrate (Curran, Montvalo, Yagita, & Allison, 2010). Therefore, one might expect anti-PD-1 blockade to show a similar expansion of T cells within an anti-PD-1 partially responsive tumor like CT26. Unexpectedly, there was not an increase in total CD45⁺ cells with anti-PD-1 antibody
(32% ± 16) as compared to isotype control (32% ± 16), and only a modest 13% increase in total T cells (17% ± 7 vs 15% ± 6; figure 3). However, robust changes in T cell composition and phenotype were observed. A 28% increase in total CD4+ and statistically significant increases in Treg (+2.4-fold, \( p < 0.01 \)) and total CD8+ cells (+92%, \( p < 0.05 \)) with mDX400 treatment underscore the changes in T cell composition. The administration of mDX400 resulted in a decrease in the CD4/CD8 ratio from 1.8 to 1.2, reflecting the increased number of cytotoxic effector cells in the TME. Robust changes in functional markers were seen in both CD4+ and CD8+ compartments. Within the CD4+ population there was a 6-fold increase in IL-10 expression in total CD4+ cells (\( p < 0.001 \)), and a 5-fold increase in IL-10+ Treg (\( p < 0.01 \); data not shown). Large upregulation of the activation marker CD69 (+94%, \( p < 0.001 \)) and proliferation marker Ki67 (+2.9-fold, \( p < 0.01 \)) was also observed. Within the CD8+ compartment there was a 75% increase in CD69 expression (\( p < 0.01 \)) and a 65% increase in Ki67 (\( p < 0.01 \)). IL-10R expression, which was largely absent from CD4 cells in both isotype and antibody treated groups, was increased 2.3-fold in CD8+ cells with mDX400 treatment (\( p < 0.05 \)) from 17% ± 20 to 40% ± 24.

It is unclear why syngeneic tumors respond poorly to immunotherapy as they grow larger. As tumors co-opt mechanisms for immune evasion it stands to reason that the ratio of tumor cells to effector cells would increase, thereby overwhelming an immune response initiated at later stages of tumor growth. However, deleterious changes to existing immune cell phenotype may also hinder the ability to promote further anti-tumor immunity.
Figure 3. Flow cytometry data from small CT26 tumors treated with mDX400. Data represents mean + SEM. A. CD45+ and CD45- cells as percentage of live singlets, B. Phenotypic marker expression as percentage of CD4+, C. CD4+ and CD8+ T cells as percentage of CD3+, D. Phenotypic marker expression as percentage of CD8+, E. Total myeloid and T cells as percentage of CD45+, F. Phenotypic marker expression as percentage of CD11b+. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.
Here, treatment in larger tumors (average size 250mm³), which respond poorly to anti-PD-1 blockade, unexpectedly led to increases in total CD45⁺ (48%) and total T cells (45%, \( p < 0.05 \); figure 4). However, as mentioned previously, these tumors consisted of significantly less total CD45⁺ cells (5% isotype, 7% mDX400) compared to smaller tumors (32% isotype, 32% mDX400), which therefore also increased the overall tumor to effector cell ratio. A similar increase in total CD4⁺ cells with mDX400 (23%, \( p < 0.05 \)) was observed in large tumors, but unlike small tumors there was no increase in CD8⁺ cells (32% ± 10 vs. 29% ±10), resulting in an increase in CD4 to CD8 ratio from 1.5 to 2.0. A significant increase in CD25⁺ T_{reg} (63%, \( p < 0.001 \)) was seen, and the overall percentage of CD25⁺ T_{reg} was increased several fold in both mDX400 (2.8-fold) and isotype (4.2-fold) treated animals as compared to smaller tumors.

Changes in functional markers in the CD4⁺ compartment were notably different than that observed in small tumors, where small but not statistically significant increases in CD69 (35%), Ki67 (13%), and IL-10 (23%) were observed. While there was not an increase in the size of the CD8⁺ compartment with mDX400 treatment the phenotype of CD8⁺ cells was largely consistent with that seen in smaller tumors, with large increases in CD69 (81%, \( p < 0.001 \)) and Ki67 (65%, \( p < 0.001 \)). Although antibody treatment induced a robust 8.5-fold increase in IL-10R (\( p < 0.001 \)) the percentage of CD8⁺ cells expressing the receptor were markedly lower than in small tumors (-35-fold isotype, -10-fold mDX400)
Figure 4. Flow cytometry data from large CT26 tumors treated with mDX400. Data represents mean + SEM. A. CD45+ and CD45- cells as percentage of live singlets, B. Phenotypic marker expression as percentage of CD4+, C. CD4+ and CD8+ T cells as percentage of CD3+, D. Phenotypic marker expression as percentage of CD8+, E. Total myeloid and T cells as percentage of CD45+, F. Phenotypic marker expression as percentage of CD11b+. * = p < 0.05, *** = p < 0.001, **** = p < 0.0001.
While checkpoint inhibitor therapy has proven to be a breakthrough in many different cancer types, there are still significant populations of cancer patients that do not respond to this kind of treatment as monotherapy. This creates the need to explore additional therapies that combine in beneficial ways with drugs like Keytruda, something that is currently an intense focus of the pharmaceutical industry. The poor response to anti-PD-1 antibody treatment in large CT26 tumors creates an opportunity to explore combination therapies that work in an additive or synergistic fashion. One modality that logically pairs with the adaptive immune activation seen with PD-1 pathway blockade is to stimulate the innate immune response, and a promising candidate mechanism is the STING pathway. cGAS (cyclic GMP-AMP synthase) is a cytosolic double stranded DNA sensor that upon activation leads to the generation cyclic GMP-AMP (cGAMP) which binds to STING (stimulator of interferon genes) leading to the generation of type I interferon (figure 5). Type I interferon in turn serves to facilitate cross priming of antigens by APCs and promotes their migration to lymph nodes, further enhancing activation of cytotoxic CD8\(^+\) cells (Corrales, McWhirter, Dubensky Jr., & Gajewski, 2016). While PD-1 pathway blockade removes the brakes from tumor resident T cells a STING agonist would create newly educated and primed T cells by enhancing APC function, and these two functions could create an anti-tumor response greater than either alone.
Figure 5. STING pathway schematic. Chen (2016) Nature Reviews Immunology.
Mice bearing small CT26 tumors and dosed intratumorally with 500 μg cGAMP achieved 103% tumor growth inhibition (Two-way ANOVA, time $p < 0.0001$, treatment $p < 0.0001$, interaction $p < 0.0001$) including 10-20% complete regressions. Whole tumor lysate taken from these mice at four hours shows a robust induction of pathway specific cytokines of IL-10 ($p < 0.05$), IFNβ ($p < 0.0001$), and IL-6 ($p < 0.05$), with all three cytokines returning to baseline at 24 hours (figure 6). A 360 μg dose of DMXAA (5,6-dimethylxanthenone-4-acetic acid; a mouse specific STING agonist) achieves similar results (data not shown).

Figure 6. Tumor cytokine data following cGAMP administration. Data represents mean + SEM. A. IL-6, B. IL-10, C. IFNβ. * = $p < 0.05$, **** = $p < 0.0001$.

While it has been shown that IL-10 can directly activate and expand tumor resident CD8$^+$ T cells (Mumm, et al., 2011) (Emmerich, et al., 2012), there is another beneficial role for this cytokine in this context. IL-10 production in the TME, which came primarily from T$_{reg}$ and was generated in a type I interferon dependent manner, has been shown to limit oncogenic T$_h$17 inflammation (Stewart, et al., 2013). A subset of CD4$^+$ T cells called T helper 17 (T$_h$17) cells promote antibacterial, antifungal, and wound healing activities, and in many cancers the TME exhibits high levels of T$_h$17 inflammation.
However, Th17 cells remain highly plastic and can be induced to express CD39 and CD73 which in combination convert ATP to adenosine, suppressing T cell responses and in turn promoting cancer growth (Martin, Apetoh, & Ghiringhelli, 2012). Therefore the generation of type I interferon could serve multiple beneficial purposes including promoting enhanced APC function, and generating IL-10 which both suppresses Th17 inflammation and activates tumor resident CD8+ T cells.

While STING agonists generate robust anti-tumor responses in small CT26 tumors, this treatment is also less effective in larger CT26 tumors. Mice bearing CT26 tumors with an average size of ~250mm³ and dosed intratumorally with 360 μg DMXAA achieved 88% tumor growth inhibition but no complete regressions 11 days after the initiation of treatment (figure 8). Large CT26 tumors are therefore an ideal setting to study the combination of an anti-PD-1 antibody and a STING agonist.

![Figure 7. CT26 efficacy data with cGAMP treatment. Data represents mean +/- SEM.](image-url)
Combination treatment with STING agonist and anti-PD-1 antibody

In order to assess the potential combination benefit of a STING agonist and an anti-PD-1 antibody mice bearing CT26 tumors with an average size of ~250mm$^3$ were used. Experiments were repeated at least once and produced consistent data across replicates. Mice were dosed according to the paradigm in table 3. Mice dosed with control agents PBS and IgG1 isotype control antibody began to reach endpoint tumor burden on day 11, at which point PBS + mDX400 had achieved 35% TGI, DMXAA + IgG1 had achieved 88% TGI, and the combination of DMXAA + mDX400 had achieved 114% TGI including three complete regressions (figure 8).

Table 3. Treatment paradigm for CT26 DMXAA mDX400 combination study.

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</tbody>
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Two-way ANOVA revealed statistical significance (time $p < 0.0001$, treatment $p < 0.0001$, interaction $p < 0.0001$), with multiple comparison tests indicating changes relative to control in group 4 beginning on day 4 ($p < 0.05$), in group 2 beginning on day 7 ($p < 0.0001$), and in group 3 on day 11 ($p < 0.001$). Four hours after the first dose five mice from each group were euthanized and tumors were collected for cytokine analysis (figure 9). Robust, statistically significant increases in IL-12p70, IL-2 and STING pathway cytokines IL-6, IL-10, and IFN$\alpha$ were observed in groups 2 and 4. Group 4 showed increases over group 2 (i.e. a combination benefit) in IFN$\alpha$ (9%), IL-2 (25%), and IL-12p70 (57%).
Figure 9. Cytokine data from CT26 DMXAA mDX400 combination study. Data represents mean + SEM. * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$. A. IL-17A/F, B. IFNα, C. IL-6, D. IL-12p70, E. IL-2, F. IL-10.
IL-2 is a potent inducer of T cell survival, activation, and proliferation, and can signal in an autocrine positive feedback loop whereby T cells both secrete the cytokine and respond to it. IL-12 is secreted by activated myeloid cells and facilitates the conversion of naïve CD4^+ T cells into a T_h1 phenotype, resulting in the generation of IFNγ and an enhanced a CD8^+ response to intracellular antigens (Hsieh, et al., 1993), with IL-12p70 being the active subunit. Contrary to expectation IL-17A/F, an indicator of T_h17 inflammation, was not elevated in control tumors and was instead significantly increased with DMXAA treatment in both groups 2 and 4.

Eight days post the first dose 10 animals from each group were sacrificed and tumors were collected for cytometric analysis (figure 10). Consistent with the preliminary experiment mDX400 treatment alone did not induce robust signs of immune activation. This treatment resulted in a small (+10%) increase in total CD8^+ T cells, which had a 48% increase in CD69 expression \( (p < 0.01) \), and a 39% increase in Ki67. DMXAA treatment alone led to an increase in total CD45^+ (68%, \( p < 0.0001 \)) and total T cells (+37%) and to decreases in total CD4^+ (-55%; \( p < 0.01 \)) and total CD8^+ (-47%; \( p < 0.05 \)). Robust upregulation of immune activation markers was observed on T and myeloid populations. On CD4^+ cells CD25 expression was increased 4.4-fold \( (p < 0.001) \), CD69 was increased 6.8-fold \( (p < 0.01) \), and IL-10 was increased 95%. On CD8^+ cells CD69 expression was increased 2.4-fold \( (p < 0.0001) \), while IL-10R – not typically associated with immune activation – was increased 12-fold \( (p < 0.0001) \). Similarly, CD69 expression was increased 56% \( (p < 0.001) \) on myeloid cells.
Figure 10. Flow cytometry data for CT26 DMXAA mDX400 combination study. Data represents mean ± SEM. A. CD45+ and CD45- cells as percentage of live singlets, B. Phenotypic marker expression as percentage of CD4+, C. CD4+ and CD8+ T cells as percentage of CD3+, D. Phenotypic marker expression as percentage of CD8+, E. Total myeloid and T cells as percentage of CD45+, F. Phenotypic marker expression as percentage of CD11b+. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.
Interestingly, while CD25 and CD69 were upregulated on CD4$^+$ and CD8$^+$ T cells, these populations had reductions in proliferation marker Ki67; -54% for CD4$^+$ and -94% for CD8$^+$ ($p < 0.05$). DMXAA + mDX400 administration produced the most robust tumor growth inhibition, including several complete regressions, and the cytometric profile of this group was very similar to DMXAA alone. This treatment led to an increase in total CD45$^+$ (64%, $p < 0.001$) and total myeloid cells (13%). Total T cells were relatively unchanged, while CD4$^+$ (-33%) and CD8$^+$ (-49%; $p < 0.01$) subsets were reduced, resulting in an unusually large population of CD4/CD8 double negative cells. On CD4$^+$ cells CD25 expression was increased 4-fold ($p < 0.0001$), CD69 was increased 15-fold ($p < 0.001$), and IL-10 was increased 2.5-fold. On CD8$^+$ cells CD69 expression was increased 2.3-fold ($p < 0.0001$), while IL-10R was increased 13-fold ($p < 0.0001$). Similarly, CD69 expression was increased 45% ($p < 0.01$) on myeloid cells. Proliferation marker Ki67 was slightly decreased on CD8$^+$ T (-9%), but slightly increased on CD4$^+$ T cells (63%).

Results Summary

Anti-PD-1 antibody treatment resulted in robust tumor growth inhibition (89%; 4/10 complete regressions) when administered to animals bearing small CT26 tumors but demonstrated less tumor growth inhibition (35%; 0/10 complete regressions) when administered to animals bearing large CT26 tumors. Cytometric data generated eight days after the onset of treatment in small tumors showed that the administration of mDX400 generated robust induction of activation markers CD25 and CD69, and of proliferation marker Ki67, in tumor infiltrating T cells, and an increase in total CD8$^+$ T cells, the
primary cells responsible for anti-tumor activity. Additionally, induction of IL-10 is observed in both CD4⁺ T cells and myeloid cells, and upregulation of IL-10R is observed on CD8⁺ T cells. On the other hand, administration of mDX400 in large CT26 tumors generated a partial immune activation profile at eight days after the onset of treatment. In the preliminary experiment CD4⁺ T cells showed a moderate increase in CD25, smaller increases in CD69 and Ki67, but no increase in IL-10. CD8⁺ T cells retained increases in CD69, Ki67, and IL-10R, but there was not an increase in total CD8⁺ T cells. Myeloid cells showed a robust increase in CD69 but not IL-10. In the combination experiment the PBS + mDX400 treatment led to a small (+10%) increase in total CD8⁺ T cells, which had a 48% increase in CD69 expression ($p < 0.01$), and a 39% increase in Ki67.

Similar to anti-PD-1 treatment, administration of STING agonist in small CT26 tumors generated robust tumor growth inhibition (103%; 2/10 complete regressions) but exhibited less tumor growth inhibition (88%; 0/10 complete regressions) when administered to animals bearing large CT26 tumors. STING agonist administration generated increases in pathway relevant cytokines IL-6, IL-10, and type I IFN at 4 hours post dose in both small and large tumors. Additionally, in large CT26 tumors STING agonist led to activation but not proliferation of tumor infiltrating T cells, robust induction of IL-10R on CD8⁺ T cells, and an increase in IL-10 in CD4⁺ cells.

Combination treatment of mDX400 and DMXAA in large CT26 tumors led to greater tumor growth inhibition (114%, 3/10 complete regressions) than either agent alone. Cytokine data showed a combination benefit on levels of type I IFN, a potent mediator of innate immunity; IL-2, a key stimulator of T cell activity; and IL-12p70, a
cytokine that increases CD8+ T cell activity. Cytometry data showed that the immune activation profile that resulted from the combination treatment was very similar to DMXAA treatment alone including increased CD25 and CD69 expression, as well as IL-10 production in CD4+ cells and increased CD69 and IL-10R expression on CD8+ cells, but did not indicate clear signs of combination benefit.

These data demonstrate that in tumors in which there is a robust anti-tumor response driven by pharmacological intervention with either anti-PD-1 antibody, STING agonist, or the combination of both the IL-10/IL-10R pathway is upregulated. This suggests that in the CT26 tumor microenvironment IL-10 is a component of immune activation rather than immune suppression. The data supports the hypothesis that IL-10 generated by STING agonist is a component of the combination benefit observed on tumor growth inhibition when co-dosed with anti-PD-1 antibody in large tumors. However, the hypothesis that type I interferon dependent IL-10 generated by STING agonist constrains T_h17 inflammation was not supported. There was no evidence of T_h17 inflammation in CT26 tumors treated with control agents, and STING agonist treatment alone or in combination with anti-PD-1 antibody exacerbated IL-17 cytokine levels. Lastly, while IL-10+ T_reg were observed (data not shown), they were not the sole source of IL-10 within the tumor microenvironment.
Chapter IV

Discussion

The aim of this study was to characterize the changes in immune infiltrate following treatments that elicit tumor growth inhibition, and to determine if the generation of IL-10 following STING agonist treatment may be a factor contributing to a combination benefit with anti-PD-1 antibody. IL-10 has long been characterized as an immunosuppressive cytokine given its involvement in suppression of the T cell stimulating cytokine IFNγ (Fiorentino, et al., 1991) (D'Andrea, et al., 1993), disruption of IL-10/IL-10R signaling causing animal models of colitis (Sellon, et al., 1998) and human inflammatory bowel disease (Kotlarz, et al., 2012), and its ability to convert naïve CD4+ T cells into becoming immunosuppressive Treg cells (Fu, et al., 2004). However, when researchers tried to further elucidate the immunosuppressive functions of IL-10 in the tumor microenvironment they unexpectedly discovered that it contributed to a number of mechanisms essential to effective tumor control, including enhancing the function and activity of CD8+ T cells, which they note display preferential upregulation of IL-10 receptor (Mumm, et al., 2011). Later work by the same group expanded upon this line of inquiry by showing that IL-10 can directly activate and expand tumor resident CD8+ T cells (Emmerich, et al., 2012). These findings were supported by the current study. So it appears that IL-10 plays contradictory roles depending on the specific milieu, contributing to immune suppression in the gastrointestinal tract, while contributing to
immune activation in the tumor microenvironment. IL-10 is commonly associated with regulatory T cells (T_{reg}). T_{reg} cells are the arbiter of immune self-tolerance, preventing immune responses against self-antigens, and play an important role in autoimmune disease. T_{reg} cells express the transcription factor forkhead box P3 (foxp3). The precise function of foxp3 is not well understood, nor is its expression unique to T_{reg} cells. However, an X-linked mutation in the production of foxp3 protein product scurfin results in a fatal lymphoproliferative condition in mice (Brunkow, et al., 2001), and a similar condition called immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans (Brunkow, et al., 2001), demonstrating the critical function of foxp3 in restraining immune response. In addition to the effect of IL-10 on naïve CD4^{+} T cells, IL-10 secretion is one of the mechanisms by which T_{reg} cells inhibit other immune cells (Annacker, Asseman, Read, & Powrie, 2003), particularly in the GI tract. However, while T_{reg} cells have long been a logical target of tumor immunologists, research has also shown that T_{reg} cells can play a beneficial role in an effective anti-tumor response. Inflammation caused by CD4^{+} T helper (T_{h}) 17 cells, defined by their production of IL-17, can promote tumor growth, and Stewart and colleagues showed that type I interferon-dependent IL-10 secreted by intratumoral T_{reg} cells can restrain this type of response (Stewart, et al., 2013). However, the current study did not show evidence for T_{h}17 inflammation, and while we were able to show type I interferon dependent IL-10 production, STING agonist treatment also drove the production of IL-17. These discrepancies could be explained by differences in mouse syngeneic models used, as different tumor lineages display a high degree of heterogeneity.
It is interesting to note that we observed upregulation of the IL-10/IL-10R pathway on T cells in small CT26 tumors following anti-PD-1 antibody treatment. While the immune activation signature in these tumors is strong, we are not aware of other research that has demonstrated a connection between these two pathways, and it is not immediately clear how they interact. In fact, it has been suggested that IL-10 secretion by inhibitory immune cells is one of the mechanisms that contribute to resistance to PD-1 pathway blockade (O'Donnell, Long, Scolyer, Teng, & Smyth, 2017). Further studies should investigate the mechanism by which these pathways crosstalk and determine the relative contribution of the IL-10 pathway to anti-tumor responses driven by PD-1 pathway blockade.

The current study was designed to characterize what factors may contribute to a combination benefit with STING agonist and anti-PD-1 antibody. Cytokine measurements taken from whole tumor lysate at 4 hour post dose showed combination benefit with three cytokines: IFNα, IL-2, and IL-12p70. Type I interferon, consisting of both IFNα and IFNβ, is the primary product of STING pathway activation, and is a potent inducer of innate anti-viral immune response (see figure 11). Blocking the PD-1 pathway does not produce type I interferon (figure 9). While it has been shown that in vivo treatment with STING agonist (Ghaffari, et al., 2018) or IFNα (Terawaki, et al., 2011) can upregulate the expression of the PD-1 pathway, it is not clear if blocking the PD-1 pathway can modulate the STING pathway. Unpublished internal gene expression profiling data has shown upregulation of a similar innate viral sensing pathway following anti-PD-1 antibody treatment in mice (data not shown). It is therefore plausible that mDX400 is capable of modulating a member of the STING pathway resulting in
increased type I interferon production, and this possibility should be explored in future studies.


IL-2 is the primary cytokine responsible for T cell survival and proliferation, and is produced by activated T cells resulting in an autocrine signaling feedback loop. It is therefore not surprising that treatments that generate robust T cell activation would generate high levels of this cytokine. The effect on IL-12 is particularly interesting. Conceptually, one advantage to combination therapy with STING agonist and anti-PD-1 antibody is that they each modulate one of the two primary aspects of immunity, innate and adaptive, respectively, and IL-12 is a cytokine that bridges the two (see figure 12). IL-12 is normally produced by activated myeloid cells, one of the key modulators of
innate immunity. It directly induces the production of IFNγ (Trinchieri, 1995) and promotes the polarization of naïve T cells into T_h1 cells (Hsieh, et al., 1993), both of which enhance CD8^+ T cell activity, a primary component of adaptive immunity. An important mechanism by which PD-1 pathway blockade modulates its anti-tumor effect is by un-inhibiting exhausted antigen specific cytotoxic CD8^+ T cells, the primary tumor killing effector cells. Therefore enhancing the activity of cytotoxic CD8^+ cells is a function where these two pathways may be directly synergizing.

Figure 12. IL-12 pathway schematic. Trinchieri (2003) Nature Reviews Immunology.

The current study did not show a difference in the combination treatment versus either STING agonist or anti-PD-1 antibody alone by flow cytometric protein analysis at eight days post the initiation of treatment. It is worth noting the difference in collection
times for cytokines and cytometry: four hours post a single dose versus eight days post the initiation of treatment (three doses of DMXAA, two doses of mDX400). These time points were optimized separately for STING agonist (four hours) and anti-PD-1 antibody (eight days). The kinetics of response vary considerably for different cytokines, so while four hours post dose is the peak for type I interferon the other cytokines measured will have their own kinetic profiles. The kinetics of turning cytokine production into functional cellular responses is also complex. Similarly, eight days post the initiation of single agent mDX400 treatment is the peak of CD8$^+$ T cell infiltration in the tumor. But this time point may not be optimal for CD8$^+$ T cells with the combination treatment, and is likely not optimal for other cell types.

Another significant limitation of the current study is the use of flow cytometry to perform this type of protein expression analysis. While the cell types and functional markers explored here did not indicate a combination benefit that does not exclude the possibility that differences in immune cell phenotype exist. Flow and mass cytometry each have benefits and drawbacks, and for various practical reasons flow cytometry was predominantly utilized here. However, flow cytometry panels are significantly constrained in size due to overlap of emission spectra, and the largest panel capable of being run on the available equipment is 14 parameters. In order to explore more markers several separate flow cytometry panels would need to be run, which would require larger samples yielding more cells. The present study began treatment in larger tumors for two reasons: they respond with less efficacy to single agents alone allowing a window to observe a combination benefit, and they provide a higher probability of yielding more tissue for analysis at the time of collection. However, the robustness of the efficacy of the
combination treatment yielded tumors that were very small, significantly constraining sample size. Two methods could be employed to address this in future studies: generate gene expression data using broad panels to determine which transcripts show the largest changes thereby informing the design of more specific cytometry panels; or redesign in vivo studies (adjusting dose concentration, dose frequency, collection time, etc.) to allow for enough sample to remain for multiple flow cytometry panels or the use of mass cytometry. Mass cytometry uses antibodies labeled with heavy metal isotopes. After staining of single cells with labeled antibodies cells are analyzed in a mass spectrometer. Mass spectra are exquisitely specific allowing for single Dalton resolution, and mass cytometry panels can be as large as 135 parameters. Such large panels allow for much broader phenotyping which would increase the likelihood of finding changes in whole cell populations or functional markers within cell lineages. For example, the current study suggests STING agonism and PD-1 blockade may have combination benefit on CD8+ T cells. Cytotoxic CD8+ T cells primarily mediate their cell killing activities through the secretion of cytotoxic granules containing the factors perforin and granzyme. Upon release perforin oligomerizes in a calcium dependent manner forming pores in the target cell membrane which allows for the entry of the pro-apoptotic serine proteases called granzymes (Trapani, 1995). Increased cytotoxic CD8+ T cell activity is easily determined using cytometry via intracellular staining of antibodies raised against perforin and granzyme. However, large mass cytometry panels require considerably more cells for multiple technical reasons. Additionally, the current study utilized the activation marker CD69, which is an early activation marker broadly expressed by most white blood cells (Ziegler, Ramsdell, & Alderson, 1994). Nonetheless, many activation markers exist, and
the kinetics of expression, and specificity to cell type vary considerably. While perforin and granzyme are markers for increased cytotoxic CD8\(^+\) T cell activity, myeloid cells upregulate CD40, CD80/86, and MHC class I and II upon activation. While these are all markers that are upregulated upon activation the kinetics (early versus late) and duration (brief versus prolonged) of expression, as well as the function of the actual protein vary considerably. Larger panels would be able to identify more cell types and include more functional markers increasing the likelihood of isolating differences between treatments.

In conclusion, we demonstrated a combination benefit in large CT26 tumor with co-administration of anti-PD-1 antibody and STING agonist on both tumor efficacy and multiple tumor cytokines. Cytometric profiling of tumor infiltrating immune cells revealed a role for IL-10/IL-10R on immune activation in this setting. Future studies are required to better understand the interaction of the IL-10 pathway and the PD-1 pathway in the context of tumor clearance.
References


