AUGMENTING COMBINATION IMMUNOTHERAPIES WITH VACCINATION OR COSTIMULATORY/INHIBITORY ANTIBODIES FOR THE TREATMENT OF CANCER

By

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Abbreviations

ADCC- Antigen-dependent cellular cytotoxicity

Ag- Antigen

APC- Antigen presenting cell

BTLA- B and T lymphocyte attenuator

CAR- Chimeric antigen receptor

CTLA-4- Cytotoxic T lymphocyte-associated protein 4

DC- Dendritic cell

DRiP- Defective ribosomal products

ELISA- Enzyme-linked immunosorbent assay

GITR- Glucocorticoid-induced TNFR-related protein

GM-CSF- Granulocyte-macrophage colony-stimulating factor

GVAX- GM-CSF-secreting vaccine

H&E- Hematoxylin and eosin

HRP- Horse radish peroxidase

i.m.- Intermammary

ICOS- Inducible T cell costimulator

ICS- Intracellular cytokine staining

IDO- Indoleamine-pyrrole 2,3-dioxygenase

IFN-γ- Interferon gamma

IHC- Immunohistochemistry

IL- Interleukin

KLRG1- Killer cell lectin-like receptor G1

LAG-3- Lymphocyte activation gene 3

LN- Lymph node

MCA- Methylcholanthrene

MHC- Major histocompatibility complex

MMP-9- Matrix metallopeptidase 9

MMTV-PyMT- Murine mammary tumor virus-polyoma middle T

NK- Natural Killer cells

NSCLC- Non-small cell lung cancer

PD-1- Programmed death-1 receptor

PD-L1- Programmed death ligand 1

s.c.- Subcutaneous

SLiP- Short-lived proteins

ROS- Reactive oxygen species

TAA- Tumor-associated antigen

TAM- Tumor-associated macrophage

TCR- T cell receptor

TGF-β- Transforming growth factor-beta

Th- T helper

Th1- Type 1 helper response

Th2- Type 2 helper response

TIL- Tumor-infiltrating lymphocytes

TIM-3- T cell immunoglobulin and mucin-domain containing-3

TNF- Tumor necrosis factor

T_{reg}- Regulatory T cell

TSA- Tyramide signal amplification

VEGF- Vascular endothelial growth factor

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Abstract

Recently, antibodies that block T cell inhibitory checkpoints have proven to be a beneficial treatment for patients with solid tumors. However, when given as monotherapies a substantial portion of patients remain refractory to treatment. Given the complexities of the interface between tumors and the immune system, alternative therapies are required to treat these patients and elucidating novel therapeutic combinations remains an urgent need. This body of work explores two potential ways to impact tumors that are either devoid of T cell infiltrate or refractory to checkpoint blockade. The first approach addresses cancer vaccination, which can impact patients with "cold" tumors (that lack infiltrating T cells) by priming a new T cell response to tumor-specific antigens. It is poorly understood whether booster vaccinations, commonly used with vaccines against pathogenic microorganisms, inhibit the efficacy of a single vaccination with whole-cell tumor vaccines. We determined that tumor immunogenicity (defined by the protection offered by a single prophylactic vaccination) or addition of the adjuvant GM-CSF (also known as a GVAX vaccine) did not diminish the efficacy of multiple vaccinations, and did not increase inhibitory regulatory T cells. Importantly, the addition of GM-CSF did significantly increase the amount of T cells in the tumors of multiply vaccinated animals. This suggests that the addition of GM-CSF to vaccines correlates with improved trafficking of T cells to the tumor, a benefit that may be useful in cancer vaccination in the future.

The second approach addresses tumors that may have tumor T cell infiltrate, but remain refractory to monotherapy checkpoint blockade such as anti-PD-1. We found that in an orthotopically transplanted mammary tumor model we could significantly impact tumor growth by stimulating the OX40 costimulatory receptor with an agonist antibody. Surprisingly, when we combined this treatment with PD-1 blockade, we reduced therapeutic efficacy compared to anti-OX40 monotherapy and saw dramatic increases in

serum cytokines as well as increased expression of peripheral T cell inhibitory receptors. This reduced anti-tumor effect correlated with an early burst of T cell proliferation, but long-term reduction in T cell proliferation in the tumor, suggesting dysfunctional activation and differentiation. By sequencing our combination treatment, giving anti-OX40 first followed by anti-PD-1, we were able to avoid this dysfunction. Sequential combination not only delayed tumor growth significantly longer than the concurrent combination or anti-OX40 alone, but ~30% of treated mice completely regressed their tumors and survived over 200 days. This effect proved to be dependent on both $CD4^+$ and $CD8^+$ T cells although $CD8^+$ cells were only required after ~25 days for long-term tumor delay and regression. This work suggests that the timing of combination antibody therapy targeting inhibitory and costimulatory receptors is critical to their success. Together these data provide support for varying methods of augmenting single agent immunotherapy in patients, and provide key insights into mechanisms that may impact patients who are refractory to checkpoint blockade or require a novel tumor-specific T cell priming event.

CHAPTER ONE: INTRODUCTION

The human immune system's relationship with cancer is incredibly complex. However, suggestions that the immune system can treat cancer have kept scientists searching for potential ways to augment immunity for over 100 years. We hypothesize that overcoming immune tolerance and activating a functional anti-tumor T cell response in cancer patients requires sufficient antigenic targets, tumor-specific primed T cells, the proper costimulatory signals, and the right cytokine milieu. Patients lacking any of these necessary components (or patients who have generated immune mechanisms that suppress or inhibit them) may necessitate therapeutic intervention to correct their deficiencies, with many patients likely requiring multiple immunotherapies in combination for an optimal response. As our understanding of the intricacies of the immune system and the complexities of cancer have grown exponentially over the last few decades, only recently have researchers begun to grasp why earlier attempts at immunotherapy failed and how truly difficult it is to fine tune an immune response to eliminate malignancies in patients with diverse tumor microenvironments and immune inhibitory mechanisms.

With the recent proliferation of agents targeting various molecules of the immune system, including myeloid, T and B, and Natural Killer (NK) cells, there is great interest in combining novel therapies with standard of care treatments. These include radiation, chemotherapy, and surgery as well as more mature immunotherapies that have not yet seen broad clinical success like vaccination and adoptive T cell transfer. As our understanding of the immune system continues to grow, combination immunotherapies will very likely have a major role in cancer treatments of the future. This thesis explores multiple types of combination therapies using vaccination, cytokines, checkpoint blockade and costimulation antibodies, and the mechanisms of action that allow these combinations to be most effective in priming and maintaining or reinvigorating an anti-tumor response.

Adaptive Immunity

The mammalian immune system is divided into two main factions: innate immunity and adaptive immunity. Innate immunity immediately recognizes specific molecular patterns or molecules from non-mammalian cells such as lipids, RNA, and peptidoglycans and initiates a rapid response to attempt to control a pathogen. This can be done in a number of manners, including sequestration within phagocytic cells such as macrophages and dendritic cells that can act as antigen presenting cells (APCs). Innate lymphocytes also produce cytokines and chemokines that lead to inflammation and the recruitment and activation of APCs that are capable of initiating adaptive immunity. This intricate link between the innate and adaptive immune systems is critical and was highlighted by observations that vaccination without some form of an adjuvant, such as bacteria or chemical compounds like aluminum oxide that activate the innate immune system, failed to instill adaptive immunity. Charles Janeway famously described this requirement for innate immunity when inducing adaptive immunity as the "immunologist's dirty little secret", cleverly referencing bacteria's role as an adjuvant to vaccines (1).

Adaptive immunity is the source of immunological memory and is initiated by exposure to a foreign substance and the subsequent innate immune response. A foreign substance or "intruder" into the body is phagocytosed by APCs such as dendritic cells (DCs), which are activated and matured by innate immune-generated cytokines. Other phagocytic cells such as macrophages and B cells can also act as APCs. Pathogens are then broken down into small fragments known as Ags, and these Ags are processed intracellularly, loaded onto major histocompatibility molecules (MHC), and then presented on the surface of the APC surface. The adaptive immune system is split into two categories: humoral immunity made up of B cells and the antibodies they produce and cellular immunity, mainly made up of T cells. T cells are generated in the bone marrow but mature in the thymus (giving them their

"T" name), while B cells are generated and mature in the bone marrow. Importantly, T and B cells differ in their unique receptors; the T cell receptor (TCR) recognizes both a self-MHC molecule and the foreign Ag being held by it, while the B cell receptor (BCR) does not require MHC-associated presentation and can directly bind to its antigenic target. The BCR can also become soluble in more mature B cells, which are then referred to as antibodies or immunoglobulins (Ig). T cells are divided into $CD4^+$ or $CD8^+$ during maturation in the thymus, with $CD4^+$ T cells recognizing Ag presented in MHC class II molecules, and $CD8^+$ T cells recognizing Ag presented in MHC class I molecules. This TCR recognition and the subsequent downstream signaling of the TCR is termed "signal 1", and is the first step in commencing a T cell priming event. Upon TCR stimulation a variety of costimulatory and inhibitory receptors are upregulated on the surface of the T cell within 24 hours.

When upregulated, costimulatory receptors on the surface of Ag-experienced T cells recognize specific ligands on APCs (or other myeloid, tumor, or T cells) (Table 1). This ligand-costimulatory receptor interaction is termed "signal 2". Thus Ag alone is not sufficient to initiate a proper priming event, and the requirement for signal 2 (or peripheral tolerance) is a major protective measure for the immune system to prevent immune response against self-Ags (2,3). Subsequently T cells that survive peripheral tolerance and only see Ag in the absence of costimulatory signals can become dysfunctional and are termed "anergic" (4,5). The second signal on T cells is much more complicated than signal 1, as a wide variety of surface receptors, some of them also capable of sending inhibitory signals (Table 1), are expressed on the surface of an Ag-stimulated T cell. These receptors are expressed at different times after TCR stimulation, and the ligands present around the T cell shape the signals the T cell is given. Receptors capable of positive signaling leading to T cell proliferation and expansion are the first to be expressed (some such as CD28 are even constitutively expressed on naïve T cells at the time of Ag stimulation), while receptors

	Receptor (T cell)	Ligand (APC, Tumor)	Reference
Negative Signaling Inhibition			
	PD-1	PD-L1 (B7-H1), PD-L2	(6)
	CTLA-4	CD80 (B7-1), CD86 (B7-2)	(7)
	LAG-3	MHC II	(8)
	TIM-3	Galectin 9	(9)
	BTLA	B7-H4, HVEM	(10)
	VISTA (PD-1H)	Unknown	(11)
	TIGIT	PVR (CD155)	(12)
Positive Signaling Costimulation			
	CD28	CD80, CD86	(13,14)
	OX40	OX40L	(15)
	GITR (TNFRSF18, AITR)	GITRL	(16)
	4-1BB (CD137)	4-1BBL	(17)
	CD27	CD70	(18)
	ICOS	ICOS-L (B7-H2)	(19)
	CD40L	CD40	(20)

Table 1: T cell costimulation and inhibition surface receptors

that provide T cells negative, inhibitory signals appear at a slightly delayed time point and initiate immune contraction. The most well characterized T cell costimulatory molecule is CD28, a key component of signal 2 that ligates with either CD80 (B7.1) or CD86 (B7.2) expressed on the surface of activated APCs. Other costimulatory molecules and their ligands are listed in Table 1.

After signal 2 and the subsequent intracellular signaling, T cells begin a process known as clonal expansion. During clonal expansion the initial singular, Ag-experienced T cell proliferates to produce daughter cells sharing the exact same TCR, and thus the same Ag specificity. This process requires the production and consumption of Interleukin-2 (IL-2), which was initially called T cell growth factor due to its necessity for in vitro T cell growth and persistence (21-23). Daughter cells differentiate into effector cells, which are highly functional and generated to destroy an immediate target, and memory cells, which are much longer lived and allow T cells to expand more quickly and increase functionality upon a secondary exposure. The expression of specific cell surface markers/receptors is used to

identify subsets of murine T cells at various stages of differentiation and maturation. CD44 and CD62L (L-selectin) are the two most commonly used markers to designate naïve, effector memory, and central memory T cells, with $CD62L^{+}CD44^{-}$ cells classified as naïve, $CD62L^{+}CD44^{+}$ cells classified as central memory, and $CD62L^{-}CD44^{hi/+}$ classified as effector memory or effector cells (24,25). Surface receptors such as KLRG1 or BTLA can also distinguish effector cells differentiation status, with late state, terminally differentiated effector cells expressing KLRG1 and losing BTLA expression (26,27). It is important to note that BTLA is expressed on type 1 helper (Th1) $CD4^{+}$ cells, but not type 2 helper (Th2) $CD4^{+}$ cells (10). These late stage cells are capable of effector functions, such as secreting cytokines, but have lost their proliferative capacity. Most of the T cells generated through clonal expansion will contract after the initial immune response (many by utilizing signaling through inhibitory receptors), but some will remain as long-term effector and central memory cells, protecting against future exposure.

In addition to conventional $CD4^{+}$ and $CD8^{+}$ cells, a distinct subpopulation of suppressive $CD4^{+}$ T cells has emerged: the regulatory T cell (T_{reg}). This population of cells was first identified as "suppressor cells" in the 1970s (28), and was later narrowed down to $CD4^{+}$ cells (29,30). But a unique marker for these cells remained unidentified until Shimon Sakaguchi identified a subpopulation of $CD4^{+}$ cells that that were suppressive and additionally expressed the high affinity IL-2 receptor α chain CD25 (31-33). Years later the transcription factor FoxP3 was identified as a more reliable intracellular marker for T_{reg} cell identification (34,35), as CD25 could incorrectly identify $CD4^{+}FoxP3^{-}$ effector cells which were activated ($CD25^{+}$) but had no regulatory functions. However FoxP3 is not constitutively expressed in human T_{reg} cells as it apparently is in murine T_{reg} cells (36). Regulatory T cells can be categorized into natural (or thymic) T_{reg} cells, which develop in the thymus to control against autoimmunity, or induced (or adaptive or peripheral) T_{reg} cells.

Induced T_{reg} cells are naïve $CD4^+$ cells converted to T_{reg} cells outside of the thymus by exogenous Ag in transforming growth factor-β (TGF-β)-rich or IL-35-rich microenvironments (37-39).

T_{reg} cells can suppress through a variety of mechanisms. They constitutively express the inhibitory receptor cytotoxic T lymphocyte antigen-4 (CTLA-4, CD152) that can interrupt antigen presentation through downregulation of costimulatory receptors on APCs (40-42). They also secrete immunosuppressive cytokines like TGF-β, IL-10, and IL-35 (43-45). Additionally, T_{reg} cells can induce apoptosis of effector cells via granzymes and/or perforin (46,47). The transfer of cyclic adenosine monophosphate (cAMP) (48) and cell surface contact of molecules like Galectin-1 have also been shown to suppress effector T cells (49). Given the high expression of CD25 on T_{reg} cells, some groups have suggested IL-2 consumption as a mechanism of suppression (50), but this mechanism remains controversial (51).

Malignancies can utilize both natural and induced T_{reg} cells due to their expression of both self- and neo-antigens (52-54). Indeed, $CD4^+FoxP3^+$ T_{reg} cells have been associated with reduced overall survival in a majority of solid tumors, but also were associated with improved survival in colorectal and head and neck cancer (55,56), suggesting that T_{reg} cells may also be used as a surrogate marker for an anti-tumor response. Logically, depleting T_{reg} cells to improve immune responses against tumors has been an active area of research (57), and how to handle T_{reg} cells (both those already present and any that might be generated by treatment) is still a question that needs to be addressed with each novel therapy introduced. For example it has been demonstrated that, in addition to blocking inhibitory signaling, certain clones of anti-CTLA-4 antibodies (including the FDA-approved Ipilimumab) are able to deplete T_{reg} cells, which have constitutive expression of CTLA-4 on their surface (58).

During T cell differentiation, $CD4^+$ T cells can be induced to make specific cytokines and not others. This feature of $CD4^+$ T cells (along with their interactions with B cells) caused them to gain the nickname "helper" T cells, as the cytokines they produce help activate cytotoxic $CD8^+$ T cells. Interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) are considered Type 1 (or Th1) cytokines, which lead to a more inflammatory immune response capable of eradicating intracellular pathogens. Type 2 (or Th2) cytokines, including IL-4 and IL-10, shift an immune response towards humoral immunity and are effective against parasites. These cytokines have both positive and inhibitory effects, with Th1 cytokines inducing more Th1 cytokines and dissuading a Th2 response, and Th2 cytokines doing the opposite. Transcription factors have been a useful way to differentiate between the types of CD4 cells, with T-bet highly expressed in Th1 and GATA-3 highly expressed in Th2 (59,60). More recently a third type helper $CD4^+$ T cell that secretes IL-17 (Th17) has been identified (61) that can be identified by high expression of the RORγT transcription factor (62). Thus a cytokine profile is often referred to as being "skewed" one direction or the other as once an immune response is initiated it tends to go in only one direction, unless a new immune stimulus alters it. In a similar way macrophages can be polarized by Th1 or Th2 cytokines to produce either type 1 (M1) or type 2 (M2) cytokines, respectively. It has also been shown to work in the reverse direction, with cytokines secreted from polarized macrophages affecting T cell polarization (63). Inbred mouse strains are known to polarize specific ways, with C57BL/6 mice skewing towards a Th1 response and BALB/c and FVB/NJ mice skewing towards type 2 cytokines (63). In the tumor microenvironment, it has been well documented that Th2 cytokines favor tumor growth, whereas Th1 cytokines are integral for T cell-mediated tumor regression (64-67). Thus the ability to repolarize an anti-tumor immune response from Th2 to Th1 has been a key focus of tumor immunology research over the last few decades.

Despite tight regulation of T cell differentiation and expansion, T cells can become dysfunctional. For immune responses that do not fully clear a pathogen or tumor, chronic stimulation with antigen can lead to T cell exhaustion. Initially characterized in viral infection models, T cell exhaustion is identified by antigen-specific cells that have lost the capacity to produce cytokines like IL-2 and TNF-α (68,69). The loss of other effector functions, such as the ability to proliferate, lyse target cells, or secrete IFN-γ, have also been identified as effects of exhaustion (70). Eventually these dysfunctional, exhausted cells can be deleted (70,71). Initial research on T cell exhaustion focused on $CD8^+$ T cells, but it was later confirmed that $CD4^+$ T cells could also become dysfunctionally exhausted through chronic antigen stimulation (72,73). Intriguingly, recent publications suggest that exhausted cell populations are made up of both less exhausted cells (perhaps central memory cells) that have a capacity to regain functionality and a subset that is truly exhausted and unrecoverable (74-76). Cell surface markers of exhausted T cells and therapeutic attempts to rescue them in the context of cancer will be discussed below.

Malignant Transformation and Innate Immunity

Malignancies arise when a cell obtains genetic mutations that cause abnormal or uncontrolled proliferation. While activating mutations to oncogenes and inactivating mutations to tumor suppressor genes are always requirements for tumor initiation, local inflammation provided by innate immune cells is also a critical component of tumor promotion and growth. Tumor promoting inflammation has even been added to Hanahan and Weinberg's seminal "Hallmarks of Cancer" (77), demonstrating its importance in tumor development and growth. However this association is not new, as it was first suggested over 150 years ago that inflammation was a contributing factor to tumor development (78).

Components of the innate immune system such as neutrophils, mast cells, and macrophages can secrete cytokines and chemokines capable of creating a chronically inflamed microenvironment leading to eventual dysplasia (or aberrant proliferation of an abnormal cell type) (79-81). Premalignant cells can thrive in this environment, which shares many similarities in microenvironment, cytokine milieu, and increased cellular proliferation with a site of wound healing. As a tumor grows, access to nutrients provided in the blood becomes crucial and here too macrophages play a major role, secreting known promoters of angiogenesis like vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP-9) (82,83). Additionally, TAM-secreted MMP-9 has been suggested as a key promoter of lung metastases, leading to tumor outgrowth from the site of primary development (84).

In later stages of tumor growth, cells of the innate immune system impact adaptive immune cells in the tumor microenvironment. Innate phagocytic cells like DCs and macrophages engulf dying tumor cells and present tumor Ags within MHC molecules on their surface to prime tumor-specific T cells. However, as these T cells are then recruited into the growing malignancy, tumor-associated macrophages (TAMs) can limit their effect by secreting inhibitory cytokines like IL-10, TGF-β, arginase, and reactive oxygen species (ROSs) (85-89). TAMs can also secrete chemokines that recruit inhibitory T_{reg} cells into the tumor (90). Inhibitory TAMs are often referred to as having an M2 phenotype, with their ability to secrete type two cytokines like IL-4 and IL-10. Due in part to these suppressive capabilities, high densities of TAMs are associated with reduced overall survival in a number of solid tumors, including breast cancer {Zhang:2012dn, (91). However, as discussed above, macrophages can also be polarized to an M1 phenotype, with M1 TAMs secreting type 1 cytokines that help stimulate an effective anti-tumor immune response. Local type 1 cytokine inflammation in the tumor at later stages of tumor growth can induce

an efficient immune response with effector T cells. Interestingly certain inflammatory cytokines can have opposite roles in tumor progression at different stages of development. For instance, TGF-β has anti-tumor properties at early stages of tumor development, but in the malignant setting, TGF-β is immunosuppressive and pro-metastatic and blockade of it can augment anti-tumor immunity (92-94). Thus elements of the innate immune system have an incredibly complex role in tumor initiation, development, growth, and also immune-induced regression.

Clinical reagents targeting myeloid-oriented mechanisms of tumor growth and dissemination have had a checkered history, with some like MMP-9 inhibition all but abandoned due to lack of clinical efficacy (although new insights into drug specificity may offer promise going forward) (95-97). Alternatively, anti-VEGF reagents have experienced broad success with multiple drugs obtaining FDA approval (98). Other promising interventions for myeloid-based inhibitory mechanisms currently being investigated include anti-CSF1R (99,100) and inhibition of the immunosuppressive molecule indoleamine-pyrrole 2,3-dioxygenase (IDO) (101,102). Importantly for combination therapy, the diversity and plasticity of myeloid cells, as well as their abundance in not only tumors, but also normal tissue, demands that they be taken into consideration when discussing other types of immunotherapeutic cancer treatment beyond ones that specifically target myeloid cells and the innate immune system.

Cancer Immunoediting

Aberrantly proliferating tumor cells in malignancies can contain non-mutated self-Ags that are normally minimally expressed. Overexpression of these Ags or of non-mutated Ags that are normally not expressed (some do not consider these types of Ags “self”, despite the fact that they are non-mutated) can be immunogenic (103). Indeed, many top ranked

cancer antigens of clinical interest are non-mutated self-Ags (104). Tumors also often contain neo-antigens or mutated Ags, which if presented on the surface of APCs or tumor cells can also prime a specific T cell response. Mutational burden varies greatly across cancer types (105), but an increased number of mutations has been correlated to improved response to immunotherapy (106,107). Through adaptive immunity targeting either of these tumor associated antigens (TAAs) and the process of cancer immunosurveillance (108), tumor cells can be either eliminated or maintained in a state of equilibrium with the immune system. Equilibrium occurs when a tumor is neither growing or regressing, and is simply kept in check by the immune system (109). In humans, data supports the concepts of immunosurveillance. Patients who receive organ transplants, and out of necessity are put on immunosuppressive drugs, are significantly more likely to be diagnosed with a variety of unrelated cancers (110). This was elegantly demonstrated in the preclinical setting by Robert Schreiber and colleagues, who showed that RAG2$^{-/-}$ mice lacking T and B cell compartments grew methylcholanthrene (MCA) carcinogen-induced tumors at a faster rate and greater percentage than mice with an intact adaptive immune system (111). This work demonstrated a role for the adaptive immune system in the prevention of malignancies. The innate immune system, through Natural Killer (NK) cells and their polarization of macrophages, is also capable of eliminating tumors in this manner of immunosurveillance (112). Some tumors however transition to an escape phase, where the immune system is no longer able to keep the tumor in check and the equilibrium is disrupted (113). These tumors have previously been screened by the immune system and subsequently, may lack obvious, high affinity Ags that would mark them for destruction (111), allowing them to grow. This system of immune screening of tumors has been coined “immunoediting”, as tumors that reach the escape phase have been edited to not have easily recognizable immune response-

generating antigenic targets, making them much harder for the immune system to eliminate at later phases without outside intervention.

The concept of immunoediting suggests that some tumors that have escaped the immune system have done so because of a failure to initially prime T cells specific to Ags on that tumor. Alternatively "edited" tumor cells have most likely been selected when immune-activating Ags are lost. Progressing tumors also utilize other mechanisms to avoid the immune system such as downregulation of Ag-presenting MHC molecules, upregulation of inhibitory ligands like PD-L1, reduction or loss of chemokines capable of recruiting T cells, recruitment and differentiation of suppressive myeloid cells, secretion of type 2 polarizing cytokines like IL-4 and IL-10 and vascularization-inducing VEGF, and the induction of T_{reg} cells. Therefore it is not only sufficient to determine the correct tumor Ags (and multiple Ags are surely better than a single one, given the possibility of Ag loss) and prime a specific T cells response against them, but methods capable of overcoming these tumor-induced suppressive mechanisms are crucial to a successful anti-tumor immune response.

A Brief History of Cancer Immunotherapy

In the 1890's, William Coley, a New York surgeon since dubbed the "Father of Immunotherapy", noted that patients who had post-surgical infections seemed to correlate with improved response to their cancer (114). Hypothesizing that infection was critical for to prevent recurrence, he utilized "Coley's toxins", consisting of killed pathogenic bacterial strains, to vaccinate cancer patients and trigger the, unknown at the time, innate immune system. Results of Coley's toxins have been retrospectively reported, with estimates of ~50% of patients achieving an anti-tumor response (115), but inconsistencies in treatment administration, recordkeeping, and follow up, as well as the introduction of radiation therapy pushed the field away from the concept. Paul Ehrlich also contributed substantially

to the field of cancer research at the turn of the 20th century, hypothesizing that the immune system dictated tumor growth (later termed "cancer immunosurveillance" by Burnet (108)). Ehrlich is also credited for pioneering drug screens and introducing the first chemotherapy agents, two critical components of clinical research and cancer treatments over the last century (116).

It would not be until the 1950's when research would again seriously focus on the role of the immune system in cancer, with work utilizing novel murine models of tumor induction reestablishing a role for the immune system in cancer treatments. Edward Foley demonstrated that murine methylcholanthrene (MCA)-induced tumor cells could be used as vaccines to protect from subsequent tumor challenge (117). Methylcholanthrene was used to generate tumors for its ease of administration (injection or painting on the skin) and consistent, relatively rapid tumor induction. However, Prehn and Main showed that MCA-induced tumors can have unique antigens (Ags) that did not allow for cross protection by immunizing with a tumor that differed from the one used for live, challenge, an early glimpse at the difficulty in finding common tumor Ags the field would face (118). George Klein et al. contributed by documenting that the autochthonous host maintained immunity against its own passaged primary tumor (119). These pioneering studies were the first to establish reproducible preclinical models that could be used to study the vaccination and the immune response to cancer, and coincided with an unprecedented period of immunological discovery in the 1950's and 1960's. Many MCA-induced tumor models are still used in contemporary cancer research.

In the 1960's, the adoptive transfer of lymphocytes to treat tumors was first attempted clinically (120), although adoptive transfer had been successfully used to treat preclinical tumors dating back to 1955 (121). This technique was improved with the discovery of the cytokine IL-2 (initially termed "T cell growth factor"), which allowed T cells

to be expanded in vitro for transfer back into the patient in much greater numbers (21,122). These pioneering experiments hypothesized that T cells in the tumor were not present in great enough numbers to impact tumor growth, and that by removing these tumor-infiltrating lymphocytes (TIL) and expanding them ex vivo in the absence of tumor immune suppression, a much larger amount of tumor-specific T cells would be effective when reintroduced into a patient (123). These transferred T cells also have a greater likelihood of being tumor-specific compared to T cells from another patient (even if they had similar HLA types), as they were initially isolated from autologous tumor expressing similar Ags. Currently this technique has been expanded upon and TIL are screened for tumor-specific clones that have both high affinity and effector function specific to a patient's autologous tumor before expansion and reinfusion with IL-2 support (124,125). Adoptive cell therapy has demonstrated dramatic results for some patients, but limitations in generating TIL from patients as well as the need for centers with expertise in the therapy has restricted its use in the clinic.

Over the last two decades an exciting new area of immunotherapy research has appeared that utilizes adoptive transfer techniques to create and re-infuse chimeric Ag receptor (CAR) T cells (126,127). To generate these modified cells, T cells are removed from patients' blood and genetically engineered to express a tumor-specific variable fragments, derived from monoclonal antibodies, attached directly to costimulatory signaling domains. Thus when the T cell recognizes the specific Ag it does not require any additional costimulatory signals to be activated. These cells have shown great proliferative potential in some patients. In many patients CAR T cells are persistent, but by bypassing normal mechanisms of costimulation and inhibition they can also be dangerous if the targeted Ag is expressed outside of tumor cells (128,129). To prevent adverse, off-target effects preclinical CAR T cells have been engineered with a "suicide" gene that when activated can rapidly kill

off the cells (130), but this technology has not yet been broadly adapted in the clinic. CAR T cells have been successful in liquid tumors like leukemia that have a specific and common Ag target (such as B cell Ag CD19 in B cell leukemia) (131,132), but moving this technology into solid tumors has proven extremely difficult (133,134). Hurdles include avoiding off target effects by finding common tumor Ags that are exclusive to tumors, trafficking into the tumor, and avoiding immune suppression in the tumor microenvironment. Nevertheless, as our understanding of ways to counteract immune suppression in the tumor grows, CARs remain an attractive option for further research in solid tumors.

Costimulation and Checkpoint Blockade as Cancer Therapy

More recently cancer immunotherapy has seen rapid advances with antibodies targeting T-cell receptors (135-137). Initially these therapies have used antibodies to block inhibitory receptors (or their ligands) or "checkpoints" on T cells, but preclinical work has progressed to investigate a much wider variety of both inhibitory and costimulatory receptors over the last few decades. As discussed above, after TCR stimulation costimulatory receptors provide crucial second signals for T cell activation and function. The prototypical T cell costimulatory receptor is CD28 (13), which interacts with CD80 or CD86 on an APC to positively signal for T cell proliferation and IL-2 secretion (138,139). Increasing expression of CD80 on either tumors or APCs can augment anti-tumor immunity (140-142). Additionally, soluble CD80 also boosts anti-tumor immune responses, but this effect is due to both costimulation of T cells and sequestering PD-L1, preventing it from binding to PD-1 (143,144). Clinically, CD80-specific antibodies have not shown significant effect in liquid tumors (145), however the mechanism of action with this antibody is targeted at blocking costimulation of malignant lymphocytes instead of acting as an agonist on tumor-specific T cells. Currently in the clinic the CD28/CD80 costimulatory pathway is

mainly being utilized in either the expansion of T cells for adoptive transfer or transfected into CAR-signaling domains (146,147). Other costimulatory receptors can induce anti-tumor immunity in murine models with agonist monoclonal antibodies, including 4-1BB (CD137), ICOS, CD40, and GITR (148-151). However, like CD28, the clinical use of these agents as monotherapies has been limited. Instead they are more commonly applied in combination with vaccination or incorporated into the signaling domains of CAR T cells (131,152).

Another costimulatory receptor with translational interest as a target for cancer immunotherapy is OX40 (CD134 or TNFRsF4), a TNF family receptor that is upregulated on T cells after the TCR recognizes its specific antigen (15,153). The receptor is ubiquitously expressed on $CD4^+FoxP3^+$ T_{reg} cells and activated, Ag-experienced conventional $CD4^+FoxP3^-$ T cells, transiently expressed on activated $CD8^+$ T cells, and can also be upregulated shortly after re-activation of primed effector T cell (154,155). OX40 stimulation results in increased T cell proliferation, activation, differentiation, and survival (15,154,156,157). Agonist antibodies specific to OX40 can induce significant anti-tumor effects in preclinical models (158-160) and despite OX40 expression occurring mainly on $CD4^+$ T cells, anti-tumor immune responses have been credited to both $CD4^+$ and $CD8^+$ cells (155,161,162). OX40 costimulation has also demonstrated enhanced preclinical anti-tumor effects when combined with anti-CTLA-4 and either adjuvants, vaccination, or radiation (163-166). Supported by this preclinical data, OX40 is currently being evaluated as a monotherapy in clinical trials in a variety of solid tumors (167).

Working in tandem with T-cell costimulation, inhibitory or checkpoint signaling is a crucial component of a normal immune response and plays a strong roll in T cell exhaustion. During T cell priming and clonal expansion, inhibitory receptors help regulate growing T cell populations. After expansion of an Ag-experienced T cell, the process of contraction

eliminates short-lived effector cells through apoptosis and reduces the population to only long-lived memory cells (both central memory ($CD62L^+$) and effector memory ($CD62L^-$)). This contraction maintains T cell homeostasis and allows for the expansion of other T cell clones as needed to respond to new Ag sources. Two of the most thoroughly studied and characterized inhibitory receptors, program cell death protein 1 (PD-1, CD279) and CTLA-4, are currently targeted with blocking antibodies to reverse suppression and activate (or reactivate) T cells capable of eliminating solid malignancies. This section will focus mainly on CTLA-4 and PD-1, with brief mentions of two other inhibitory receptors with emerging clinical relevance, lymphocyte activation gene 3 (LAG-3) and T cell immunoglobulin and mucin-domain containing-3 (TIM-3).

CTLA-4 competes with costimulatory molecule CD28 on the surface of T cells for binding to CD80 or CD86 (B7-1 or B7-2) expressed on the surface of APCs (168-170). CTLA-4 has a ten fold higher affinity for CD80/86 than CD28 (171) and when upregulated it outcompetes CD28, preventing this important costimulatory interaction from occurring. CTLA-4 can also trans-endocytose CD80/86 from the surface of APCs and degrade it within the T cell (172). CTLA-4 is upregulated within 24 hours of T cell activation (169), but it's effects are optimized on cells who have already experienced CD28-mediated costimulation, indicating that CD28 expression is more prevalent (and thus functional) at the initiation of T cell activation (170). Despite its identification in 1987 (7), it took seven years before CTLA-4 was suggested to have inhibitory effects on T cells (173,174). Upon CD80/86 stimulation, CTLA-4 activates serine/threonine phosphatase PP2A, which inhibits serine/threonine kinase Akt signaling within the T cell (175). This results in halting T cell proliferation via the prevention of IL-2 accumulation (176).

Early work involving the mechanisms of CTLA-4 inhibition were done hand in hand with research utilizing CTLA-4-specific antibodies to treat tumors in preclinical models.

James Allison's group published work demonstrating anti-tumor effects with anti-CTLA-4 antibodies shortly after they had shown CTLA-4 to be an inhibitory molecule (177,178). Supported by this preclinical data, anti-CTLA-4 moved to phase I clinical trials, showing both efficacy and autoimmune-like toxicity (179,180). Interestingly, both of these trials utilized patients who were either previously vaccinated or were vaccinated in conjunction with antibody dosing, which will be discussed further in sections below. Eventually, anti-CTLA-4 (Ipilimumab), showed significant objective responses in a phase III clinical trial of late stage melanoma patients, leading to it's FDA approval in 2011 (135).

In contrast to CTLA-4 and another early discovered inhibitory receptor LAG-3 (8), PD-1 was never assumed to be a costimulatory molecule. Instead it was discovered when looking for genes in T cells undergoing programmed cell death, thus giving it its name (6). By studying autoimmunity in PD-1 knockout mice, its role in T cell inhibition, and not just apoptosis, was later identified (181). PD-1 is upregulated on the surface of T cells within 24 hours of TCR stimulation, allowing it to double as a marker for Ag-experienced cells (182). In the context of cancer, Steven Rosenberg's group also identified PD-1 as a marker for tumor-specific TIL (183). However, the receptor has also been suggested as a marker for exhausted T cells in environments of persistent antigen and inflammation (184,185). Recently it was reported that exhausted PD-1$^+$ cells cannot be rescued with PD-1 pathway blockade to differentiate into normal memory T cells (186). When ligated to either programmed death-ligand 1 (PD-L1, B7-H1, CD274) or programmed cell death-ligand 2 (PD-L2, B7-DC, CD273), PD-1 initiates an attenuation of T cell proliferation and cytokine production (187-189). Although these inhibitory effects are similar to those seen with CTLA-4 inhibition their signaling mechanisms differ, with PD-1 directly inhibiting phosphatidylinositol 3-kinase (PI3K), downstream from the CD3 and CD28 receptor, but upstream from Akt, which CTLA-4 indirectly inhibits (175). PD-L1 is upregulated by IFN-γ,

granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-4 and is expressed on a wide variety of immune cell types including T cells, B cells, DCs, and macrophages (190). PD-L2 is not as widely expressed, and is found only on DCs and macrophages, although there is evidence that expression of either ligand is dictated by the type of T cell or macrophage response (type 1 or type 2) (191). In an interesting twist, PD-L1 can also bind to CD80 (but not CD86), although there are conflicting reports of whether this interaction results in T cell inhibition or costimulation (192-194).

The discovery of PD-L1 or B7-H1 occurred simultaneously in two different groups; one led by Tasuku Honjo (whose lab initially identified PD-1 in 1992) in collaboration with Gordon Freeman and Arlene Sharpe and the other by Leiping Chen, respectively. Each group gave the ligand a different name, but PD-L1 is the most commonly used term currently. These two groups also simultaneously demonstrated a role for PD-L1/B7-H1 and the PD-1 receptor in tumor immune escape (195,196). PD-L1 expression can be a direct mechanism of tumor immune suppression, with tumor cells expressing the ligand. This has been demonstrated clinically in a wide variety of solid malignancies (195,197-200). However, either PD-L1 or the receptor PD-1 can be effectively blocked by antibodies to generate a preclinical anti-tumor response (201,202). On the back of these data, anti-PD-1 and anti-PD-L1 antibodies entered clinical trials, and initially showed efficacy and similar toxicity profiles compared to anti-CTLA-4 (136,137,203). Anti-PD-1 (nivolumab) is currently FDA approved for melanoma, non-small cell lung cancer, renal cell cancer, and Hodgkin's lymphoma, while pembrolizumab (another anti-PD-1 antibody) is approved in melanoma, non-small cell lung cancer, and head and neck cancer. Lagging a bit behind these anti-PD-1 drugs, anti-PD-L1 atezolizumab is currently approved for bladder cancer and non-small cell lung cancer. Despite the early reports on similar toxicity levels highlighted above, anti-PD-1/PD-L1 has reduced incidences of toxicity compared to anti-CTLA (204). This is supported

by previous data demonstrating much less severe immunological-induced effects in PD-1 versus CTLA-4 knockout mice (181,205).

PD-L1 expression has been suggested as a biomarker for response to PD-1 pathway-targeted drugs, but the data have thus far been mixed (206). The majority of reports have shown PD-L1 expression as a positive predictor of response to PD-1/PD-L1 (136,207-211), but some others have found no correlation (212). Nevertheless, responses are also seen in PD-L1$^-$ patients (although not at the levels seen in PD-L1$^+$ patients) (213). Complicating the problem, many of these studies use different methods and reagents for staining for PD-L1 and even don't agree on a standard cutoff for PD-L1 positivity. Additionally, some count all cells that stain for PD-L1 (including lymphocytes and myeloid cells), while others only count PD-L1 expression on tumor cells. Aside from technical and analytical discrepancies, expression of PD-L1 is inducible and can be transient and can also be associated with TILs and an active immune response (with effector cytokine IFN-γ a key inducer of PD-L1) (214,215). Thus while PD-L1 expression may play a substantial role in predicting not only responses to PD-1 pathway blockade but also overall survival (216), standardized, uniform methods of evaluating PD-L1 expression are still necessary. Efforts are currently underway to meet this need, with a partnership between multiple pharmaceutical and diagnostic companies working on the "Blueprint" project in non-small cell lung cancer (NSCLC) (217).

This importance of PD-L1 as a biomarker was highlighted recently with the results of two phase III clinical trials in NSCLC that used differing eligibility criteria based on PD-L1 expression in the tumor. One trial set a 50 percent or greater cut off for PD-L1 expression, and in this more stringently selected set of patients, PD-1 blockade as a first line therapy proved to be significantly more effective than standard of care chemotherapy (218). The other trial enrolled patients who had PD-L1 expression on as little as one percent of their tumor (as well as patients with brain metastases), making this trial open to a much broader

range of patients (219). The latter trial failed to show any benefit compared to chemotherapy for patients whose tumor cells were at least five percent PD-L1$^+$, while the former trial led to FDA approval of anti-PD-1 as a first line therapy in NSCLC only a few months later. Despite these results, standardization of PD-L1 expression as a biomarker remains a major unresolved issue, but these discrepant clinical trials are a great example of the potential role that biomarkers can play in selecting immunotherapies for patients.

TIM-3 and LAG-3 are two additional inhibitory receptors currently being evaluated as targets for cancer immunotherapy. TIM-3 is expressed on Th1 CD4$^+$ cells, stimulated with galectin 9, regulates macrophage activation, and is expressed, along with PD-1, on populations of exhausted T cells (9,220,221). TIM-3 also is expressed on myeloid cells such as dendritic cells, and can play a major role in innate immune responses (222-224). Interestingly, but not surprisingly given their co-expression on exhausted T cells, the first reference of anti-TIM-3 antibodies providing an anti-tumor effect was implemented in combination with anti-PD-1 treatment (225). LAG-3 was identified over a decade prior to TIM-3, but similar to CTLA-4 it was initially suggested to be an activating receptor, and transfecting LAG-3 into tumor cells actually inhibited their growth in vivo (8,226). This is believed to occur because of stimulation to LAG-3's ligand, MHC II expressed on APCs, and because of this it was even suggested that LAG-3 could make a good vaccine adjuvant (227,228). Later LAG-3 was shown to also be a T cell exhaustion marker that is functionally distinct from PD-1 and is associated with T_{reg} cells (229-232), but can also be targeted in combination with PD-1 for an enhanced anti-tumor effect {(229-234). Thus the LAG-3 and TIM-3 inhibitory receptors, in addition to CTLA-4 and PD-1, present immunotherapy researchers with a variety of targets in attempts to reverse exhaustion and re-activate T cells.

In addition to agonist and blocking capabilities, some antibodies can induce antibody-dependent cellular cytotoxicity (or antibody-dependent cell-mediated cytotoxicity, ADCC), with NK cells, macrophages, and some granulocytes targeting and lysing (or deleting) antibody-covered cells. The Fc portions of bound antibodies ligate to specific Fcγ receptors expressed on ADCC effector cells, triggering the release of cytotoxic granules like Granzyme B and perforin and often the secretion of effector cytokines like IFN-γ. With high amounts of Fcγ receptors expressed on both tumor and intratumoral immune cells (235,236), ADCC is a component of the anti-tumor response for many antibody-based therapies (237,238). Macrophages are most often the effector cell induced to perform ADCC (239,240), but some reports suggest that NK cells play a necessary role with certain antibodies (241).

It is becoming clear that antibody isotype plays a major role in the mechanism of action of antibodies, whether that is blockade, stimulation, or depletion/ADCC. Certain antibody isotypes, like Human IgG1 or murine IgG2a, which activatory Fcγ receptors have a higher affinity for, are much more likely to induce ADCC (242,243). Ipilimumab, an IgG1 antibody, is an example of this, and depletion of CTLA-4-expressing T_{reg} cells has been proposed as a major mechanism for the drug's anti-tumor effects (244). Inversely, antibodies with mouse IgG1 or rat IgG2a isotypes are more likely to interact with inhibitory FcγRIIB receptors, avoiding ADCC (245,246). Indeed many agonist antibodies like anti-CD40 and anti-OX40 require FcγRIIB receptors to provide a costimulatory effect (247,248), although this is likely due to crosslinking that allows for TNFR synapses to form, rather than any signaling to the FcγRIIB-expressing cell (247,248). Antibodies that block but may not engage any Fcγ receptors, like human IgG2 and IgG4 or antibodies engineered without Fc portions, are a third category of antibodies to show anti-tumor efficacy. While these isotypes could possibly bind Fcγ receptors, a recent report demonstrated that PD-1

pathway blocking antibodies are more effective when they do no engage Fcγ receptors (249). Thus, in addition to the specific costimulatory or inhibitory receptor a monoclonal antibody targets, the isotype of the antibody can play a major role in the immune mechanisms it engages and its overall anti-tumor effect.

Therapeutic Cancer Vaccination and "Cold" Tumors

Despite the promising results achieved with antibodies targeting T cell inhibitory receptors such as CTLA-4 and PD-1, many patients remain refractory to these therapies. A substantial portion of these patients has minimal T cell infiltrate (209), which has been shown to correlate negatively with both survival and response to conventional therapies and immunotherapies (250,251). These tumors devoid of immune infiltrate appear to subsist while being ignored by the immune system and have been coined "cold" tumors. Shifting them to "hot" tumors, which contain tumor-specific T cells, will require more than checkpoint blockade, which mainly enhances previously activated T cells expressing the specific checkpoint receptors. An anti-tumor immune response needs to be initiated or re-initiated in patients with "cold" tumors, and vaccination seems a logical solution to prime a new response.

Vaccination, with the proper adjuvants, can prime both novel tumor-specific T cells as well as shift the cytokine milieu to re-invigorate T cells that may have been previously primed by the tumor, but have become lost or dysfunctional due to exhaustion or anergy (252). Adjuvants or other immune stimulating modalities appear to be key to a response to vaccination. Indeed one recent preclinical report suggests that vaccination and radiation can be used to "warm" PD-L1 refractory tumors, making them susceptible to PD-L1 blockade (253). Thus vaccination, despite being dismissed by many in the oncology

community because of decades of unrealized promise, remains a strong contender to treat "cold" tumors.

With complicating negative factors such as MHC downregulation and reduced antigen processing in tumor cells, as well as a suppressive immune environment, effective therapeutic cancer vaccines have proven to be a tough nut to crack in the clinic. In a 2004 report Rosenberg et al. performed a meta analysis on 35 clinical trials to determine that only 3.3 percent of cancer patients had an objective response to multiple types of common cancer vaccination (254). Many approaches to cancer vaccination have been taken over the last 50 years, including autologous or allogeneic whole-cell tumor vaccines, dendritic cell-based vaccines (DC-based), peptide vaccines, and DNA vaccines. Some vaccines can also be modified to secret cytokines or express costimulatory molecules, both of which aid in priming events induced by the vaccines.

The uniqueness of each tumor has made common antigenic targets much harder to distinguish than the foreign Ags in vaccines targeting pathogens that are made up of essential components of bacteria or viruses. Thus there has been great interest in utilizing whole-cell vaccines that express a wide variety of tumor antigens (potentially 100 percent of them in the case of autologous vaccines) versus a peptide vaccine that will only express a single antigen. Unlike vaccines made up of killed or weekend bacterium or peptide vaccines, whole-cell tumor vaccines also contain many self-Ags, which are often either ignored by the immune system or detrimentally induce an inhibitory immune response. As a testament to the difficulty researchers have faced in tumor vaccine development, only a single vaccine is currently FDA-approved for use in cancer patients. Sipuleucel-T (Provenge) is a DC-based vaccine that uses a patients own DCs transfected to express a common prostate cancer protein (PAP) and the cytokine GM-CSF, and was approved based off of a 4.1 month increase

in overall survival, despite only 1 of 341 patients demonstrating a partial objective response by CT scan (255).

In preclinical models, 3-methylcholanthrene-induced tumors have been used for decades as whole-cell tumor vaccines, mainly in prophylactic vaccination-challenge experiments (117-119). Three-methylcholanthrene is a known carcinogen, which is commonly found in cigarette smoke, making it a useful tool for studying mutated tumors (256). However methylcholanthrene-induced tumors rarely share Ags capable of inducing an effective immune response (118,119,257), demonstrating the difficulty in finding common antigenic targets even in tumors that are induced by the same carcinogen in a syngeneic mouse. The theory of immunodominance of tumor antigens has been proposed to explain this phenomenon (258). This theory suggests that tumors have dominant antigens that can limit a response to subdominant antigens. This suggests that some tumors may be effective as vaccines if the dominant tumor antigens are sufficient tumor rejection antigens. Robert Schreiber's group eloquently demonstrated this at the molecular level by showing that the loss in some clones of a specific mutation that had previously lead to T cell-induced tumor rejection resulted in those tumors ability to grow out with the loss of this dominant antigen (259). But on the contrary if vaccines are made up of dominant antigens that are not tumor rejection antigens, they can limit the immune effects due to subdominant antigens (258,260).

Short-lived proteins (SLiPs): a novel class of cancer antigens

Arguably the greatest asset of a whole-cell vaccine is its antigenic complexity, yet there is a limitation in the immunologically relevant antigenic repertoire associated with these vaccines. Whole-cell vaccines generally rely on cross-presentation of stable long-lived proteins to effectively prime an immune response against tumor-associated antigens,

including many on the National Cancer Institute's top 75 cancer antigens of interest list (104). However, a significant proportion of class I binding epitopes are derived from short-lived proteins (SLiPs) or defective ribosomal products (DRiPs) including mutated, cryptic, misfolded and neo-antigens which are inefficiently cross-presented due to their transient nature (261-263). Instead of being substrates for cross-presentation, these SLiPs and DRiPs are quickly degraded by the proteasome, loaded onto MHC molecules and delivered to the cell surface (261). SLiPs and DRiPs presented on the surface of the tumor as peptide:MHC complexes (pMHC) are excellent targets for a primed cytotoxic T lymphocyte, yet normally are not effectively cross-presented to prime a specific T cell response. Thus a vaccine rich in SLiPs and DRiPs, that can be effectively cross-presented could potentially prime a broader repertoire of T cells, specific for pMHC on tumors, and provides intriguing therapeutic potential.

Our institute has developed a novel strategy to enrich tumor-derived SLiPs and DRiPs through blockade of the proteasome. This process shunts SLiPs and DRiPs to the autophagy pathway where they accumulate in autophagosomes. We then treat with NH_4CL to block the fusion of these autophagosomes with the lysosome, preventing their degradation. These cells are harvested, lysed, enriched by differential centrifugation and the contents enriched for SLiPs and DRiPs are then used as a vaccine (DRibble vaccine) (263,264). Using a prophylactic 3-methylcholanthrene (MCA)-induced sarcoma model, we have previously demonstrated that vaccination with DRibbles vaccine provides cross-protection against a panel of non-homologous syngeneic MCA-induced sarcomas while irradiated whole-cell tumor vaccination was only effective at protecting against a challenge with the homologous (vaccinating) tumor (265). These findings, along with data from a model antigen system (264), support the hypothesis that a DRibble vaccine contains a shared repertoire of SLiP antigens. Further reinforcing this concept, recently both syngeneic

and allogeneic DRibble vaccines have been successfully combined with anti-OX40 costimulation for the therapeutic treatment of preclinical mammary tumors (266). This vaccine offers one alternative for targeting a class of tumor antigens that may not be effectively cross-presented in the tumor-bearing host, and allow priming of tumor-specific T cells against a range of common over-expressed antigens that is not obtained with cancer vaccines currently used in clinical trials. A DRibble vaccine for adjuvant treatment of definitively treated non-small cell lung cancer has just completed a phase I/II clinical trial with evidence of safety, priming of broad humoral immunity against relevant cancer antigens and expansion of T cell receptor repertoires (267). A combination immunotherapy trial in patients with advanced cancer is planned.

Combination Immunotherapy in Cancer

As described above, both the innate and adaptive systems are critical to an optimal immune response and there are multiple requirements for activation of the adaptive immune system. Given the need for so many specific signals, it is not surprising that single agent immunotherapy often does not work or only impacts a minority of patients. As our understanding of immunological mechanisms has increased over the decades, new discoveries of inhibitory and costimulatory receptors, cytokines, and suppressive cell populations have offered opportunities to augment monotherapies, even ones that have seen success as monotherapies such as checkpoint blockade. When designing these combinations however, it is critical to understand what immune suppression initiated by the tumor or compensatory mechanisms initiated by the immune system need to be overcome to improve response. This section will briefly discuss some of the more common suppressive mechanisms and combinations of immunotherapies that have been successful at overcoming these mechanisms. While many immunotherapies have been combined with

conventional treatments that do impact the immune system, such as chemotherapy and radiation, this section will only discuss combinations involving T cell targeted immunotherapies.

T cell costimulation and inhibition plays a major role in the priming phase of an immune response. Thus when priming new T cells after vaccination a wide variety of targets for combination treatment can potentially augment a vaccine's effect. With their ability to shape immune responses and directly contribute to T cells survival and function, cytokines have been an attractive target for combination immunotherapy. In support of this, three cytokines were ranked in the top five of a list of immunotherapeutic agents with potential use in treating cancer (268). Early efforts focused on the combination of effector cytokines such as IL-2 combined with TNF and the combination of IL-2, TNF, and IFN-α (269-271). However, many of these cytokines proved to be too toxic when given to patients as systemic therapy, and most were later abandoned as practical clinical cancer therapies (272). Combined with vaccination though, cytokines critical to T cells growth and expansion IL-2 and IL-12, in addition to GM-CSF, proved to be very effective preclinically. Vaccination with tumor cells transfected to secret IL-2 proved to be successful in altering the cytokine milieu and augmenting vaccination-induced priming (273). IL-12 combined with IL-15, CD80 costimulation, or an IL-2 producing vaccine proved to be effective combinations (274-276). GM-CSF, a cytokine which activates and matures DCs allowing for improved antigen presentation, initially proved to be the most effective cytokine to pair with preclinical tumor vaccination when used to treat poorly immunogenic B16 melanoma (277). GM-CSF-secreting vaccines (GVAX) are still used in both clinical and preclinical research (278,279), and IL-2 is mainly used clinically as a monotherapy, requiring careful management of its potentially severe toxicity (280).

Tumor vaccination has also been partnered with therapies targeting novel costimulatory molecules to help provide "signal 2" during the priming event. Initially, tumor cells were transfected to express the costimulatory molecules, in an attempt to keep the costimulation localized to where antigen presentation occurred. For example, CD80 (B7.1)-transfected tumors have been used to successfully augment preclinical vaccination (281-283). Tumor cells transduced with OX40 ligand also have shown the ability to augment preclinical vaccination(284). However, as reagents improved, researchers moved to successfully combining vaccination with antibodies targeting costimulatory molecules such as CD40, OX40, GITR, and 4-1BB, which is much easier than having to select for transfected tumor cells (162,285-288).

Antibodies targeting inhibitory receptors have also demonstrated synergy with vaccination. Anti-CTLA-4 antibodies can augment vaccination with tumor vaccines with or without GM-CSF (289-291). Interestingly in the clinical setting though, the addition of a gp100 peptide vaccine to anti-CTLA-4 did not augment the effect of anti-CTLA-4 alone in a phase III clinical trial of metastatic melanoma patients (135). Preclinical vaccination combined with antibodies targeting PD-1 or soluble PD-1 have also demonstrated the ability to augment vaccination (292,293). Dual PD-1 and CTLA-4 blockade have been successfully combined with vaccination (294,295), as have combinations of costimulation and checkpoint blockade with anti-4-1BB or OX40 agonist added to either anti-PD-1/PD-L1 or anti-CTLA-4 (165,201,296). Combined, these studies show that in a broad range of tumor models and with a wide assortment of vaccines, targeting "signal 2" can effectively augment tumor vaccination.

Especially when it comes to vaccination, regulatory T cells have been a major thorn in the side of cancer immunologists (297). Recognizing their ability to limit T cell responses by secreting inhibitory cytokines or inducing apoptosis, a variety of unique approaches

have been applied in combination immunotherapies to counteract T_{reg} cells, especially when priming a new T cell response through vaccination. Initial observations that $CD4^+$ depletion augmented vaccination, led to the eventual use of CD25-specific depletion (the majority of the $CD25^+$ population is made up of $FoxP3^+$ T_{reg} cells (298)), which augments the anti-tumor effect of anti-CTLA-4 and GM-CSF-secreting vaccine (57). In another report, an IL-2-linked toxin depleted $CD25^+$ T_{reg} cells, and given in combination before vaccination augmented T cell responses with a DC vaccine in renal cancer patients (299). Another way to deplete T_{reg} cells is with cyclophosphamide (Cytoxan), a form of chemotherapy (300), and this form of depletion was shown to also synergize with tumor-localized CTLA-4 blockade (301). Another group was able to target T_{reg} cells for depletion with an antibody specific for folate receptor 4, which augmented therapeutic efficacy of anti-CTLA-4 and a GM-CSF-secreting vaccine (302). And finally, T_{reg} cell depletion through genetically engineered diphtheria toxin receptor (DTR) ablation (which was a mechanistic study that cannot be easily translated to the clinic) was successfully combined after radiation therapy, but checkpoint blockade (either anti-CTLA-4 or anti-PD-1/PD-L1) failed to augment the effect (303). This body of work demonstrates a variety of ways to efficaciously combine T_{reg} cell depletion with both vaccination and checkpoint blockade, further augmenting these T cell-oriented immunotherapies.

It is important to point out that many of these reports of combination immunotherapy were performed in dissimilar tumor model systems with differing tumor immunogenicities (discussed below) and used different reagents, sizes of tumors at initialization of treatment, and treatment schedules. While a certain combination may be effective in one model, in another it might offer no benefit over monotherapy or potentially even inhibit the effect of monotherapy. For example, anti-CTLA-4 combined with anti-PD-1 is far more effective against CT26 colon carcinomas than PD-A pancreatic carcinomas

(295,304). This is complicated by the fact that negative data is rarely published, unless the lack of synergy can be attributed to specific mechanisms and these provide insights into improving the combination. Thus results of preclinical work with combination immunotherapies can be difficult to interpret when reviewed as a whole. However there are success stories of combinations transitioning from bench to bedside. Recent work with the combination of anti-PD-1 and anti-CTLA-4 is one of these success stories of promising preclinical data being effectively moved into the clinic and eventually leading to FDA approval.

PD-1 and CTLA-4 blockade proved to be effective in combination in the preclinical setting and the success of clinical trials with either drug as monotherapy lead to their combination in the clinic shortly after (135,136,294). Initials reports of the phase I clinical trial in melanoma patients were impressive, with 40% showing objective responses and 16 of those 25 patients experiencing tumor reduction of 80% or more (212). The anti-tumor response with combination treatment also occurred faster than had been reported when either agent was given as a monotherapy. In subsequent phase II and phase III trials objective response rates were 61% and 58%, respectively, with 11.5% of patients demonstrating a complete response in the phase III trial (213,305). Surprisingly, in this larger cohort of patients the combination was only more effective than anti-PD-1 alone in patients whose tumors were PD-L1 negative. Given that there was a higher level of toxicity in combination–treated patients, this work suggests that PD-L1 can be used as a biomarker in melanoma patients to receive either anti-PD-1 or a combination of anti-PD-1 and anti-CTLA-4. Despite the risks of toxicity, the September 30th, 2016 accelerated FDA approval of the combination of anti-CTLA-4 and anti-PD-1 as a frontline treatment for advanced melanoma patients has further piqued interest in other combinations of antibodies that target both inhibitory molecules, but also costimulatory ones.

MMTV-PyMT Tumor Model and Tumor Immunogenicity

The transgenic MMTV-PyMT (PyMT) mouse was first developed in 1991, by injecting a plasmid encoding polyoma virus middle T antigen under the direction of the mouse mammary tumor virus promoter/enhancer into fertilized mouse embryos (306). Middle T antigen has been shown to associate and activate a number of tyrosine kinases (307-309). Female PyMT mice develop multifocal adenocarcinoma in each of their 10 mammary glands by 8-10 weeks of age. These primary tumors follow a similar progression to malignancy as human breast cancer (310), and these transgenic animals also develop lung metastases after primary tumor development (306). As opposed to cell line transplantable tumor models, the PyMT model develops tumors and a subsequent immune response in a progressive fashion that establishes immune suppression and T cell tolerance (311), more closely mimicking the tumor/immune environment in human cancer. PyMT tumors are heavily infiltrated with T lymphocytes as well as myeloid cells (312).

Orthotopically transplanting these spontaneously derived tumors to naïve animals allows for more uniform and controlled growth within experiments, as well as the potential to actually clear the primary tumor in a host that we hypothesize is not centrally tolerized to the middle T antigen and genetically predisposed to incessantly drive oncogenesis. However this model does present an interesting problem in the comparison or combination of repeated experiments, as entirely different spontaneous tumors are used in each experiment, with varied mutations, antigenic repertoire, growth rates, and metastatic potential. On the other hand, this model does present a more translationally relevant scenario similar to the clinic where treatment is given to patients with tumors that differ in all of these characteristics. Thus if the patterns of significant anti-tumor effect hold up across multiple experiments in this model the data may arguably be more translationally

relevant than if they are performed in experiments utilizing a consistent, in vitro-passaged tumor cell line.

Despite being used for decades as a model in the study of tumorigenesis, the PyMT tumor model has only more recently been utilized to investigate immunological questions about the roles that myeloid and T cells play in establishing a protumor microenvironment and suppressing an anti-tumor response. Autochthonous PyMT tumors contain suppressive $CD11b^{+}GR\text{-}1^{+}$ myeloid cells (313,314) and metastasis-enhancing $CD11b^{+}GR\text{-}1^{-}F4/80^{+}$ cells, which become polarized in a Th2-dependent manner by $CD4^{+}$ T cells (312). MicroRNA miR-155 may also play a role in enabling these protumor inflammatory TAMs by skewing towards a Th2-phenotype and reducing $CD11c^{+}$ TAMs (315). In the transplantable PyMT model, the efficacy of taxol-based chemotherapy was enhanced in a $CD8^{+}$ T cell-dependent manner with the depletion of macrophages (316). Protumoral macrophages were later demonstrated to inhibit $CD8^{+}$ T cell-dependent responses to chemotherapy through a mechanism involving IL-10 repression of IL-12 secretion by dendritic cells (317). T_{reg} cells can also play a significant role in tumor growth, and their ablation resulted in growth attenuation and an enhancement of radiotherapy (303). Interestingly, this therapeutic effect appeared dependent on conventional $CD4^{+}$ cells and not $CD8^{+}$ cells. Together these reports demonstrate a complex microenvironment of myeloid and lymphoid cells in PyMT tumors.

Transplanting spontaneous tumors presents a problem in identifying the immunogenicity of the tumors, as each tumor will be unique. The term “immunogenicity” is currently poorly defined in tumor immunology. Generically, the term often refers to tumors that are capable of inciting some kind of positive or successful immune response. However, we would argue that generating an exhausted or dysfunctional immune response should also fit into the definition and disagree with the term “nonimmunogenic” tumors. For some tumors that fall into this category, T cell-targeted monotherapies (like anti-PD-1 antibodies

or T_{reg} depletion) may be sufficient to circumvent or block suppressive mechanisms. And as discussed above, for truly "cold" tumors devoid of any T cells, a new T cell priming event is necessary. Our laboratory operates under a much more specific definition of immunogenicity. We classify tumor immunogenicity by how well vaccination with 10^7 irradiated tumor cells protects mice from a live challenge of twice the TD100 (the minimum dose of tumor with 100% tumor induction) given 14 days later (64). Strongly immunogenic tumors protect roughly 70-100 percent of vaccinated mice, while poorly immunogenic tumors protect 30 percent or less. Tumors in between are categorized as moderately or weakly immunogenic. We believe this approach allows for an objective assessment of a tumor's ability to prime an effective immune response, which is strongly determined by the tumor antigens available in the irradiated tumor vaccine. Consequently, using this method to establish the immunogenicity of spontaneous transplanted MMTV-PyMT tumors, which may have variable antigenic repertoires between the vaccinating and challenging tumor, is problematic. However, in unpublished data we have measured a cell line (FAT) derived from an MMTV-PyMT tumor to be poorly immunogenic (providing protection in only 2/11 vaccinated mice). These data, along with a lack of response to checkpoint blockade suggests that at least some of these spontaneously derived tumors are poorly immunogenic.

Others have suggested alternative means of quantifying tumor immunogenicity, including resection of transplanted and simultaneous transplant of a second tumor or immune profiling of transplanted tumors with quantitative PCR, IHC and flow cytometry(318,319). Mechanism of and immunogenic cell death induced by heat shock proteins has also been suggested as a key factor of tumor immunogenicity (320). Thomas Blankenstein has suggested that only tumors that are rejected in naive mice should be considered highly immunogenic, and a requirement of vaccination for tumor rejection would classify tumors as "intermediately" immunogenic (321). While this makes sense, it is

important to remember that in preclinical tumor models the dose of tumor that a naive mouse is challenged with is a critical factor in how quickly a tumor grows or if it is rejected. Thus we believe that in addition to immunogenicity, each tumor has a "tumorigenicity" that defines how well a tumor grows (or what dose it takes to grow) after transplant into naïve mice. We identify tumorigenicity by determining a TD100 for each tumor cell line.

Many factors can influence a tumor's immunogenicity, including tumorgenicity, tumor growth rate, antigenic repertoire, MHC expression, cytokine secretion, chemoattractants for both lymphoid and myeloid cells, and expression of inhibitory ligands like PD-L1. Although clinical work has correlated the presence of TIL with improved patient survival, this does not mean that all tumors with T cell infiltrate are strongly immunogenic (250). As reviewed above, tumors have many ways to stall an immune response and some of these are so effective that they cannot be overturned by whole-cell vaccination, thus using the presence of TIL as a marker for tumor immunogenicity may be misleading. Indeed, two untreated tumors that are commonly classified as poorly immunogenic, 4T1 mammary tumor and B16 melanoma, both have $CD3^+$ and $CD8^+$ T cell infiltrate when transplanted into naïve mice (322). Yet response to some monotherapies such as anti-PD-1 may in fact be a more rapid, although perhaps less precise, means of measuring tumor immunogenicity. Success with these therapies would propose that sufficient tumor antigens (antigenicity) and T cells were present, and that T cells were simply exhausted by immune suppression induced by a potentially strongly immunogenic tumor. Conversely, tumors that do not respond to PD-1 monotherapy may be poorly immunogenic and need additional priming events provided by vaccination or even costimulation. In the case of spontaneously derived tumors such as MMTV-PyMT where our normal method of using the tumor cell line itself as a whole-cell vaccine isn't practical, the efficacy of anti-PD-1 monotherapy may provide a surrogate method for loosely determining tumor immunogenicity.

Multispectral Imaging of Tumors

As pointed out above, tumor immune infiltrate is beginning to be recognized as a critical component of both overall survival and response to immunotherapy, as well as some conventional therapies. Accordingly, standardized methods capable of both identifying and quantifying TIL are critical to the field of oncology. We have adopted a novel method of immunohistochemistry (IHC) that utilizes multispectral imaging to allow for the identification of a variety of lymphocytes on a single fixed slide of tissue. IHC has been a critical component of cancer research for many decades. Initial diagnosis of solid tumors currently relies on a pathologist to determine that neoplastic cells have transformed to a malignant state and that a tumor is not benign. IHC is used to determine not only the shape of cells, but also what types of cells are present in a sample. Hematoxylin and eosin (H&E) have long been the gold standard for grossly identifying different types of cells by histology, with hematoxylin staining basic cell components such as DNA/RNA in the nucleus and eosin staining acidic components collagen and mitochondria. Using these stains and analyzing the size and proportion of the nucleus and other intracellular bodies, pathologists can easily identify different cell types. Nonetheless this method is not particularly specific, and other antibody-conjugated stains are needed to further phenotype lymphocytic and monocytic cells. Identification of specific lymphocytes, especially $CD8^+$ T cells, has become more important recently as the value of evaluating T cells in the tumor has become more clear (discussed above) (250,251). However these methods can only identify a single receptor target at a time, making the higher level of cell phenotyping and quantification obtained from flow cytometry unachievable. To circumvent this problem new techniques in multispectral imaging have recently been introduced which allow for visualization and quantification of up to seven different markers on a single slide of fixed tissue.

Tyramide signal amplification (TSA) has played a key role in this optimization of multispectral imaging. TSA molecules bind to all tyrosine residues adjacent to a specifically bound secondary antibody with streptavidin-horse radish peroxidase (HRP), which provides a much brighter signal than traditional staining methods utilizing only streptavidin-HRP. Using this technique, primary antibodies can serially be applied to a single tissue (and cell), stained, and stripped off. After all staining is complete slides can be imaged with a microscope capable of detecting the 6-7 wavelengths of fluorescent light emitted by the different fluorophore-bound TSA molecules. The end result allows for multiple markers to be identified on the same cell, fox example allowing for the distinction between a non-proliferating $CD3^+CD4^+FoxP3^+Ki67^-$ T_{reg} cell and a $CD3^+CD4^+FoxP3^+Ki67^+$ proliferating T_{reg} cell. This method, along with zinc fixation, has proven to also be successful in murine tissue, which traditionally has been difficult to stain for CD4 and CD8 markers (323).

Utilizing these techniques, spatial relationships of cells within the tumor microenvironment can now be visualized and quantified. "Nearest neighbor" analysis can be done to quantify how may suppressive T_{reg} cells are around a $CD8^+$ effector cell, for example. Importantly in the tumor, cells can now be quantified to a much more sophisticated degree without the need to digest the tumor as is typically required for flow cytometric analysis. Flow cytometry has been shown to both undercount cells (324) and tumor enzyme digestion has prompted questions about cleaving off surface receptors (325,326). Multispectral IHC and TSA staining are already being utilized to evaluate new biomarkers and segregate patients into groups more or less likely to respond to certain therapies. Thus treatments could potentially be better tailored to patients they are most likely to impact, allowing patients to pursue other treatment options, saving patients time and money.

Additionally they also provide an excellent platform for preclinical researchers to gauge the impact of immunotherapies on the quantity and quality of TIL.

Chapter 2: Multiple vaccinations of GM-CSF-secreting murine tumor vaccines (GVAX) maintain prophylactic efficacy and increase T cell tumor trafficking.

Abstract:

Despite decades of use in clinical trials, the efficacy of booster vaccinations with whole-cell tumor vaccines remains poorly understood. We have reported that booster vaccinations with a poorly immunogenic GM-CSF-secreting (GVAX) murine tumor induce high levels of regulatory T (T_{reg}) cells, which limited the therapeutic efficacy of tumor-specific effector cells. To determine whether multiple vaccination-induced immunity is limited by a GVAX tumor vaccine's inherent immunogenicity or the adjuvant GM-CSF, we compared one and three vaccinations with either irradiated strongly immunogenic MCA-304 or weakly immunogenic MCA-310 sarcoma tumor cells. Triple vaccination maintained single vaccine protection with the strongly immunogenic tumor and significantly augmented single vaccine protection with the weakly immunogenic tumor, with no increase in T_{reg} cells between one and three vaccinations of either tumor. Splenic effector T cells from mice receiving multiple vaccinations secreted almost 10 fold more tumor-specific IFN-γ compared to animals multiply vaccinated with GM-CSF-secreting tumor vaccines. However, triple vaccination with GM-CSF-secreting tumors did not alter the protection we saw without GM-CSF, and importantly the addition of GM-CSF increased $CD3^+$ and $CD8^+$ T cell tumor infiltrate in triply vaccinated animals compared to MCA-310 alone. Our results demonstrate that tumor immunogenicity does not impact the efficacy of multiple vaccinations of GM-CSF-secreting whole-cell sarcoma vaccines, and show that GM-CSF alters T cell trafficking and importantly augments tumor infiltration. Our data supports the continued use of GM-CSF with cancer vaccination, and although GVAX vaccines have not seen clinical success thus far, we believe that in combination with new immunotherapies they will continue to have a role in immuno-oncology moving forward.

Introduction:

As our understanding of immune suppression mechanisms in the tumor microenvironment has substantially increased over the last decade, researchers are beginning to elucidate some of the hurdles preventing effective therapeutic tumor vaccination. Recently, the demonstration that immunotherapy can overcome some of these suppressive mechanisms (described above) has renewed interest in cancer vaccines as a way to augment current immunotherapies such as checkpoint blockade. In a wide range of clinical trials, checkpoint blockade or immunotherapies targeting T cell inhibitory receptors (CTLA-4, PD-1, and PD-L1) have proven effective against solid tumors (135-137). At the same time other groups have shown that therapeutic benefit and increased survival appear to correlate with the extent of tumor immune infiltrate at the initiation of treatment (250,327). For patients whose "cold" tumors are bereft of T cells, who will likely see little benefit from T cell-activating therapies like checkpoint blockade, tumor vaccines offer the chance to prime a novel tumor-specific immune response. These newly primed immune responses could then be further augmented by combination with immunotherapies such as checkpoint blockade (328). Indeed, vaccination using granulocyte macrophage-colony stimulating factor (GM-CSF)-secreting tumor vaccines has been augmented by checkpoint blockade in multiple preclinical models (295,296,329). Furthermore it has been suggested that vaccine-primed T cells may in fact condition the tumor microenvironment to allow endogenous, suppressed or exhausted anti-tumor T cells to regain functionality (252).

With future cancer treatments likely relying on combination therapies, whole-cell tumor vaccines continue to be of interest in cancer research because of their broad diversity of potential shared tumor antigens that may prime an immune response. The make up of tumor antigens is a significant determinant in a tumor's immunogenicity, defined by a tumor's ability to provide protection from tumor challenge when given as a prophylactic

irradiated whole-cell vaccine. However whole-cell vaccines also contain many overexpressed self antigens, which may have the potential to induce and activate suppressive T_{reg} cells (330,331). T_{reg} cells play a central role in the maintenance of self-tolerance by suppressing immune responses to self-antigen, and work by our group and others has shown them to be a major impediment to anti-tumor immunity (31,332-334). Thus, booster vaccinations, commonly used with pathogen-directed vaccines, may in fact be ineffective when using tumor vaccines due to the generation of T_{reg} cells (254). Preclinical work with multiple vaccinations remains perplexing as some reports have shown booster vaccinations to be effective in the prophylactic settings (335,336), while others have found them to be detrimental to priming an anti-tumor immune response (334,337).

Multiple clinical studies utilizing booster vaccinations have shown modest objective responses with GM-CSF-secreting whole-cell cancer vaccines (338,339), but promising preclinical data showing the effectiveness of these types of vaccines has proven to be difficult to consistently translate to the clinic (340,341). GM-CSF stimulates the growth and maturation of dendritic cells, macrophages, neutrophils and eosinophils and used as an adjuvant it has been shown to recruit dendritic cells, a key source of tumor antigen phagocytosis and presentation, to the site of vaccination (277,342). Our group has shown that vaccinations with a GM-CSF-secreting clone of the B16BL6-D5 (D5) melanoma cell line primed tumor-specific, functional effector cells only when $CD4^+$ T cells were depleted during the first and second booster vaccinations (334). $CD4^+Foxp3^+$ T_{reg} cells were increased after multiple D5-G6 vaccinations but significantly diminished with $CD4^+$ depletion. Adoptively transferring T cells from $CD4^+$-depleted, multiply vaccinated mice into D5 tumor-bearing mice delivered effective therapy, while T cells from mice given multiple vaccinations without $CD4^+$ T cell depletion were much less effective.

The D5 melanoma is a poorly immunogenic tumor and thus single vaccinations without GM-CSF do not prime immune protection from subsequent tumor challenge (343). Therefore we sought to determine whether the efficacy of multiple (booster) vaccinations with GM-CSF-secreting vaccines is negatively impacted by poor tumor immunogenicity, or whether GM-CSF itself has an inhibitory effect with multiple vaccinations. To address this we utilized MCA-induced fibrosarcomas with differing immunogenicities (MCA-304 and MCA-310)(65), which we transfected to stably secret GM-CSF. Here we demonstrate that GM-CSF does not alter the protective immunity primed by a strongly immunogenic (MCA-304) or weakly immunogenic (MCA-310) whole-cell tumor vaccine, regardless of whether booster vaccinations are given. This work provides rationale for the continued use of booster vaccinations with GM-CSF-secreting tumor vaccines in combinatorial therapy regimens against solid tumors, and suggests that the addition of GM-CSF can increase T cell infiltration after multiple vaccinations.

Materials and Methods:

Mice and Tumor Cell Lines

Female C57BL/6J mice were purchased from Charles River Laboratories (San Diego, CA, USA) and maintained in a specific pathogen-free environment at the Earle A. Chiles Research Institute. Mice were 8-10 weeks old at the initiation of experiments. MCA-304 and MCA-310 are 3-methylcholanthrene (MCA)-induced fibrosarcoma cell lines of C57BL/6J mice. MCA-304GM and MCA-310GM are stable clones of MCA-304 and MCA-310, respectively, transduced with a murine granulocyte–macrophage colony-stimulating factor (GM- CSF) MFG plasmid. MCA-304GM cells secrete approximately 126 ng/10^6 cells/24 hours of GM-CSF, and MCA-310GM secretes approximately 135 ng/10^6 cells/24 hours.

GM-CSF Transfection

GM-CSF plasmid containing a puromycin-resistance gene was added at a ratio of 2:1 to transposase (both gifts from HM Hu, Earle A. Chiles Research Institute). Metafectene (Biontex Laboratories) in serum-free, antibiotic-free Complete Media (CM, consisting of (Lonza RPMI-1640 with 1% L-Glutamine (Lonza), 1% Sodium Pyruvate (Lonza), 1% Non-essential Amino Acids (Lonza), 0.1% Beta Mercaptoethanol) was added and incubated at 15 degrees Celsius for 30 mins. Plasmids were added to 50% confluent MCA-304 or MCA-310 cells in 6 well plate and incubated at 37° Celsius for 14 hours. 2 ug/mL of puromycin was added at 14 hours and 24 hours. Puromycin resistant clones were selected after 48 hours and grown in tissue culture flasks (Corning) with CM. GM-CSF secretion was confirmed by ELISA as described below.

Vaccination

Mice were vaccinated by subcutaneous injection with 10^7 irradiated (10,000 rads of gamma irradiation) tumor cells. Mice were injected with 2.5 x 10^6 cells into each of the four flanks. Triple vaccination groups were vaccinated on days 0, 14, and 28. Single vaccination groups were only vaccinated on day 28. All mice were sacrificed 10 days afterwards (day 38), and splenocytes were used for analysis and to generate effector T-cells for ELISA.

Tumor Challenge

Ten days after vaccination, mice were injected with 2 x 10^5 (twice the lethal dose) cells of live MCA-304 tumor or 2 x 10^4 (twice the lethal dose) cells of live MCA-310 subcutaneously on the flank. Tumors were measured thrice weekly with calipers, and tumor area was determined by multiplying height x width. Animals were sacrificed when tumor area was greater than 150 mm^2.

Effector T Cell Generation

Vaccinated splenocytes were harvested ten days subsequent to final vaccinations and effector T cells were generated as previously described (344). Briefly, single cell suspensions were prepared by washing spleens through cell strainers with Complete Media. Cells were activated for 2 days at 2 x 10^6 cells/mL in complete media in 24-well plates with 5 ug/mL of 2c11 antibody (anti-CD3) at 37 ° Celsius. T-cells were then harvested and expanded at 2 x 10^5 cells/mL in complete media with 60 IU/mL of Interleuken-2 (IL-2, Chiron) in LifeCell Tissue culture flasks (Baxter) for 3 days at 37 ° Celsius.

ELISA

2 x 10^6 activated/expanded (as described above) effector T cells were co-cultured with 2 x 10^5 target tumors (10:1 ratio) in 2 mL of complete media containing 60 IU/mL IL-2 in 24-well flat bottom plated (Corning) for 24 hours at 37° Celsius. Effector cells cultured with or without anti-CD3 (10 ug/well) were used as positive and negative controls, respectively. Supernatants were assayed by ELISA using purified rat anti-mouse IFN-γ antibodies at 2 μg/mL, rat anti-mouse anti-IFN-γ biotin-labeled antibodies at 1 μg/mL (BD Biosciences). Absorbance at 450 nm was read on a Wallac Victor2 plate reader. IFN-γ concentration was determined by linear regression using WorkOut 2.5 software (PerkinElmer). GM-CSF secretion of MCA-304GM was measured by ELISA using purified rat anti-mouse anti-GM-CSF antibodies at 2 μg/mL, rat anti-mouse anti-GM-CSF biotin-labeled antibodies at 1 μg/mL (BD Biosciences).

Intracellular FoxP3 Staining

10^6 splenocytes from single cell suspensions were surface stained using anti-CD4 APC-H7 (clone RM4-5, BD Biosciences), anti-CD8 V500 (clone 53-6.7, BD Biosciences), and anti-CD3 PerCP-efluor 710 (clone 17A2, eBioscience), followed by the manufacturer's protocol for fixation and permeabilization (ebioscience). Cells were then intracellularly stained using anti-FoxP3 efluor450 (clone FJK-16s, ebioscience). Samples were run on LSRII cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar). $CD3^+CD4^+FoxP3^+$ T_{reg} cells were gated through singlet lymphocytes, followed by $CD3^+$ cells, followed by $CD4^+$ cells.

Immunohistochemistry and Multispectral Imaging

Mice were vaccinated one or three times with 10^7 irradiated whole-cell vaccines of either MCA-310 or MCA-310GM seven days apart, and then challenged with a high tumor dose of 7

x 10^5 MCA-310 cells (> 70 time the lethal dose). Twelve days later tumors were harvested, Zinc fixed (0.1 M Tris buffer, pH 7.4, 0.5 g/L Calcium Acetate, 5.0 g/L Zinc Acetate, 5.0 g/L Zinc Chloride) for 48 hours, paraffin-embedded, and stained for CD3 (clone SP7), CD4 (RM4-5), CD8 (53-6.7), F4/80 (CI:A3-1), FoxP3 (FJK-16s), and DAPI with TSA amplification (Perkin Elmer). Tumors were imaged on a *Vectra* microscope (PerkinElmer) and quantified with *InForm* multispectral imaging analysis software (Perkin Elmer) using 2-5 T cell hot spot panels from each tumor and extrapolating to full sample size using the formula: cell count/area of tumor (pixels) * 4,000,000 (cells/mm^2). Pseudo H&E images were generated with *InForm* software from multispectral images.

Statistics

Statistical analysis was implemented using Prism6 (GraphPad Software). Gehan-Breslow-Wilcoxon test was performed for Kaplan-Meier survival curves, and $p < 0.05$ (denoted as *) was deemed significant. For ELISA, T_{reg}, and infiltrating lymphocyte experiments one-way (T_{reg} cells and tumor infiltrates) or two- way ANOVA with Tukey's multiple comparison test (ELISA) were utilized. All graphs show mean with standard deviation error bars.

Results:

Single vaccination-primed protection is maintained when given with multiple booster vaccinations

To address the question of whether tumor immunogenicity dictates multiple vaccination efficacy, we vaccinated naïve mice with irradiated, strongly immunogenic MCA-304 fibrosarcoma cells or weakly immunogenic MCA-310 either once or three times at 14 day intervals. They were then challenged 10 days later with twice the TD100 dose (tumor dose where 100 percent of unvaccinated mice grow tumors) of MCA-304 or MCA-310 (Figure 2-1A). As demonstrated previously, single vaccination with MCA-304 provided complete

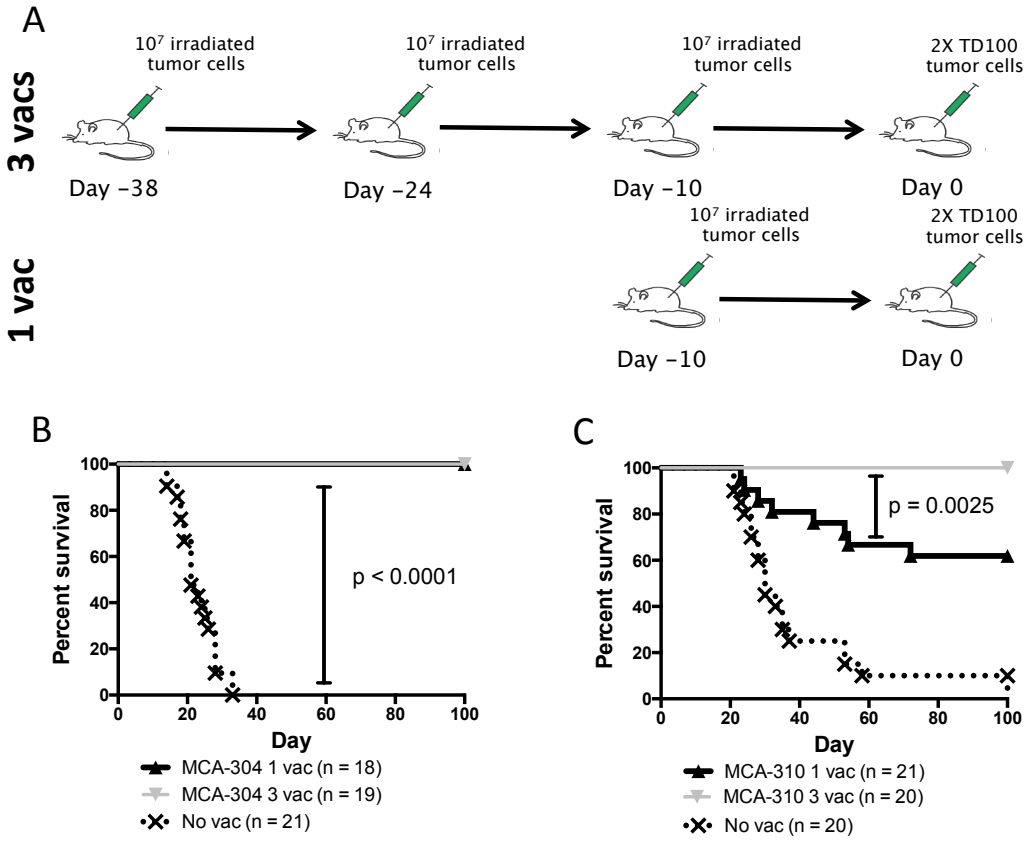

Figure 2-1: Multiple vaccinations maintain or augment single vaccination protection. (**A**) Experimental model of vaccination schedule. Naïve, C57BL/6 mice were give either one or three vaccinations of irradiated whole-cell MCA-304 vaccine (**B**) or MCA-310 vaccine (**C**). 10 days later mice were challenged with live tumor cells and monitored for tumor growth. Kaplan-Meier survival curve of three groups is shown. n = 18-21, three independent experiments combined. Gehan-Breslow-Wilcoxon tests were performed for statistical significance.

protection from tumor challenge, while MCA-310 vaccination resulted in only 60% protection from tumor challenge (Figure 2-1B, 2-1C). Triple vaccination with MCA-304 also provided complete protection from tumor challenge (Figure 2-1B). Three vaccinations with MCA-310 significantly augmented protection compared to single vaccination, also protecting 100% of mice from tumor challenge (Figure 2-1C). These data demonstrate that tumor immunogenicity does not impact the efficacy of multiple vaccinations with irradiated, whole-cell tumor vaccines.

Multiple vaccinations prime functional, tumor-specific IFN-γ-secreting effector T cells

We next sought to determine if the addition of GM-CSF to either tumor vaccine could boost the ability to prime tumor-specific effector T cells. We stably transfected a GM-CSF-encoding plasmid into MCA-304 and MCA-310 (MCA-304GM and MCA-310GM, respectively) and used these cells to compare multiple vaccinations with and without GM-CSF. The transfections did not diminish tumor cell growth rates (data not shown). To determine if irradiated tumor vaccines consisting of MCA-304GM and MCA-310GM were capable of secreting cytokine after irradiation, we irradiated cells with 10,000 rads and measured their in vitro GM-CSF secretion over 8 days. Both tumors secreted GM-CSF for at least the 8 days measured (peak of 20.7 ng/mL/10^6 cells/24 hours for MCA-304GM and an average of 14.9 ng/mL/10^6 cells/24 hours, and peak of 21.1 ng/mL/10^6 cells/24 hours and an average of 15.9 ng/mL/10^6 cells/24 hours for MCA-310GM)(Figure 2-2). The maximal level of GM-CSF secretion was similar to or exceeded the levels produced by the GM-CSF-secreting melanoma D5-G6 that we had previously used for studies on multiple vaccinations. Non-transfected MCA-304, MCA-310, and D5 tumor cells did not secrete GM-CSF (Figure 2-2).

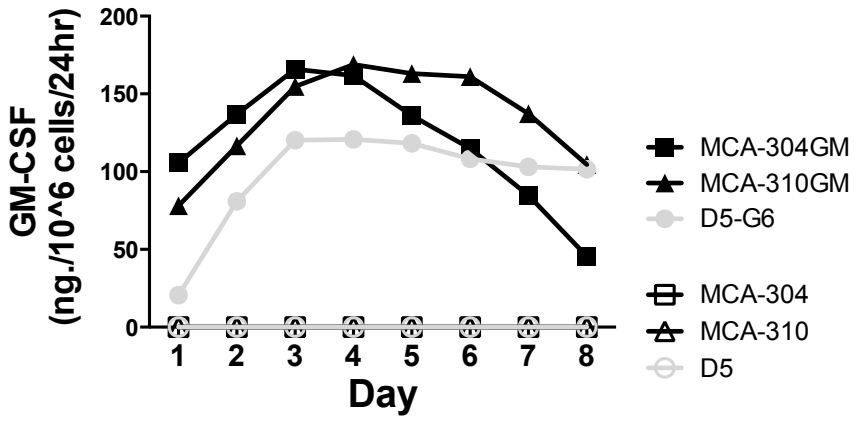

Figure 2-2: MCA-304GM and MCA-310GM secrete GM-CSF after irradiation. One million cells of three different C57BL/6 tumors, the melanoma D5-G6 and sarcomas MCA-304GM and MCA-310GM, were monitored for GM-CSF secretion for eight days post irradiation with 10,000 rads. Supernatants were collected daily and replaced with fresh media. Samples were assayed for GM-CSF via ELISA.

To confirm that a tumor-specific T cell response was primed by multiple tumor vaccinations, we in vitro activated and expanded splenocytes from animals vaccinated one or three times with MCA-304, MCA-304GM, MCA-310, or MCA-310GM. Splenocytes were harvested 10 days after the final vaccination (1st or 3rd vaccination) and cultured with anti-CD3 and then IL-2 for 2 days and 3 days, respectively. Effector T cells from MCA-304 triple vaccinated animals secreted a significantly higher quantity of MCA-304-specific IFN-γ compared to effector T cells taken from mice vaccinated with a single dose of either MCA-304 or GM-CSF-secreting MCA-304GM (Figure 2-3A). No vaccine strategy provided a significant response when effectors were cocultured with D5, an unrelated syngeneic melanoma tumor target, demonstrating the specificity of the anti-tumor IFN-γ secreted. Surprisingly, T cells from MCA-304 triply vaccinated mice generated significantly more tumor-specific IFN-γ than T cells from mice receiving triple vaccination of GM-CSF-secreting MCA-304GM. We saw a similar pattern of tumor-specific IFN-γ when comparing effector cells primed with triple vaccinations of MCA-310 or MCA-310GM (Figure 2-3B). Taken together, these data suggest that while triple vaccination increased the amount of splenic T

cell-derived tumor-specific IFN- γ, the addition of GM-CSF to vaccination altered splenic T cells functionality to tumor targets.

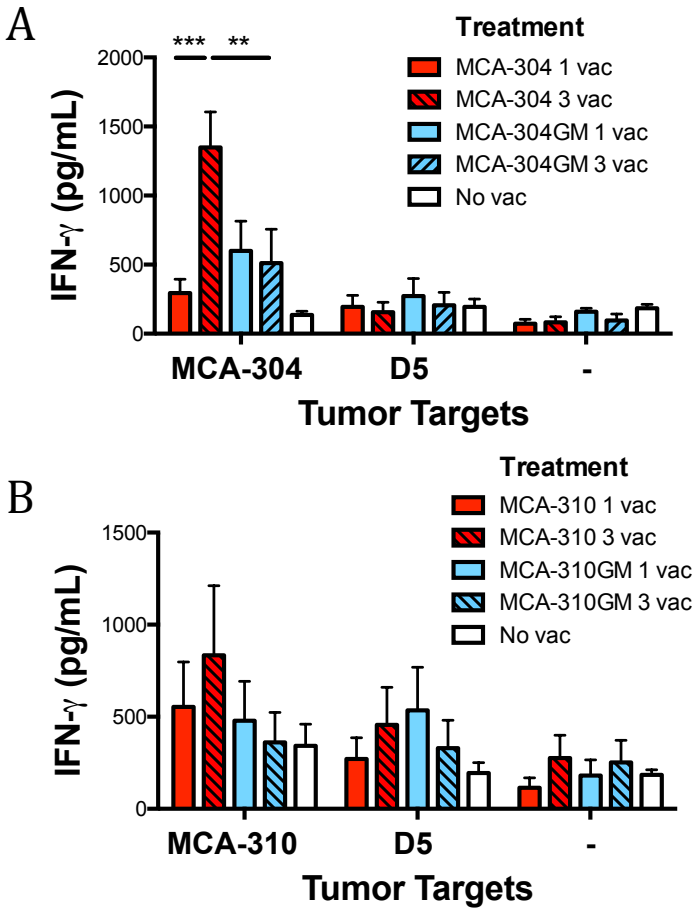

Figure 2-3: Multiple vaccinations of GM-CSF-secreting tumors result in reduction in tumor-specific IFN-γ. Splenocytes from mice singly or triply vaccinated with MCA-304 or MCA-304GM (**A**) and MCA-310 or MCA-310GM (**B**) were pooled from groups of 3-5 mice and activated/expanded on anti-CD3 for 2 days and IL-2 for 3 days. Activated T cells were cocultured with specific (MCA-304 (A) or MCA-310 (B)) or non-specific, syngeneic tumor targets (D5) for 24 hours and supernatants were analyzed for IFN-γ by ELISA assay. n = 5-6, Two combined independent experiments, mean with SEM is shown. Two-way ANOVA with Tukey's post hoc test were performed for statistical significance. ** = $p < 0.01$, *** = $p < 0.001$

Addition of GM-CSF maintains protection provided by three vaccinations

We previously demonstrated an attenuation of therapeutic efficacy from triple vaccinations compared to single vaccination using the GM-CSF-secreting weakly immunogenic melanoma D5-G6. Since tumor immunogenicity appeared to play no significant roll in multiple

vaccination efficacies with our sarcoma tumors, we investigated whether the addition of GM-CSF would alter efficacy with multiple vaccinations. To address this question we vaccinated naïve mice one or three times with either irradiated MCA-304 or MCA-310, or their GM-CSF-secreting counterparts MCA-304GM or MCA-310GM. Animals were challenged with MCA-304 or MCA-310 as described above in Figure 2-1A. Surprisingly, given the suppression we saw with triple vaccination of GM-CSF-secreting D5-G6 (334) and the

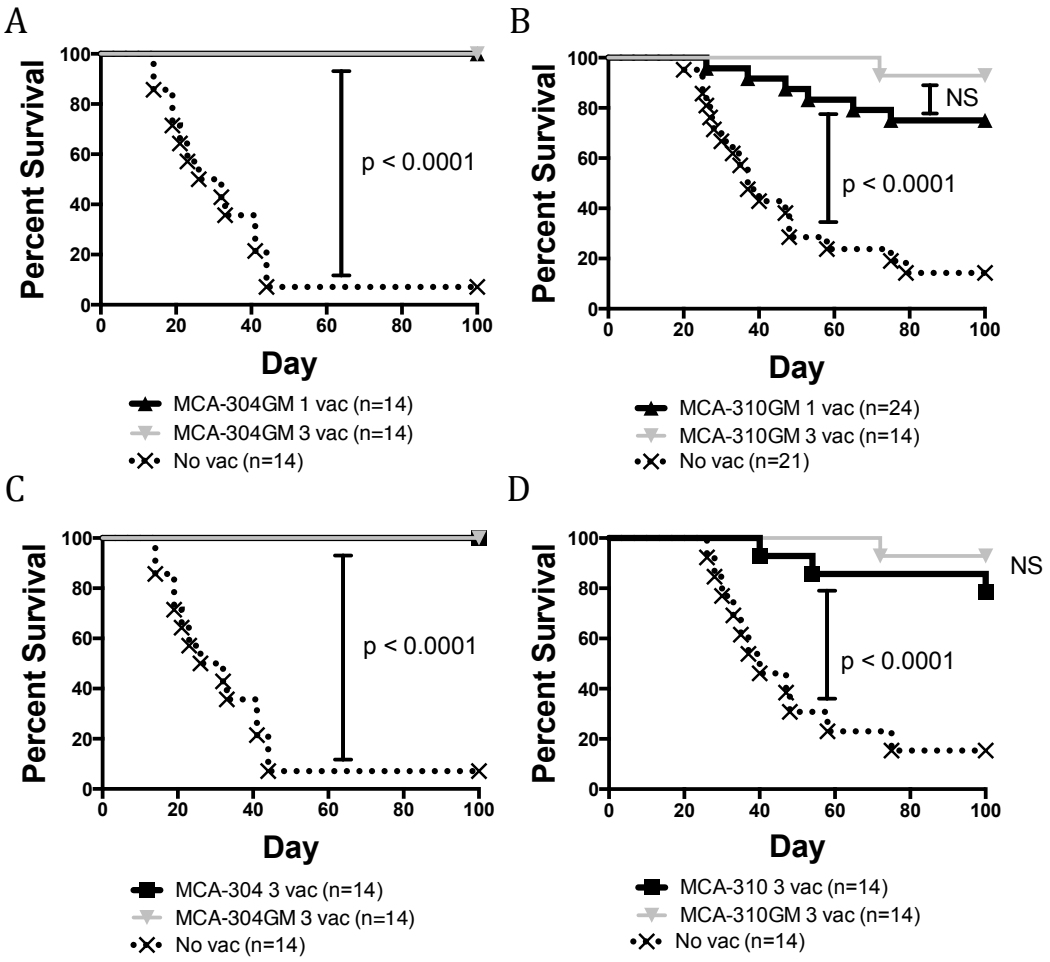

Figure 2-4: Multiple vaccinations of GM-CSF-secreting tumors maintain single vaccination protective immunity. Naïve, C57BL/6 mice were give either one or three vaccinations of irradiated whole-cell MCA-304GM (**A**) or MCA-310GM (**B**) vaccine, as described in Figure 2-1A). 10 days later mice were challenged with live tumor and monitored for tumor growth. Three vaccinations with GM-CSF-secreting tumors were compared to three vaccinations with non-GM-CSF-secreting MCA-304 (**C**) or MCA-310 (**D**). Kaplan-Meier survival curve of three groups is shown. n = 14-24, two independent experiments are combined. Gehan-Breslow-Wilcoxon tests were performed for statistical significance.

significant differences in tumor-specific IFN-γ-secretion from splenic effector T cells (Figure 2-3), single and triple vaccination with GM-CSF-secreting MCA-304GM maintained the complete protection provided by MCA-304 (Figure 2-4A, 2-4C). In concurrence with previous experiments (Figure 2-2), either one or three vaccinations with parental MCA-304 provided complete protection from tumor challenge (Figure2-2, 2-

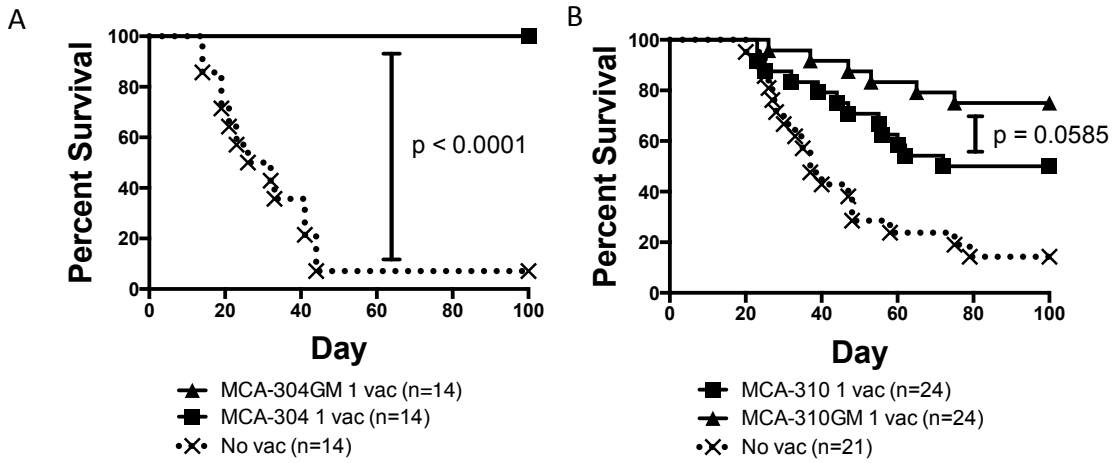

Figure 2-5: Addition of GM-CSF maintains or improves single vaccination efficacy with MCA tumors. Naïve, C57BL/6 mice were give either one vaccination of irradiated whole-cell MCA-304 or MCA-304GM (**A**) or MCA-310 or MCA-310GM (**B**) vaccine, as described in Figure 2-1A. 10 days later mice were challenged with live tumor and monitored for tumor growth. n = 14-24, combination of two (**A**) and three (**B**) independent experiments. Gehan-Breslow-Wilcoxon tests were performed for statistical significance.

4C). Three vaccinations of GM-CSF-secreting weakly immunogenic tumor (MCA-310GM) also resulted in maintenance of protection, with no statistical difference compared to three vaccinations of MCA-310 (Figure 2-4B). However, with a single vaccination the addition of GM-CSF (MCA-310GM) only marginally increased (p = 0.058) protection compared to MCA-310 singly vaccinated mice (Figure 2-5B). These results demonstrate that the addition of GM-CSF does not alter multiple vaccination efficacies in these models.

Triple vaccination with and without GM-CSF does not increase regulatory T cells

We previously demonstrated a substantial increase in total T_{reg} cells with multiple vaccinations of GM-CSF-secreting D5-G6 melanoma. Thus, we asked whether the addition of

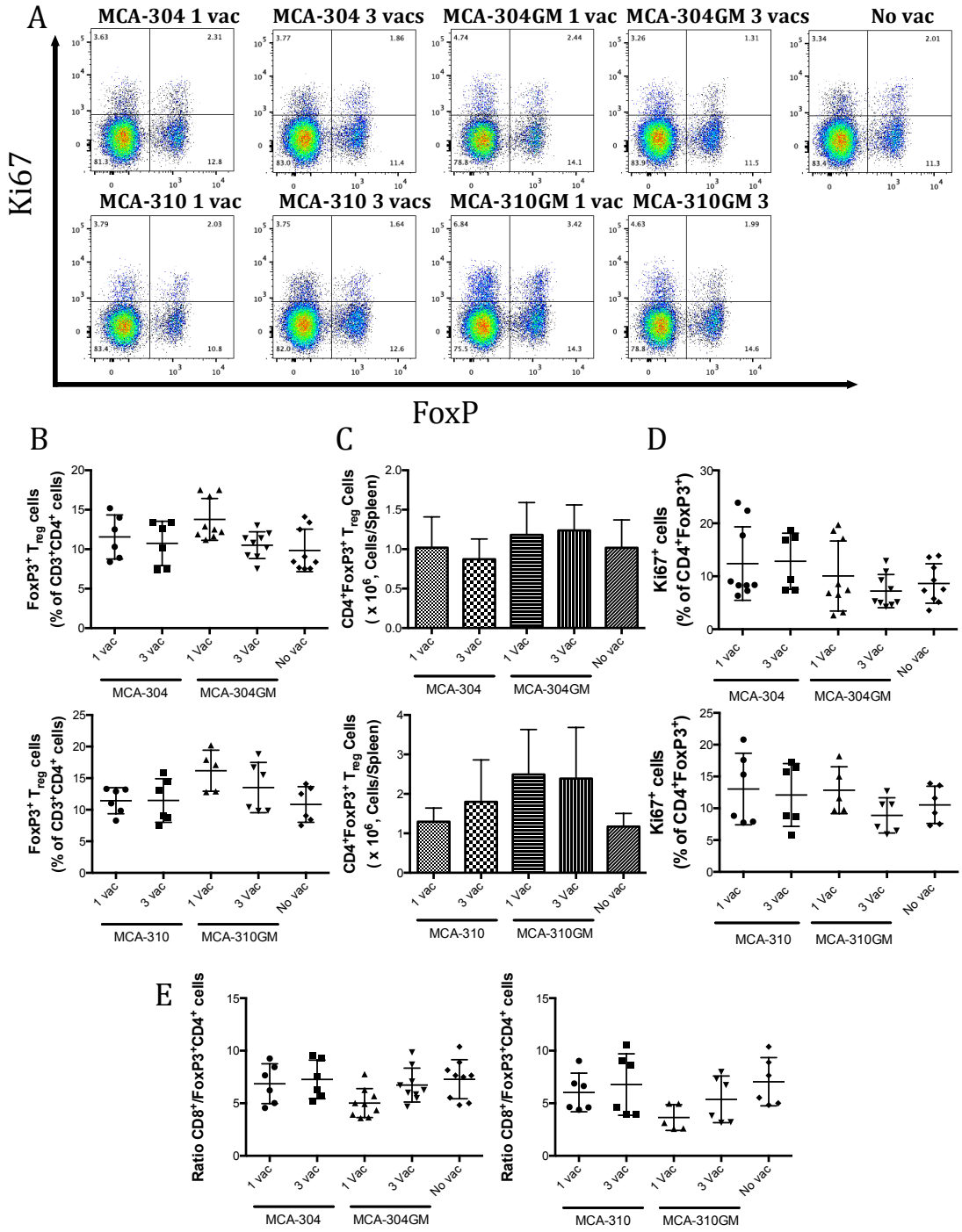

Figure 2-6: Multiple vaccinations with MCA-induced tumors do not increase regulatory T cells. Mice were vaccinated with MCA-304, MCA-310, MCA-304GM or MCA-310GM once or thrice (as described in Figure 2-1A), and 10 days after final vaccination splenocytes were taken and stained for T_{reg} cells ($CD3^+CD4^+FoxP3^+$). (**A**) Representative dot plots of $CD3^+$-gated T cells are shown. (**B**) Fox $CD3^+CD4^+FoxP3^+$ cells (as a percentage of $CD3^+CD4^+$) are quantified. (**C**) Total T_{reg} cells are quantified. (**D**) $Ki67^+$ T_{reg} cells (as a percentage of $CD4^+FoxP3^+$) are quantified. (**E**) Ratio of $CD3^+CD8^+$ cells to $CD3^+CD4^+FoxP^+$ T_{reg} cells are quantified. n = 6-9, two or three independent experiments combined. One-way ANOVA with Tukey's post hoc tests were performed for statistical significance.

GM-CSF or tumor immunogenicity significantly increased T_{reg} cells when administering three vaccinations of strongly immunogenic MCA-304 or weakly immunogenic MCA-310 with and without GM-CSF. Surprisingly we saw no significant changes in T_{reg} cell percentage, total T_{reg} cells, or proliferating $Ki67^+$ T_{reg} cells in the spleen after vaccination (Figure 2-6A-D) and no significant difference when comparing the ratios of $CD8^+$ T cells: T_{reg} cells between any of the groups (Figure 2-6E). In combination these data suggest that multiple vaccinations with MCA-induced sarcomas with or without GM-CSF does not induce T_{reg} cells in the periphery over the levels seen in a singly vaccinated or naïve animal.

GM-CSF increases T cell infiltrate in MCA-310 triply vaccinated tumors

Given the impact GM-CSF had on tumor-specific effector cells in the spleen of triple vaccinated mice, we investigated if GM-CSF altered T cell infiltration in the tumors of vaccinated mice. To determine the impact multiple GM-CSF-secreting vaccinations had on tumor-infiltrating lymphocytes (TIL), we prophylactically vaccinated mice with either irradiated MCA-310 or MCA-310GM in an abbreviated single or triple vaccination schedule (7 days apart), and then challenged with a very high dose of tumor (over 70 times the TD100 dose). The high dose was necessary to establish tumors for analysis as a majority of the triple vaccinated mice completely eliminate the lower dose tumor challenges used previously (Figure 2-4C, Figure 2-4B). Ten days later we resected the tumor and performed multispectral immunohistochemical analysis for T cells. All tumors, including those from unvaccinated mice, had some level of $CD3^+$ T cell infiltrate and a substantial $F4/80^+$ myeloid cell infiltrate (Figure 2-7A). But when we quantified TIL, we noted a significant increase in $CD3^+$ T cells in tumors from mice vaccinated with GM-CSF-secreting tumors, compared to those vaccinated with non-GM-CSF-secreting tumors (Figure 2-7B). Vaccination without GM-CSF did not lead to a significant increase in intratumoral $CD3^+$ T cells. Upon further

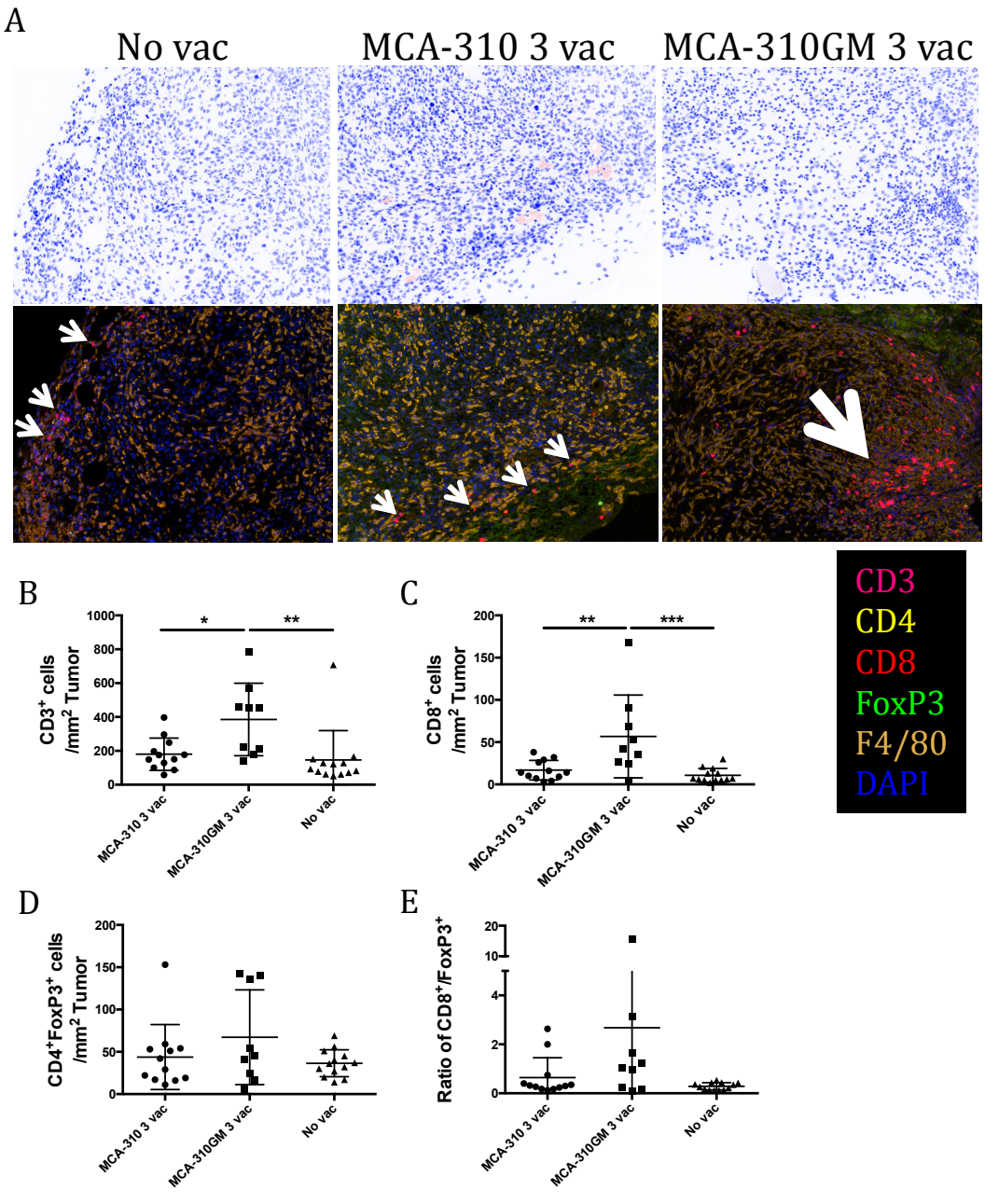

Figure 2-7: GM-CSF increases tumor infiltrating lymphocytes in multiply vaccinated MCA-310 tumors. Mice were vaccinated three times with either MCA-310 or MCA-310GM seven days apart and then challenged with tumor. Ten days later tumors were excised, fixed, and multispectral immunohistochemistry was performed. (**A**) Representative H&E (top) and multispectral images (bottom) of tumors from each treatment group. Tumors were stained for CD3 (magenta), CD4 (yellow), CD8 (red), FoxP3 (green), F4/80 (orange), and DAPI (blue). White arrows indicate $CD3^+$ T cells. (**B**) $CD3^+$ T cells were quantified from a combination of 3 independent experiments combined, total n = 9-13. (**C**) $CD8^+CD3^+$ T cell quantification (**D**) $CD4^+FoxP3^+CD3^+$ T_{reg} cell quantification. (**E**) Ratio of $CD8^+$: $CD4^+FoxP3^+$ cells. n = 9-13, combination of three independent experiments. One-way ANOVA with Tukey's post hoc tests were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

phenotypic analysis, $CD3^+CD8^+$ T cells were also significantly more prevalent in the MCA-310GM triple vaccinated tumors than those from unvaccinated or triple vaccinated with MCA-310 (Figure 2-7C). We also noted no difference in $CD3^+$ $CD4^+FoxP3^+$ T_{reg} cells (Figure 2-7D). However, despite the increase in $CD8^+$ T cells in the tumor, the ratio of $CD8^+$:T_{reg} cells was not significantly increased with the addition of GM-CSF (Figure 2-7E).

Given our previous findings showing a strong inhibitory role for T_{reg} cells with triple vaccinations of the D5-G6 melanoma, we used multispectral imaging to also analyze TIL from D5 tumor-bearing mice after multiple vaccinations of either irradiated D5 or GM-CSF-secreting D5-G6. In this model, triple vaccination with D5-G6 increased $CD3^+$ T cells numbers in the tumor (Figure 2-8A), as well as significantly boosted numbers of $CD3^+CD8^+$ T cells (Figure 2-8B), and increased $CD3^+CD4^+FoxP3^+$ T_{reg} cells compared to triple vaccination with D5 tumor (Figure 2-8C). Fitting with our previous findings using multiple vaccinations of these tumors, GM-CSF appeared to slightly reduce the CD8+:T_{reg} ratio of multiply vaccinated mice (Figure 2-8D). This was the opposite of the pattern we noted in the MCA-induced sarcoma model (Figure 2-7E). Thus we conclude that recruitment of T_{reg} cells to the tumor with multiple vaccinations of GM-CSF-secreting vaccines is likely tumor model-dependent, but not inherently dependent on tumor immunogenicity as both D5 melanoma and MCA-310 sarcomas are not strongly immunogenic tumors.

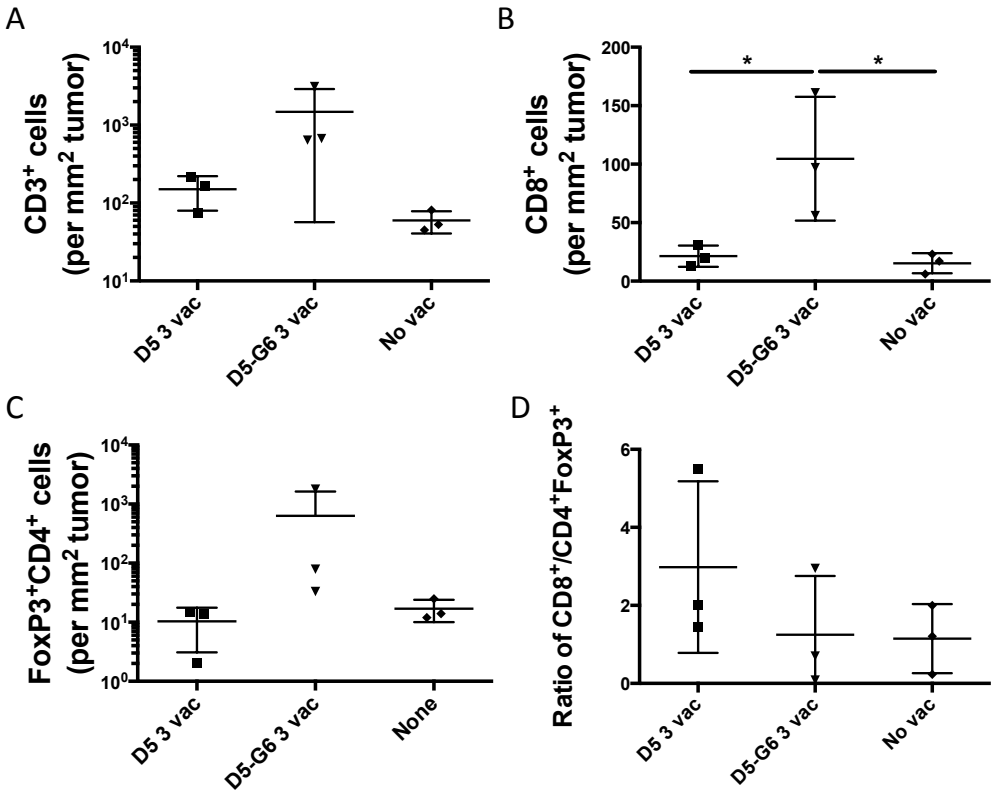

Figure 2-8: Multiple vaccinations with GM-CSF-secreting D5 tumor increases T cell infiltration, but not CD8:T_{reg} ratio. Mice were vaccinated three times with either D5 or GM-CSF-secreting D5-G6 seven days apart and then challenged with D5 tumor. Ten days later tumors were excised, fixed, and multispectral immunohistochemistry was performed. (**A**) $CD3^+$ T cells were quantified n = 3. (**B**) $CD8^+CD3^+$ T cell quantification (**C**) $CD4^+FoxP3^+CD3^+$ T_{reg} cell quantification. (**D**) Ratio of $CD8^+$:$CD4^+FoxP3^+$ cells. One-way ANOVA with Tukey's post hoc tests were performed for statistical significance, * = $p < 0.05$

Discussion:

While many groups have utilized booster vaccinations with mixed results in the clinical setting (297,345,346), few preclinical studies have investigated the efficacy of booster vaccinations with cancer vaccines. Here we demonstrate that tumor immunogenicity does not inherently impact the efficacy of multiple vaccinations with whole-cell tumor vaccines. Our results confirm that GM-CSF-secreting (GVAX) vaccines are not intrinsically incompatible with booster vaccinations in the preclinical setting. This establishes that multiple boosts of the self-peptides present in either strongly or weakly immunogenic MCA-induced whole-cell tumor vaccines are not sufficient to attenuate vaccine efficacy, which is unlike what we had previously noted in the D5 melanoma model (334).

Given the recent use of GVAX vaccines in a variety of clinical trials (279,347,348) and our previous work showing an attenuation of single vaccine priming efficacy with multiple vaccinations of the poorly immunogenic, GM-CSF-secreting tumor D5-G6, we also sought to determine if GM-CSF was detrimental when boosting with a strongly immunogenic tumor vaccine. While other studies have indicated that a high dose of GM-CSF (6000 ng/10^6 cells/24hr) can lead to immune suppression (349), the much lower levels of GM-CSF secreted by MCA-304GM and MCA-310GM (150 ng/10^6 cells/24hr and 160 ng/10^6 cells/24hr, respectively) proved to maintain or augment single vaccine efficacy based on survival data. Despite the immune protection provided by single vaccination, we surprisingly saw a reduction in the tumor-specific IFN-γ secreted by splenic T cells from mice that were triply vaccinated with GM-CSF-secreting tumors (Figure 2-3). Yet these triply vaccinated animals were equally protected from tumor challenge as mice that received triple vaccination without GM-CSF (Figure 2-4). Thus we reasoned that tumor-specific T cells must be present elsewhere in the circulation or tissue and were trafficked to

alternative locations at the particular time point we sampled. In support of this hypothesis, triple vaccination with GM-CSF-secreting MCA-310 resulted in an increase of tumor-infiltrating $CD3^+$ T cells compared to triple vaccination without GM-CSF (Figure 2-7). Importantly the addition of GM-CSF to the tumor vaccine also significantly increased $CD8^+$ T cells in the tumor. Numerous preclinical studies have demonstrated $CD8^+$ T cells to be critical for T cell-mediated tumor rejection, and others have identified $CD8^+$ T cells in the tumor as a marker for improved clinical outcome (250). So while splenic T cells primed in the presence of GM-CSF were less functional against tumor-specific targets, they ultimately trafficked to the tumor site in greater numbers. We hypothesize that combining multiple vaccinations with GM-CSF alters T cell trafficking, priming T cells that potentially remain in the blood or peripheral organs, and perhaps this allows for their more abundant recruitment to the tumor. Supporting this hypothesis, Ali et al. demonstrated that intratumoral injection of a GM-CSF-encoding herpes virus was able to generate T cells better able to traffic to the tumor rather than stay in the periphery circulation after adoptive transfer into a tumor-bearing host (350).

We previously reported a substantial increase in total splenic T_{reg} cells with multiple vaccinations of D5-G6 melanoma (334), but the role that GM-CSF played in this increase in T_{reg} cells remained an unexplored variable. Here we report that when administering our MCA-induced sarcoma vaccines with or without GM-CSF we saw no shift in percentages or totals of splenic T_{reg} cells, no difference in tumor challenge protection, and no change in tumor infiltrating T_{reg} cells (Figure 2-6). These results are congruent with previous reports, which showed no increase of T_{reg} cells with GM-CSF-secreting vaccines in other preclinical tumor models using moderately and strongly immunogenic tumors (296,351). In contrast, we only saw an increase in tumor-infiltrating T_{reg} cells with triple vaccination of GM-CSF-secreting D5-G6 melanoma compared to triple vaccination with non-GM-CSF-secreting D5

tumor (Figure 2-9). While it has been suggested that non-MCA-induced murine tumors like D5 and A20HA B cell lymphoma rely on T_{reg} cell induction for their immune evasion (54,334), the induction of T_{reg} cells may not be a critical mechanism that MCA-induced sarcomas (or at least the ones we tested) utilize or induce for immune evasion and tumor growth. Alternatively MCA-induced irradiated tumor vaccines may be unable to produce or induce the cytokine milieu necessary for T_{reg} induction, or may contain high affinity, mutated dominant tumor antigens that may not strongly induce T_{reg} cells, and thus have to rely on other mechanisms of immune suppression.

A publication by Ricupito et al. used a dendritic cell-peptide vaccine to demonstrate that homologous boosting does augment single vaccination (335). Yet, the dendritic cell-Tag-IV peptide vaccines used in this report greatly differ from the whole-cell tumor vaccines utilized in our work. Tag-IV peptide vaccines offer a very limited antigenic repertoire with far less self-peptides and a reduced potential for tolerance induction. Therefore we anticipated a different effect of homologous boosting with whole-cell vaccines in our experiments. We were surprised to see that our results supported their work and showed maintenance of vaccine efficacy with multiple booster vaccinations (Figure 2-1B and 2-4). Ricupito et al. also demonstrated the importance of correctly timing vaccine boosts, with a "tight" boost schedule (14 days apart, 1 boost) less effective than a "loose" boost schedule (28 days apart, 1 boost). Our vaccination schedule (14 days apart, 2 boosts) resembles the "tight" boost schedule, but the differences in vaccine types may explain the variation between their results and our own. Unlike peptide vaccines, whole-cell vaccines will likely prove more effective against tumors that experience antigen loss and have a better chance to prevent tumor escape via this mechanism.

Here we report that differing tumor immunogenicity of MCA-induced whole-cell tumor vaccines does not impact their protective efficacy with booster vaccinations. In

addition, supplementing the vaccine with GM-CSF did not alter protection, but importantly it did increase T cell trafficking into the tumor. In this regard, strategies entailing multiple vaccinations with tumors that do not elicit a T_{reg} cell response (perhaps due to increased mutational burden of the tumor) appear to offer an effective means of priming a tumor-specific immune response. Furthermore these tumor vaccines may be augmented with the addition of GM-CSF, as GM-CSF was not sufficient to induce T_{reg} cells. This insight should help guide tumor vaccine development and improve combinations with novel immunotherapies that are able to help augment and guide an immune response to produce the optimal anti-tumor effect.

CHAPTER 3: TIMING IS CRITICAL TO SUCCESFUL COMBINATION OF ANTI-OX40 AND PD-1 BLOCKADE

Abstract:

Recently, combination immunotherapy with two antibodies specific for inhibitory checkpoints, PD-1 and CTLA-4, was shown to increase progression-free survival compared to either agent alone in patients with advanced melanoma. These data have fueled interest in novel cancer immunotherapy combinations. Our group has focused on strategies to augment priming and amplify anti-cancer immunity using an agonist antibody against the OX-40 costimulatory receptor. As a single agent anti-OX40 has a significant therapeutic effect in the orthotopically-transplanted MMTV-PyMT mammary cancer model. However when combined concurrently with anti-PD-1 blockade, the combination significantly attenuated the therapeutic effect of anti-OX40 alone. In combination-treated mice we noticed considerable increases in both type 1 and type 2 serum cytokines and significantly augmented expression of inhibitory receptors LAG-3, TIM-3, and CTLA-4 as well as PD-L1 on T cells. In the tumor, combination treatment augmented T cell proliferation, but this was short lived and only days later T cells had significantly reduced proliferation compared to untreated mice. Since PD-1 naturally plays a role in immune contraction after T cell activation, we evaluated the effect of sequentially administering anti-OX40 followed by anti-PD-1. Sequential combination resulted in a significant increase in therapeutic efficacy compared to concurrent combination or monotherapy, complete long-term regression in ~30% of treated animals, and was dependent on both $CD4^+$ and $CD8^+$ T cells. These results highlight the importance of timing for optimized therapeutic effect with combination immunotherapies and suggest the testing of sequencing in clinical trials with combination immunotherapies.

Introduction:

The potential for immunotherapy to improve outcomes of patients with cancer, particularly through the combination of agents targeting immune inhibitory pathways, is becoming increasingly evident (212,213). Nonetheless, how to optimally combine the myriad of new immunotherapy agents currently being developed is a major question in cancer research. Antibodies targeting the Programed Cell Death protein-1 (PD-1, CD279) receptor have made a major therapeutic impact on multiple types of solid tumors (136). Given its relatively low levels of reported toxicity combined with therapeutic efficacy, PD-1 pathway blockade is currently the building block for testing clinical combinations with other immunotherapeutics. PD-1 is an inhibitory molecule upregulated after T Cell Receptor (TCR) engagement that normally plays a major role in immune response contraction, leading T cells to exhaustion and apoptosis (136,182,352-354). Cancer, however, can use the PD-1 pathway to its advantage by expressing Programmed Death-Ligand 1 (PD-L1, B7-H1, CD274) on a tumor's surface or inducing it on the surface of other tumor-associated immune cells like macrophages or dendritic cells to suppress an anti-tumor immune response, making the PD-1 receptor an attractive target for immunotherapeutic intervention (190,196,201). By blocking either the PD-1 receptor or its ligand PD-L1, exhausted tumor-specific effector T cells can then be reinvigorated to enhance their function (355).

OX40 (CD134 or TNFRsF4) is a TNF family costimulatory receptor that T cells express upon TCR stimulation (15,153). OX40 stimulation with OX40 ligand (CD252) expressed on activated APCs, leads to increases in activation, proliferation, differentiation, and survival (15,154,156,157). $CD4^+FoxP3^+$ T_{reg} cells constitutively express OX40, but activated, conventional $CD4^+FoxP3^-$ T cells also express OX40 (154). Although OX40 is mainly expressed on $CD4^+$ T cells, $CD8^+$ T cells do express the receptor, and they can also

upregulate OX40 again shortly after re-activation, which makes it an attractive target for exhausted cells in the tumor microenvironment (155). In preclinical models, anti-OX40 agonist antibodies have shown anti-tumor effects when given as monotherapy (158-160) and these effects have been attributed to costimulation of both $CD4^+$ and $CD8^+$ cells (155,161,162). OX40 costimulation has also demonstrated enhancement of anti-tumor effects when combined with checkpoint blockade (anti- CTLA-4) and either adjuvants, vaccination, or radiation (163,165,166). Given this preclinical data, clinical trials administering anti-OX40 as a monotherapy are currently underway in a variety of solid malignancies (167).

Breast cancer is the most commonly diagnosed cancer in women and improvements in patient survival rates over recent decades have been made with conventional therapies such as radiation and chemotherapy and targeted therapies like the anti-HER-2 drug trastuzumab. Nevertheless a substantial portion of patients remain refractory to these conventional treatments and over the last decade a multitude of preclinical studies demonstrating immunotherapy-mediated tumor regression, including regression with anti-OX40 (158), has renewed interest in utilizing immunotherapies in breast cancer and spawned a wide variety of clinical trials. Adding to this interest, tumor-infiltrating lymphocytes (TIL) have been shown to associate with good clinical outcome (356) and response to therapy (357). Given that PD-L1 expression on human breast cancer tumors is associated with worse prognosis (358), preliminary results of clinical trials with PD-1 blockade have shown objective responses in specific subsets of breast cancer (211). However, overall responses have been modest (5-19%), leaving a majority of patients refractory to monotherapy. OX40 expression has also been shown on breast cancer tumor infiltrating lymphocytes (158,359) and polymorphisms in OX40L (CD252) are associated with breast cancer carcinogenesis (360), suggesting that antibodies targeting these two

receptors could be effective when given in combination. Therefore we sought to combine anti-OX40 and PD-1 blockade in preclinical mammary cancer models, with the hope of further boosting the anti-tumor effect provided by anti-OX40 or anti-PD-1 monotherapy.

To address the anti-tumor effects and immunological consequences of combining immunotherapies that target both a costimulatory and inhibitory receptor, we utilized murine mammary tumor virus polyoma middle T (MMTV-PyMT) mice, a preclinical model of oncogene-driven mammary cancer (306). This model resembles luminal human breast cancer and is infiltrated with myeloid and T cells (310), making it a strong candidate for immunotherapy with T cell-targeting antibodies. Here we report that this model is refractory to PD-1 blockade, but by stimulating the OX40 receptor with an agonist antibody we significantly delayed tumor progression. However concurrent combination of anti-OX40 and PD-1 blockade diminished this effect. Along with this weakened anti-tumor effect, we noted an acute increase in serum cytokines with combination treatment, and heightened expression of T cell inhibitory receptors.

Materials and Methods:

Mice and Tumor Cell Lines

FVB/NJ MMTV-PyMT mice were provided by E. Akporiaye (Earle. A Chile Research Institute, Portland, OR). Female FVB/NJ and BALB/c mice of 6-8 weeks were purchased from Jackson Laboratories. Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 2011), and the Earle A. Chiles Research Institute Animal Care and Use Committee approved all animal protocols. All mice were maintained in a specific pathogen–free environment. The 4T1 mammary tumor cell line was acquired from ATCC. The C57MG mammary tumor used for making DRibbles vaccine was received as a kind gift from P. Mukherjee (UNC Charlotte, Charlotte, NC). Tumor cells were cultured in complete medium (CM), which consisted of RPMI 1640 (BioWhittaker) supplemented with 10% FBS (Atlas Biologicals), 50 μM 2-mercaptoethanol (Aldrich), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine and 50 μg/mL gentamicin sulfate (Lonza). Cell lines were maintained in T-225 or T-150 culture flasks (Corning) in a 5% CO_2 incubator at 37°C.

Animal Experiments

As previously described, tumors from MMTV-PyMT mice were digested with triple enzymes (Collegenase, DNAase, Hyaluronidase), agitated for 30 minutes, counted, centrifuged, and resuspended in Matrigel Matrix (Corning) (166,316). 10^6 cells were orthotopically transplanted into the mammary fat pad of naïve FVB/NJ mice (day 0). Seven days later mice were randomized into groups and injected intraperitonealy (i.p.) with either 100 μg of anti-OX40 (clone OX86), 250 μg anti-PD-1 (clone G4)(201), both (for concurrent treatment), or nothing on days 7, 9, and 11. Anti-OX40 and anti-PD-1 were a generous gift from A.D. Weinberg (Earle A. Chiles Research Institute, Portland, OR). For delayed anti-PD-1 treatment mice were given anti-OX40 on days 7, 9, and 11 followed by anti-PD-1 on days 13,

15, and 17. For delayed checkpoint blockade 250 μg anti-TIM-3 (clone RMT3-23, BioXcell) with or without 100 μg anti-CTLA-4 (clone 9D9, BioXCell) were given on days 13, 15, and 17 after anti-OX40 plus and anti-PD-1 combination on days 7, 9, and 11. For experiments combining anti-OX40 with anti-PD-L1, 200 μg of anti-PD-L1 (clone 10F.962, BioXcell) was substituted for anti-PD-1 on the same time course. For 4T1 experiments, 5 x 10^3 4T1 tumor cells were transplanted in the mammary fat pad of naïve BALB/c mice. Five days later mice were randomized and given either anti-OX40, anti-PD-1, or both on days 5, 7, and 9 (concurrent) or anti-OX40 on days 5, 7, and 9, followed by delayed anti-PD-1 on days 11, 13 and 15. For vaccination experiment mice were given either an inguinal lymph node inter-nodal injection of PBS (sham surgery) or 10 μg of autophagosome-enriched DRibbles vaccine derived from C57MG tumors. Vaccinated mice were given either anti-OX40 plus anti-PD-1 on days 5, 7, and 9 (concurrent) or anti-OX40 on days 5, 7, and 9, followed by delayed anti-PD-1 on days 11, 13 and 15. Tumors were measured thrice weekly, and mice were sacrificed when tumors reached 150 mm^2 (measured width x length). For lung metastases experiments, mice were sacrificed when primary tumor reached 150 mm^2. Lungs were inflated with India ink solution (15% diluted in DI water, with 20 drops of NH_3OH), fixed in Fekete's solution (85% 70% EtOH, 10% formaldehyde, 5% glacial acetic acid), and surface, macroscopic metastases enumerated 48 hours later by eye.

Autophagosome-enriched (DRibbles) vaccine

As previously described in *Twitty* et al. (265), autophagosome-containing vesicles (DRibbles) were harvested from cultured C57MG mammary tumor cells after treating cells with 100 nM Bortezomib (Velcade) and 10 mM of NH_4Cl in CM for 18-24 hours in a 5% CO_2 incubator at 37°C (265). The cells and the supernatant were harvested and spun at 480 x g.

The supernatant was then spun at 12,000 x g to harvest the autophagosome-containing pellet. DRibbles were resuspended at 0.5 or 1.0 mg/ml in hetastarch .

CD4/CD8 Depletion

To evaluate the necessary role of distinct cytotoxic immune cell populations in combination therapy, mice were injected i.p. with 250 μg anti-CD4 (GK1.5, BioXcell), anti-CD8 (53-6.7, BioXcell), or rat IgG (BioXcell) twice, one week apart (days 6 and 13 post tumor transplant). Mice were analyzed for confirmation of depletion by staining with different clones targeting CD4 or CD8 at 1 μg/ml for whole blood, spleen, and tumor.

Serum cytokines

For serum cytokine analysis, whole blood from treated and non-treated, PyMT tumor-bearing animals was collected on days 7, 9, 11, 13, 14, 17, and 20 post tumor transplant. Serum was isolated from whole blood by 30 minutes incubation at 37°C, 16 hours at 4°C, and 15 minute centrifugation at 2400 RCF. Serum was stained with the Th1/Th2/Th17 Cytokine Bead Array kit (BD Biosciences) according to the manufacturer's instructions, and run on an LSRII flow cytometer (BD Biosciences).

Flow Cytometry

Surface and intracellular receptors were measured from single cell suspensions of day 13 or 14 or day 19 splenocytes and tumor digest from treated and untreated MMTV-PyMT tumor-bearing mice. Cells were stained for CD3, CD8, CD4, CD45.1, ICOS, CD137 (4-1BB), GITR, PD-1, PD-L1, TIM-3, LAG-3, BTLA, CD11b, MHC II (I-A/I-E), and live/dead (Propidium Iodide, PI). All antibodies were obtained from eBioscience, Molecular Probes, or BD Biosciences. Intracellular staining of FoxP3, Ki67, and CTLA-4 was performed with FoxP3

Fixation/Permeabilization kit per manufacturer's instructions (eBioscience). Samples were run on LRSII Fortessa (BD Biosciences), and analyzed using FlowJo software (Treestar). Cells were gated on lymphocytes > singlets > live cells > $CD3^+$ > $CD4^+$ or $CD8^+$ for splenocytes or lymphocytes > singlets > live cells (Live/$Dead^-$) > $CD45.1^+$ > $CD3^+$ > $CD4^+$ or $CD8^+$ for TIL. Myeloid cells were gated on immune cells > singlets > live cells > $CD45.1^+$ > $CD3^-$ > $CD11b^+MHCII^-$, $CD11b^+MHCII^+$, or $CD11b^-MHCII^+$ for intratumoral monocytes. Total cells per mm^2 were calculated by dividing total quantified cells by tumor area (length x width) at the time of tumor resection.

Immunohistochemistry and Multispectral Imaging

Seven-day MMTV-PyMT tumor-bearing mice were treated with either no treatment, concurrent or sequential combinations of anti-OX40 and anti-PD-1 as described above. On day 20 tumors were resected, zinc fixed (0.1 M Tris buffer, pH 7.4, 0.5 g/L Calcium Acetate, 5.0 g/L Zinc Acetate, 5.0 g/L Zinc Chloride) for 48 hours, paraffin-embedded, and stained for CD3 (clone SP7), CD4 (RM4-5), CD8 (53-6.7), Ki67 (D3B5), and DAPI with TSA amplification (Perkin Elmer). Tumors were imaged on a *Vectra* microscope (PerkinElmer) and quantified with *InForm* multispectral imaging analysis software (Perkin Elmer) using 5-9 T cell hot spot panels from each tumor and extrapolating to full sample size using the formula: cell count/area of tumor (pixels) * 4,000,000 (cells/mm^2).

Statistical Analysis

All statistical analysis was performed using Gehan-Breslow-Wilcoxon test (survival) or one-way ANOVA with Tukey's or Dunnett's multiple comparison tests (tumor growth, phenotype comparisons). All statistics were done with Prism 6 (Graph Pad Software). In

figure 3-7C one outlier data point was thrown out of the No Treatment group, due to an error in cell counting.

Results:

Concurrent PD-1 blockade diminishes the therapeutic efficacy of OX40 costimulation

Given previous reports of anti-tumor effects of monotherapy with either anti-OX40 or anti-PD-1 antibodies, we sought to evaluate the therapeutic efficacy of combination therapy in murine mammary cancer models. We utilized an orthotopically-transplanted mammary tumor model where spontaneous tumors generated from FVB/NJ MMTV-PyMT transgenic mice were transplanted into naïve FVB/NJ hosts. On day 7, OX40 expression was expressed exclusively on $CD4^+FoxP3^+$ T_{reg} cells in the tumor-draining lymph node and spleen, but in the tumor, while a substantial proportion of T_{reg} cells were $OX40^+$, a small frequency of both conventional $CD4^+FoxP3^-$ and $CD8^+$ cells expressed OX40 (Figure 3-1A-C). PD-1 was expressed only on intratumoral conventional $CD4^+$ and $CD8^+$ cells (Figure 3-1B, 3-1D). Very few T cells expressed both OX40 and PD-1 (Figure 3-1B). Over half of $CD45^+CD11B^+F4/80^+MHCII^-$ myeloid/macrophage cells in the tumor expressed PD-L1, while a smaller population also expressed OX40L (Figure 3-1F, 3-1G). $CD45^-$ cells, which contain tumor cells, stromal cells, and others, expressed no PD-L1 or OX40L (Figure 3-1F, 3-1G).

Beginning on day 7, tumor-bearing mice (average tumor size of ~6 mm^2) were treated with three doses of either an agonist antibody targeting OX40 (anti-OX40), a blocking anti-PD-1 antibody, or both antibodies on days 7, 9, and 11 (Figure 3-2A). Mice that received anti-OX40 treatment exhibited a significant attenuation in tumor growth compared to untreated mice, while anti-PD-1 had no impact on tumor growth (Figure 3-2B). However when anti-OX40 was combined with anti-PD-1 blockade, instead of increasing therapeutic efficacy, there was a modest, but significant reduction in tumor control compared to anti-OX40 alone (Figure 3-2B). We also noted diminished survival in mice receiving combination therapy compared to anti-OX40 alone (Figure 3-2C). These data indicate that anti-PD-1 fails to improve the therapeutic efficacy provided by anti-OX40, and

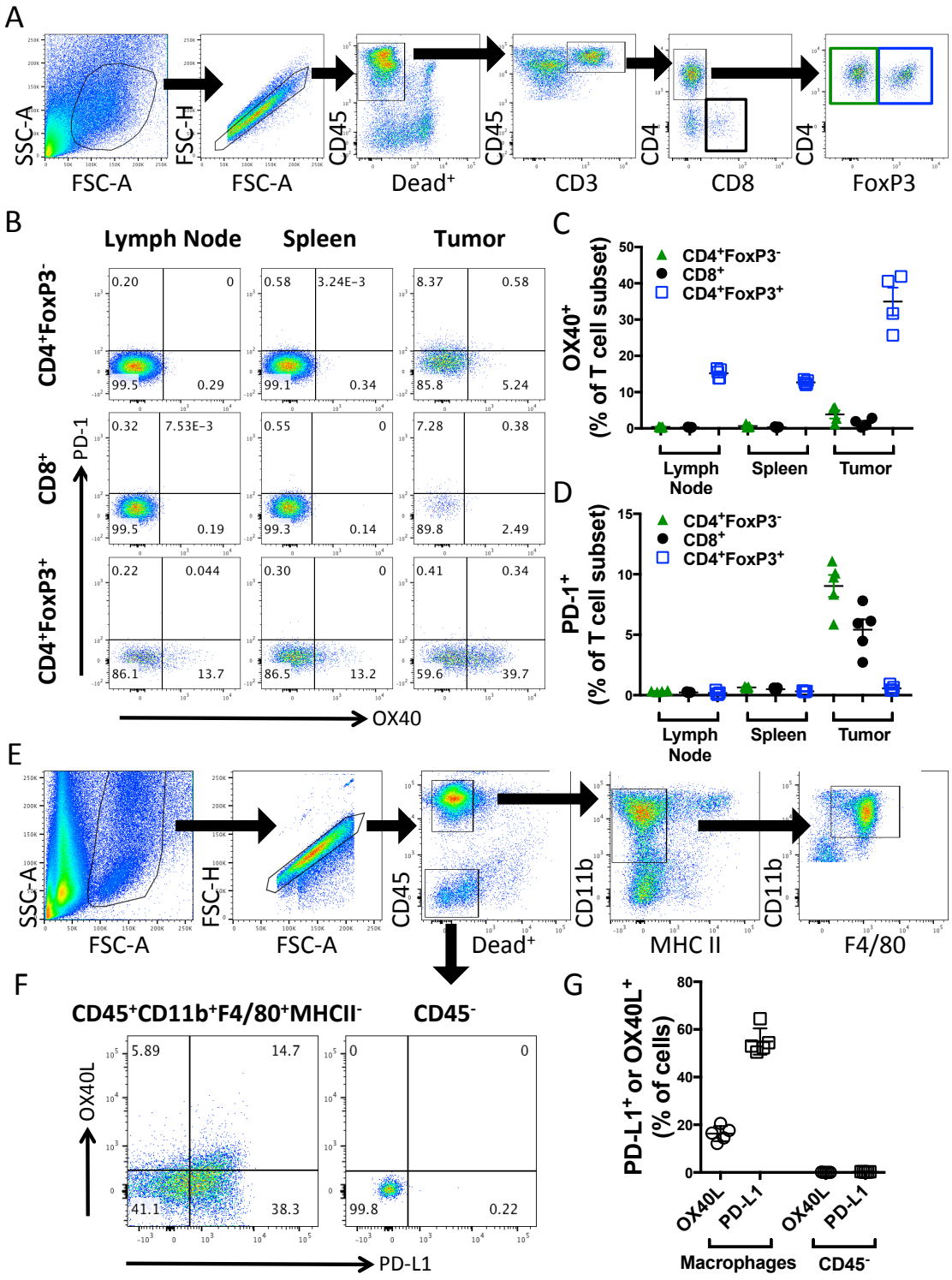

Figure 3-1: Intratumoral T cells express OX40 and PD-1. (**A**) Flow cytometry gating strategy for phenotyping CD8⁺, CD4⁺FoxP3⁻, and CD4⁺FoxP3⁺ T cells. (**B**) Representative plots of OX40 and PD-1 expression on T cell populations in the tumor-draining lymph node, spleen and tumor of 7 day MMTV-PyMT tumor-bearing mice. (**C,D**) Quantification of frequency of OX40⁺ (**C**) and PD-1⁺ (**D**) T cells. n = 4-5. (**E**) Flow cytometry gating strategy of intratumoral CD45⁺CD11b⁺F4/80⁺MHCII⁻ myeloid/macrophage and CD45⁻ cells. (**F**) Representative plots of OX40L and PD-L1 expression on

macrophage and CD45$^-$ cells. (**G**) Quantification of frequency of OX40L and PD-L1 on CD45$^+$CD11b$^+$F4/80$^+$MHCII$^-$ macrophage and CD45$^-$ cells. n = 4-5.

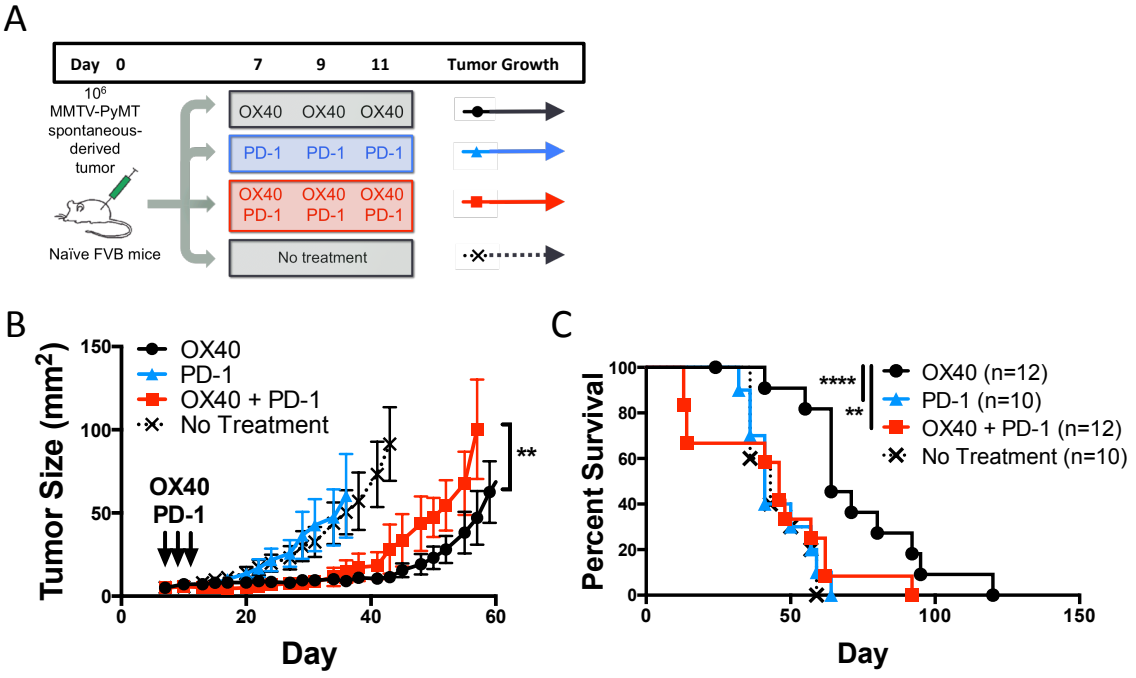

Figure 3-2: Concurrent administration of anti-PD-1 and anti-OX40 reduces anti-tumor effect of anti-OX40 alone. (**A**) Treatment schedule of MMTV-PyMT tumor-bearing mice. Mice were treated on days 7, 9 and 11 with either 100 µg. anti-OX40, 250 µg. anti-PD-1, or both antibodies. (**B**) Mean tumor growth of treated tumors, n = 6, one representative of two independent experiments is shown. Error bars represent SEM. (**C**) Kaplan-Meier survival curves of treated mice. Mice were sacrificed when primary tumor reached 150 mm^2. n =10-12, two independent experiments were combined. Error bars represent SEM. One-way ANOVA with Tukey's multiple comparison test (**B**) and Gehan-Breslow-Wilcoxon test (**C**) were performed for statistical significance. ** = $p < 0.01$, **** = $p < 0.0001$

in fact PD-1 blockade provides an adverse effect on anti-OX40-induced therapy in these models.

Concurrent PD-1 blockade and OX40 costimulation increase serum cytokines

A recent publication reported that OX40 costimulation combined with either PD-1 or PD-L1 blockade leads to excessive Interferon-gamma (IFN-γ) production and loss of parasite control in the context of plasmodium infection (361). Considering this as a possible mechanism for the reduced efficacy concurrent PD-1 blockade imparted on anti-OX40-treated mice, we measured the levels of both type 1 and type 2 cytokines in serum from

tumor-bearing animals during and after antibody treatment. A striking increase in IFN-γ was detected during treatment that peaked two days after the last antibody dose in combination-treated mice compared to mice receiving monotherapy with either agent

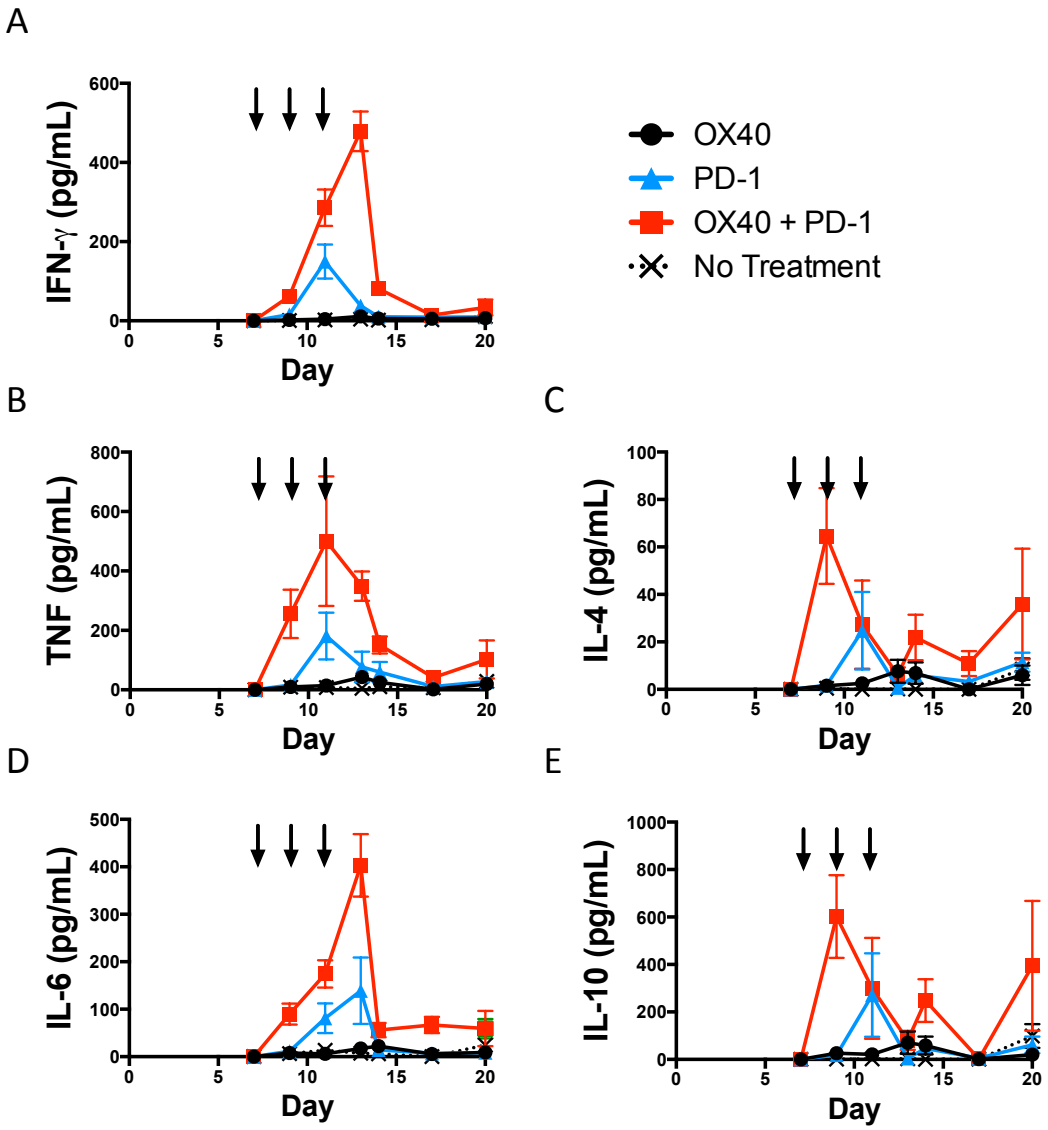

Figure 3-3: Concurrent PD-1 blockade and OX40 costimulation increase serum cytokines. Time course of serum cytokines taken from MMTV-PyMT tumor-bearing mice treated with anti OX40, anti-PD-1, or both antibodies. Black arrow (🡻) indicates antibody treatment. X-axis represents time post tumor transplant. (**A**) IFN-γ (**B**) TNF (**C**) IL-6, (**D**) IL-10, and (**E**) IL-4 were measured on days 9, 11, 13, 14, 17, and 20 post tumor transplant, n = 4-14, error bars represent SEM.

(Figure 3-3A). While anti-PD-1 alone had a substantial effect on cytokine levels compared to untreated mice, the combination treatment boosted cytokines 10-100 fold higher than in

untreated tumor-bearing mice. Serum levels of other cytokines, including IL-6 as well as both type 1 (TNF) and type 2 (IL-4 and IL-10) cytokines were also highly elevated in the combination-treated group compared to anti-OX40 alone (Figure 3-3B-E). Consistent with symptoms of a cytokine storm-like event, combination-treated mice exhibited ruffled, unkempt fur and lethargy from days ~9-14. Untreated mice had minimal or undetectable levels of all cytokines until the day 20 time point when IL-4 and IL-10 modestly increased. Intriguingly we saw consistent patterns in the kinetics of specific types of cytokines over time. Type-2 cytokines (IL-4 and IL-10) in combination-treated mice peaked directly after only one dose of antibodies, which was followed by a gradual decline but a late increase nine days after the last antibody dose. At the same time, type-1 cytokines (TNF, IFN-γ) increased during the treatment period, with TNF peaking first, and then declined after the last antibody dose. Together these data demonstrate that simultaneously stimulating the OX40 receptor and blocking the PD-1 receptor can induce an acute cytokine release of both type one and type cytokines, but the cytokine milieu transitioned from a type 2 to type 1 cytokine profile in the serum over the course of treatment.

Combination of OX40 costimulation and PD-1 blockade upregulates T cell inhibitory and costimulatory receptors

T cell activation and inhibition is a delicate balance of positive and negative signals that involves a host of transiently and constitutively expressed surface receptors, some of which are independent of each other and others that are compensatory. With the amplified levels of IFN-γ and other cytokines observed in the serum of anti-OX40 plus anti-PD-1 combination-treated mice, we anticipated substantial changes to T cell surface receptors.

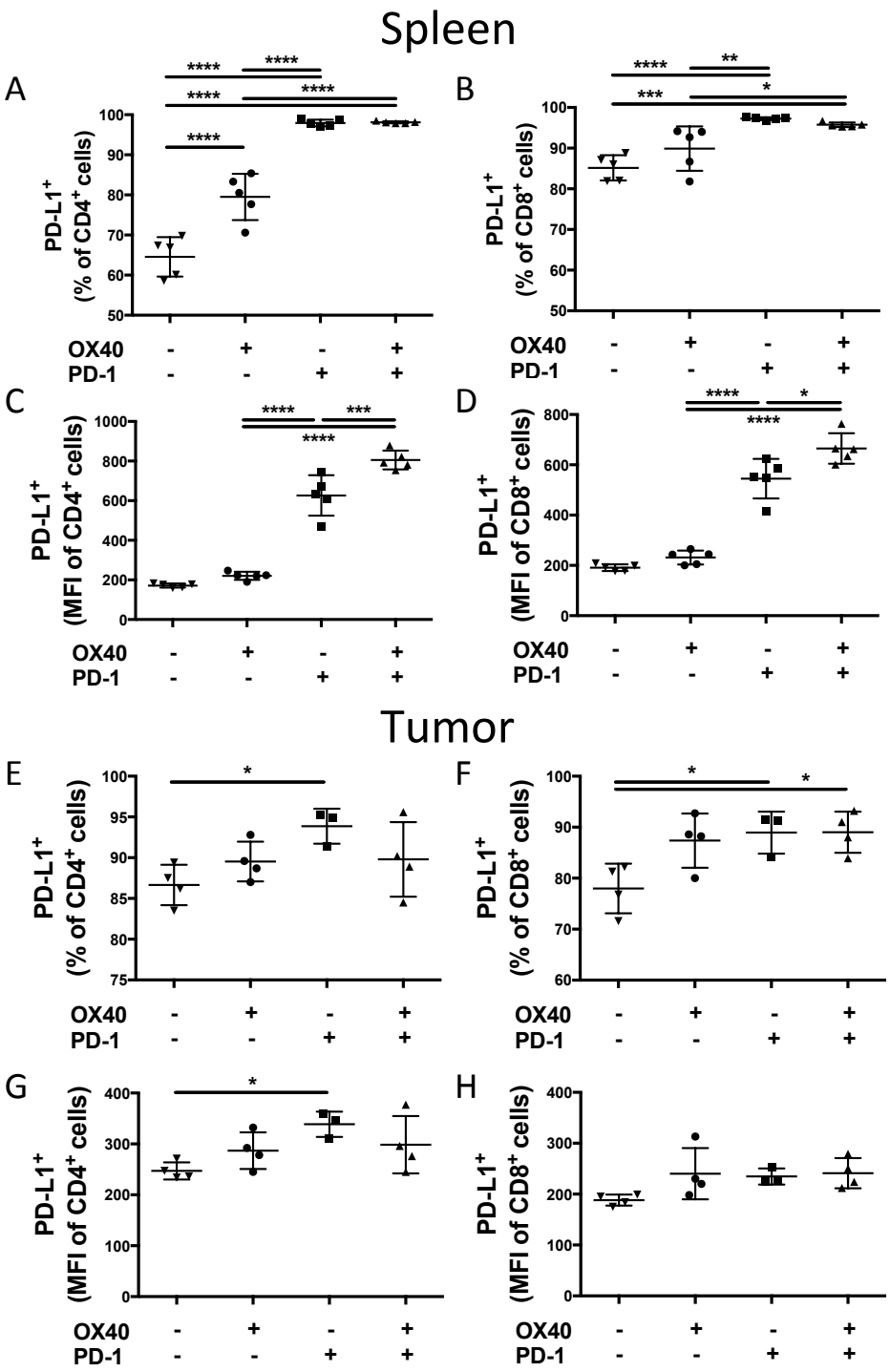

Figure 3-4: PD-L1 expression is highly expressed on T cells, and increases with anti-OX40 and anti-PD-1 treatment on peripheral T cells, but not intratumoral T cells. Flow cytometric quantification of PD-L1 expression on treated splenic and intratumoral T cells on day 13 post tumor transfer. (**A,B**) PD-L1 expression as a percentage of CD4+ (**A**) and CD8+ (**B**) splenic T cells. (**C,D**) Mean fluorescent intensity of PD-L1 expression on CD4+ (**C**) and CD8+ (**D**) splenic T cells. (**E,F**) PD-L1 expression as a percentage of CD4+ (**E**) and CD8+ (**F**) intratumoral T cells. (**G,H**) Mean fluorescent intensity of PD-L1 expression on CD4+ (**G**) and CD8+ (**H**) intratumoral T cells. n = 5, one representative of four (spleen) and two (tumor) independent experiments, error bars represent SEM.

One-way ANOVA with Tukey's multiple comparison tests were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

Similar to previous reports (362), we found that a majority of untreated splenic and intratumoral T cells expressed PD-L1 at low levels (Figure 3-4A, 3-4B, 3-4E, 3-4F). Combination treatment further increased this percentage of PD-L1$^+$ T cells on T cells in the spleen (Figure 3-4A, 3-4B), but had less impact within the tumor, only increasing the frequency of CD8$^+$PD-L1$^+$ cells (Figure 3-4E, 3-4F), although in untreated mice the frequency of intratumoral CD4+PD-L1+ cells was already very high (over 85%). IFN-γ is known to upregulate PD-L1 and after anti-PD-1 monotherapy we observed a significant increase in PD-L1 fluorescence intensity on both splenic CD4$^+$ and CD8$^+$ T cells (Figure 3-4C, 3-4D). PD-L1 expression was further increased with combination treatment (Figure 3-4C, 3-4D). In the tumor however, combination treatment did not impact PD-L1 fluorescent intensity (Figure 3-4G, 3-4H). Thus in both the spleen and tumor combination treatment lead to increased frequencies of PD-L1$^+$ T cells.

The frequency of intratumoral CD8$^+$ T cells expressing inhibitory receptors T cell immunoglobulin and mucin-domain containing-3 (TIM-3) or CTLA-4 were also significantly increased with anti-PD-1 or combination treatment, with more than double the frequency of TIM-3$^+$ cells compared to anti-OX40 monotherapy (Figure 3-5B). The prevalence of CTLA-4$^+$CD4$^+$ cells also increased in all treatment groups, but there were no changes to TIM-3$^+$CD4$^+$ cells with treatment (Figure 3-5A). In the spleen however, there were significant increases in the frequencies of both CTLA-4$^+$ and TIM-3$^+$ cells in both the CD4$^+$ and CD8$^+$ compartment with combination treatment, although these frequencies were reduced from those seen in the tumor (Figure 3-5C). The frequency of cells expressing costimulatory molecules Inducible T cell costimulator (ICOS), 4-1BB (CD137), or Glucocorticoid-induced TNFR-related protein (GITR) in the tumor were not altered with any treatment (Figure 3-5C). However in the spleen, combination treatment significantly

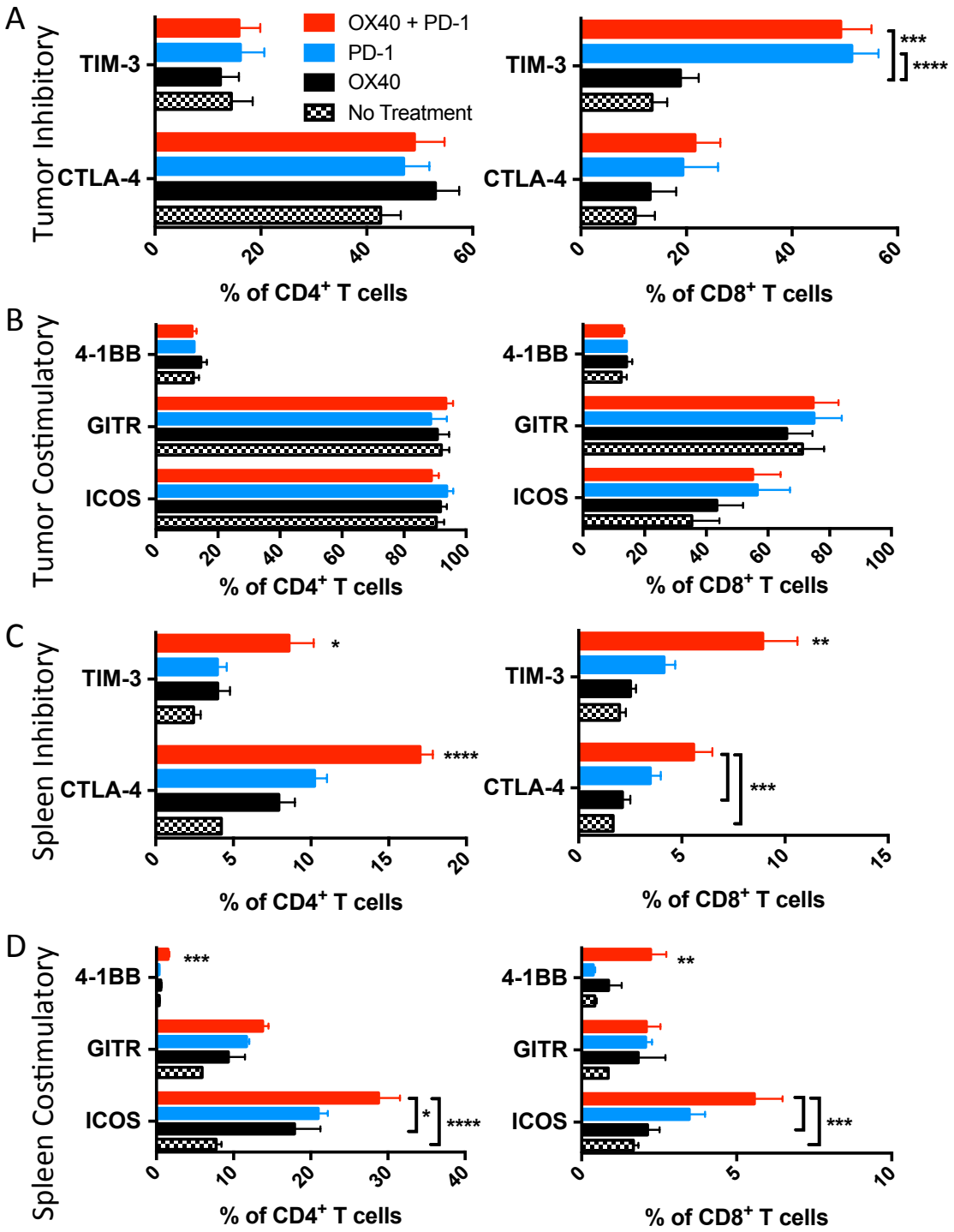

Figure 3-5: Combination of anti-OX40 and anti-PD-1 increases exhaustion of intratumoral CD8⁺ T cells. Flow cytometric quantification of surface expression of costimulatory and inhibitory receptors on CD4⁺ and CD8⁺ T cells from the tumor (**A,B**) and spleen (**C,D**) of MMTV-PyMT tumor-bearing mice treated with anti-OX40, anti-PD-1, or both, day 13 post tumor transplant as described in Figure 3-2A. (**A**) Frequency of TIM-3 and CTLA-4 inhibitory receptors expressed on intratumoral CD4⁺ and CD8⁺ T cells. (**B**) Frequency of 4-1BB, GITR, and ICOS costimulatory receptors expressed on intratumoral CD4⁺ and CD8⁺ T cells. (**C**) Frequency of TIM-3 and CTLA-4 inhibitory receptors expressed on splenic CD4⁺ and CD8⁺ T cells. (**D**) Frequency of 4-1BB, GITR, and ICOS costimulatory

receptors expressed on splenic $CD4^+$ and $CD8^+$ T cells. n = 8-10, two independent experiments combined. Significance stars without brackets indicate OX40 + PD-1 significance compared to OX40, PD-1, and No Treatment. Error bars represent SEM. One-way ANOVA with Tukey's multiple comparison tests were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

increased the prevalence of $ICOS^+$ and $4\text{-}1BB^+$ cells in both the $CD4^+$ and $CD8^+$ compartment (Figure 3-5D). Thus combination treatment appears to primarily impact $CD8^+$ T cells in the tumor, but both $CD4^+$ and $CD8^+$ T cells in the periphery. These data suggest that concurrent anti-OX40 and anti-PD-1 combination treatment impacts peripheral T cells more than either agent alone. But importantly combination treatment increases the frequency of exhausted T cells in the tumor compared to anti-OX40 monotherapy.

Concurrent combination therapy alters T cell differentiation and apoptosis

Splenomegaly has long been noted as a sign of an immunological response in preclinical cancer models (363), thus we were not surprised to find massive splenomegaly in tumor-bearing mice treated with the combination of anti-OX40 plus anti-PD-1 (Figure 3-6A, 3-6B). Mice that were untreated or treated with anti-OX40 monotherapy mice had significantly smaller spleens. Anti-PD-1 alone did have a significant effect on spleen size, however not to the levels seen with combination treatment. Kinetically, splenocyte numbers increased after just a single dose of combination antibodies, and steadily grew over time, but anti-PD-1 monotherapy did not increase splenocytes until 2-3 days later (Figure 3-6B). Given the substantial increase in serum cytokines (Figure 3-3) and splenomegaly, we hypothesized that combination treatment increases terminally differentiated T cells; a possible contributing factor to their dysfunction and attenuated therapeutic efficacy (Figure 3-2B, 3-2C). We noted significant increases in the frequency of $CD44^{hi}CD62L^-$ effector memory populations in both the $CD4^+$ and $CD8^+$ compartment in the spleen two days after the final treatment dosing (day 13) (Figure 3-6C, 3-6D). Additionally we analyzed T cells for the

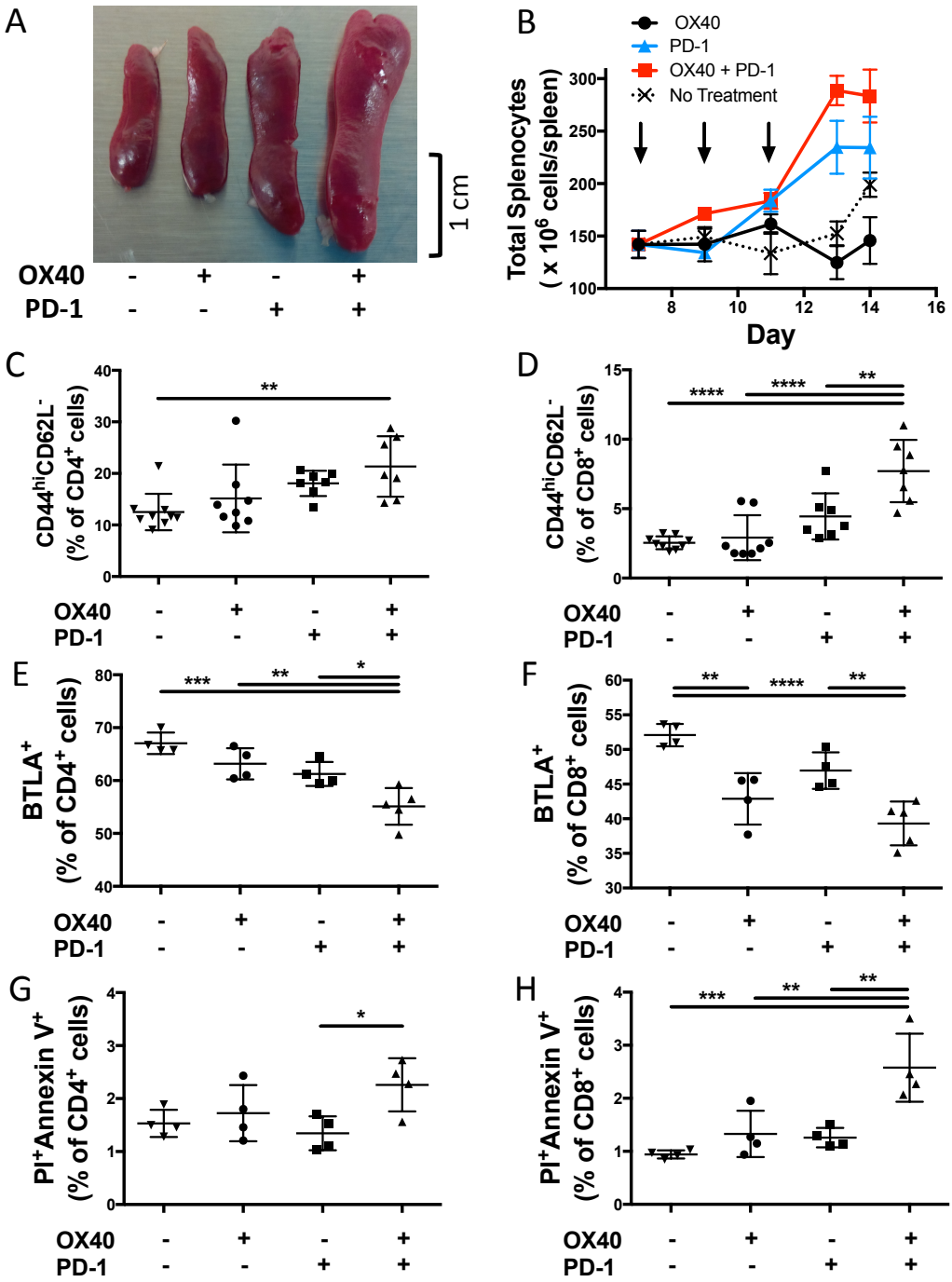

Figure 3-6: Combination of anti-OX40 and anti-PD-1 increases T cell differentiation and apoptosis. MMTV-PyMT tumor-bearing mice were treated as in Figure 3-2A. Spleens were resected on day 13 for analysis. (**A**) Representative images of spleens from treated and untreated mice. (**B**) Time course of total splenocytes Black arrow (🡻) indicates antibody treatment. X-axis represents time post tumor transplant. n = 3-5, 2 experiments combined, representative of 5 experiments for day 13. (**C-D**) Percentage of $CD44^{hi}CD62L^{-}$ $CD4^{+}$ (**C**) and $CD8^{+}$ (**D**) T cells. n= 7-9, two independent experiments combined. (**E-F**) Percentage of $BTLA^{+}$ $CD4^{+}$ (**E**) and $CD8^{+}$ (**F**) T cells, n = 4-5, one representative of two independent experiments. (G-H) Percentage of Propidium $Iodide^{+}AnnexinV^{+}$ (dead) $CD4^{+}$ (**G**) and $CD8^{+}$ (**H**) T cells. n = 4, one representative of two independent experiments,

error bars represent SEM. One-way ANOVA with Tukey's multiple comparison tests were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

expression of B and T Lymphocyte Attenuator (BTLA), an inhibitory receptor initially expressed on activated T cells. Subsequent downregulation of BTLA ($BTLA^-$), however, denotes increased T cell differentiation in the tumor and a decreased capacity to proliferate (27). With concurrent treatment of anti-OX40 combined with anti-PD-1, $BTLA^+$ T cells were significantly decreased in both the $CD4^+$ and $CD8^+$ splenic T cell populations (Figure 3-6E, 3-6F). Given that terminally differentiated, effector T cells are much shorter lived than memory populations, we investigated whether this increase in T cell differentiation correlated with an increase in T cell apoptosis. As expected we noted a greater than 2-fold increase in Annexin V^+ cells that also stained positive with Propidium Iodide (PI, Live/Dead), marking them as dead, apoptotic cells, in spleens from combination treated mice (Figure 3-6G, 3-6H). Interestingly, combination treatment seemed to affect apoptosis in the $CD8^+$ population more than $CD4^+$ cells. In combination, these data suggest that combination anti-OX40 plus anti-PD-1 increases splenic T cell differentiation excessively, resulting in cells that are more likely to be less effective and eliminated through apoptosis than those stimulated by anti-OX40 alone.

Concurrent combination therapy increases proliferating T cells in the tumor, but not overall TIL numbers

Given the increase in inhibitory receptors on T cells in the spleen, we considered the effects of combination treatment on T cell phenotypes and proliferation within the tumor. Two days after the completion of treatment (day 13 post tumor transplant), anti-OX40-treated

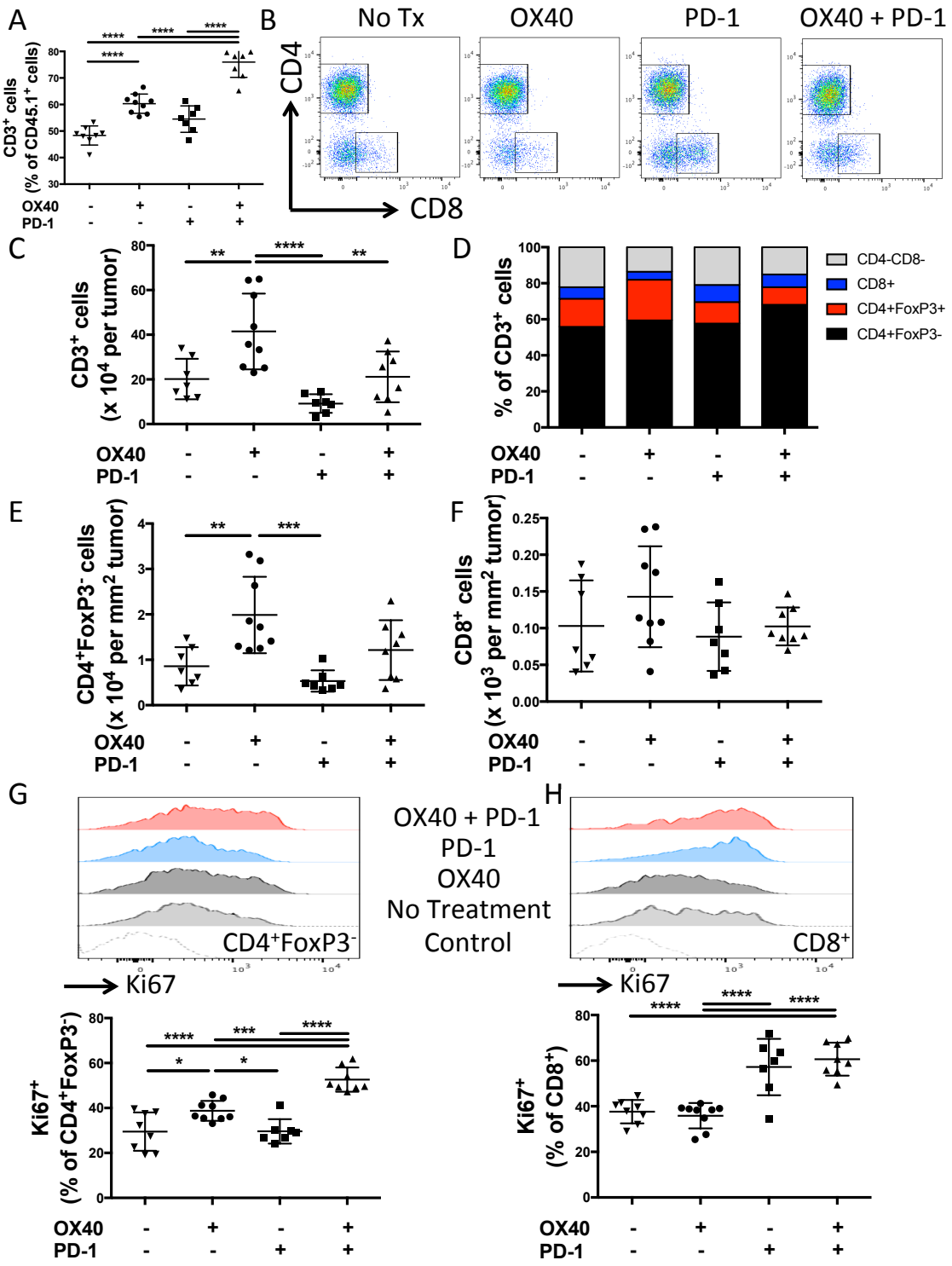

Figure 3-7: Combination of anti-OX40 and anti-PD-1 increases proliferating T cells in the tumor. MMTV-PyMT tumor-bearing mice were treated as in Figure 3-2A. Tumors were resected on day 13 for analysis. (**A**) Flow cytometric quantification of the frequency of CD3+ T cells out of CD45.1+ cell population in treated tumors, (**B**) Representative plot of tumor T cell infiltrate. (**C**) Total CD45+CD3+ cells in treated tumors, (**D**) Average T cell populations as a percentage of CD3+ cells in the tumor. (**E, F**) Total CD4+FoxP3- (**E**) and CD8+ (**F**) cells in the tumor per mm2. (**G,H**) Representative

histograms and quantification of frequency of Ki67$^+$ proliferating conventional CD4$^+$FoxP3$^-$ (**G**) and CD8$^+$ (**H**) T cells in the tumor. n = 7-10 , two independent experiments combined, error bars represent SEM. One-way ANOVA with Tukey's multiple comparison tests were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

tumors had increased CD3$^+$ T cells as a frequency of the CD45$^+$ immune cell infiltrate compared to untreated tumors, and this was further increased in mice treated with the combination anti-OX40 and anti-PD-1 (Figure 3-7A). However, only anti-OX40 treatment alone caused a significant increase in the total number of CD3$^+$ T cell infiltrating the tumor (Figure 3-7C). The predominant population of T cells in treated or untreated tumors were CD4$^+$ cells, specifically CD4$^+$FoxP3$^-$ conventional T cells, although anti-OX40 treatment caused a significant increase ($p < 0.05$) in CD4$^+$FoxP3$^+$ T_{reg} cells that did not occur with anti-PD-1 or the combination treatment (Figure 3-7B, 3-7D). Interestingly, treatment with anti-OX40 also resulted in a significantly lower ($p < 0.05$) percentage of CD8$^+$ T cells compared to anti-PD-1 or combination treatment (Figure 3-7D). Anti-OX40 monotherapy had no significant effect on the number of CD8$^+$ cells in the tumor; however, it did increase the total number of CD4$^+$FoxP3$^-$ cells (Figure 3-8E, 3-7F). Combination treatment significantly increased the frequency of both Ki67$^+$ proliferating CD4$^+$FoxP3$^-$ and CD8$^+$ T cells (Figure 3-7G, 3-7H), while anti-OX40 monotherapy moderately, but significantly increased proliferating CD4$^+$FoxP3$^-$ cells, but had no effect on proliferating CD8$^+$ cells. Thus while combination treatment can increase the percentage of tumor-infiltrating proliferating T cells, this metric does not correlate with therapeutic response.

In the spleen, anti-OX40 treatment significantly boosted both the frequency and numbers of CD4$^+$FoxP3$^+$ T_{reg} cells compared to the levels observed in untreated mice (Figure 3-8A, 3-8C). Nonetheless, in combination with anti-PD-1, the total frequency and number of of T_{reg} cells were doubled compared to anti-OX40 alone (Figure 3-8A, 3-8C). In the tumor however, the effects of treatment were much different. Anti-OX40 treatment greatly increased the total frequency and number of T_{reg} cells, compared to combination treatment

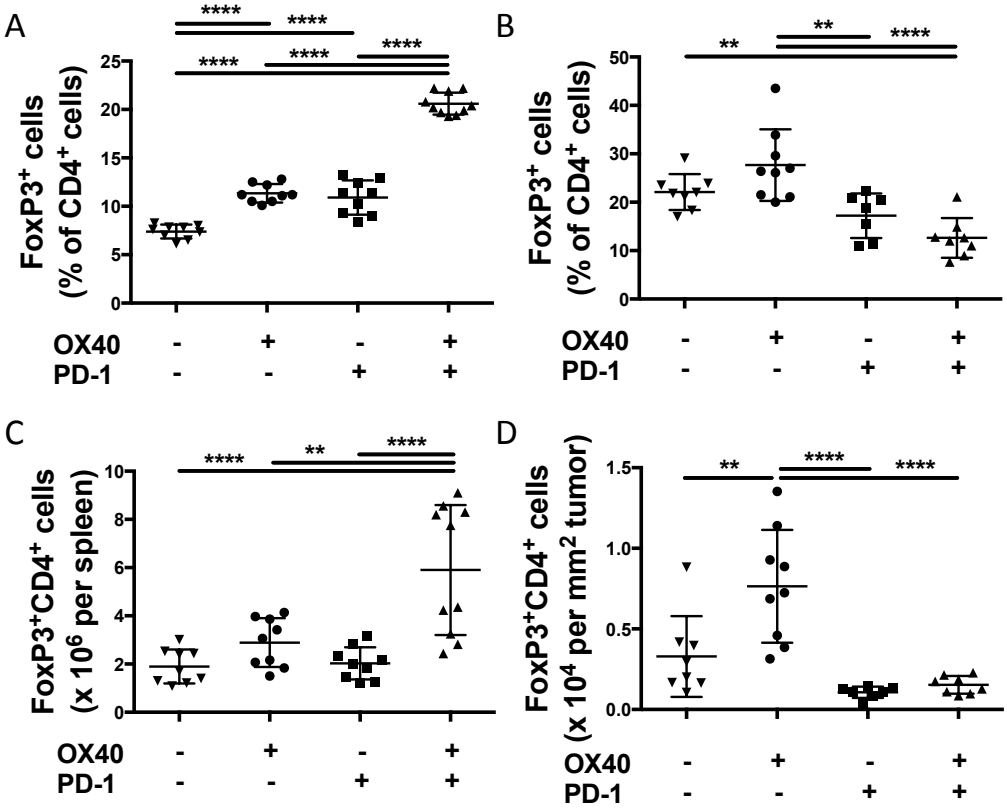

Figure 3-8: Combination of anti-OX40 and anti-PD-1 alters regulatory T cells in the tumor and spleen. MMTV-PyMT tumor-bearing mice were treated as in Figure 3-2A. Tumors were resected on day 13 for analysis. (**A,B**) Percentage of $CD4^+FoxP3^+$ T_{reg} cells in the spleen (**A**) or tumor (**B**), n = 7-10, two paired independent experiments combined. (**C**) Total $CD4^+FoxP3^+$ T_{reg} cells in the spleen. n = 4-5, one representative of four independent experiments. (**D**) Total $CD4^+FoxP3^+$ T_{reg} cells in the tumor. n = 7-9, two independent experiments combined, error bars represent SEM. One-way ANOVA with Tukey's multiple comparison tests were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$

or anti-PD-1 alone (Figure 3-8B, 3-8D). Given the unaltered number of T_{reg} cells in the tumor of combination-treated mice compared to untreated tumors, and that anti-OX40 treatment resulted in more T_{reg} cells than either, but superior therapy (Figure 3-2B, 3-2C), we conclude that intratumoral T_{reg} cells may be a compensatory response to the development of tumor-destructive T cells with anti-OX40 in the MMTV-PyMT model, as has been suggested by others (56).

Delayed PD-1 blockade augments anti-OX40 anti-tumor effects

Given the diminished anti-tumor effects generated by the concurrent combination of anti-OX40 and anti-PD-1 and the knowledge that PD-1 plays a critical role in the contraction phase of a normal immune response, we reasoned that combination treatment with anti-PD-1 antibody would be more effective if it was given after the initial T cell boost generated by anti-OX40. We noted no difference in PD-1 expression on day 13 intratumoral T cell subsets after anti-OX40 treatment compared to untreated tumors (Figure 3-9A). PD-1 expression dramatically increased in untreated tumors compared to day 7 (Figure 3-1B, 3-1D). Accordingly anti-OX40 given on days 7, 9, and 11-post tumor transplant, was followed by delayed anti-PD-1, administered on days 13, 15, and 17, providing a sequential combination treatment (Figure 3-9B). This sequential combination resulted in a significant delay ($p<0.0001$) in tumor growth compared to anti-OX40 alone with some tumors reaching complete regression (Figure 3-9C). Sequential combination therapy was also far superior to concurrent combination therapy. Despite elevated frequencies of PD-1^+ TIL, delayed anti-PD-1 treatment alone had no significant impact on tumor growth (3-9F). Anti-OX40 plus delayed anti-PD-1 correspondingly resulted in a significant increase ($p<0.001$) in survival, with ~30% of the animals experiencing complete regression of their tumors (200+ days) (Figure 3-9D). To confirm that the order of sequential treatment was crucial, we reversed the combination, giving anti-PD-1 first, followed by anti-OX40. This treatment proved to be much less effective than anti-OX40 plus delayed anti-PD-1, demonstrating that providing OX40 costimulation first and then blocking PD-1 receptor is critical to the effects of sequential combination therapy (Figure 3-9E).

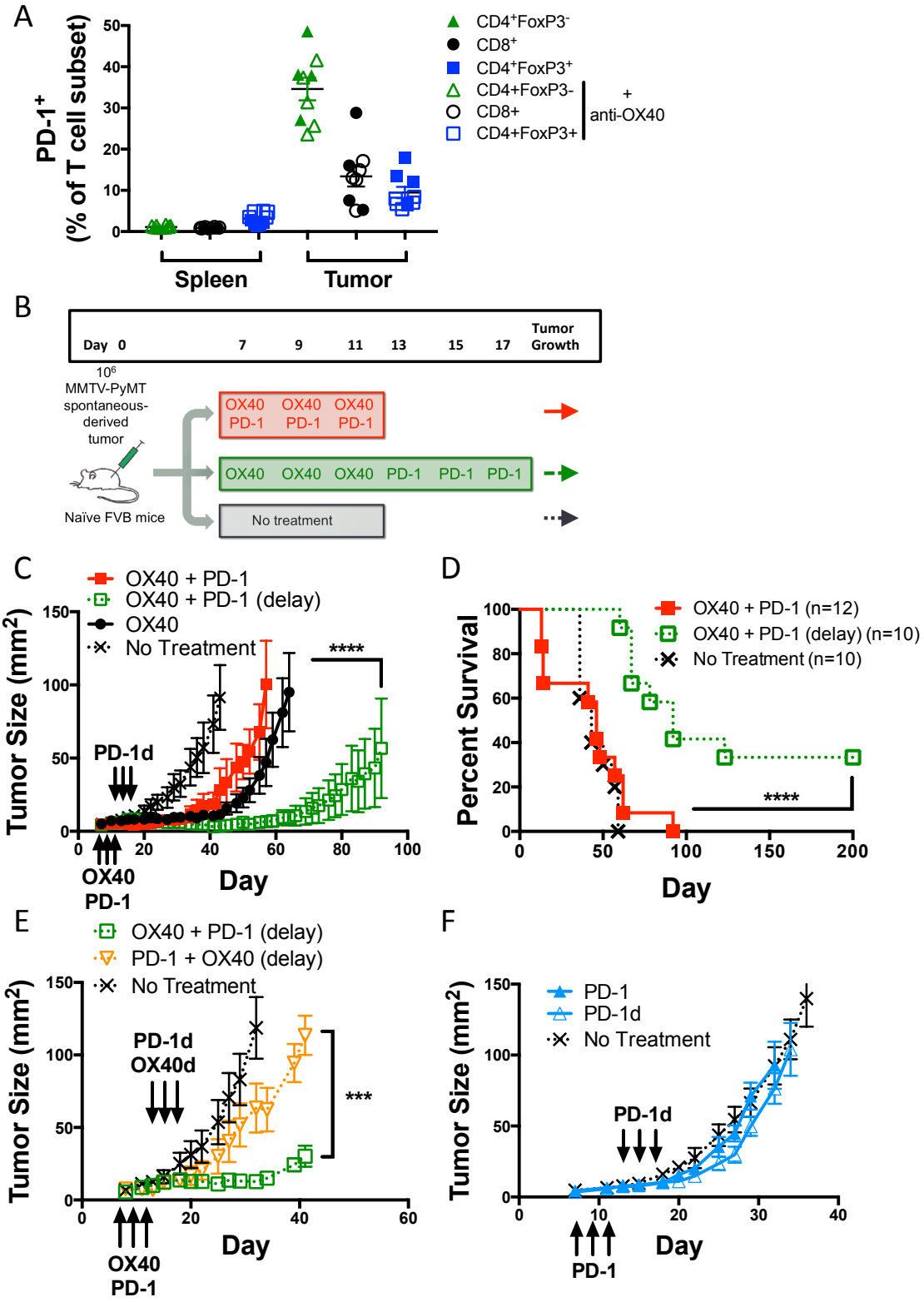

Figure 3-9: Sequential, delayed anti-PD-1 augments anti-OX40 therapeutic efficacy. (**A**) Quantification of PD-1 expression on intratumoral conventional CD4⁺FoxP3⁻, CD8⁺, and CD4⁺FoxP3⁺ T cells from MMTV-PyMT tumor-bearing mice on day 13. Open symbols represent mice given anti-

OX40 on days 7, 9, and 11. n = 4-5.. (**B**) Treatment schedule of MMTV-PyMT tumor-bearing mice comparing concurrent and delayed (delay, d), sequential therapy. Delayed anti-PD-1 was given on days 13, 15, and 17. (**C**) Mean tumor growth of treated tumors, n = 6, one representative of two independent experiments is shown. (**D**) Kaplan-Meier survival curves of treated mice. n = 10-12, two independent experiments were combined. (**E**) Mean tumor growth of tumors treated sequentially with anti-OX40 then anti-PD-1 or anti-PD-1 then anti-OX40. n = 6, one representative of two independent experiments is shown. (**F**) Mean tumor growth of tumors treated with 250 μg anti-PD-1 on days 7, 9, and 11 or 13, 15, and 17 (delayed, PD-1d) n = 7, one representative of two independent experiments is shown, error bars represent SEM. Student's T test (A), one-way ANOVA with Tukey's multiple comparison test (C, E, F) and Gehan-Breslow-Wilcoxon test (D) were performed for statistical significance. *** = $p < 0.001$, **** = $p < 0.0001$

To investigate whether sequential combination therapy was superior to concurrent therapy in a second model, we utilized the transplantable 4T1 mammary tumor model (364), which also allowed us to test this phenomenon in a second strain of mice, the BALB/c mouse. Tumors were orthotopically transplanted in the mammary fat pad and therapy was initiated five days later with concurrent combination treated mice receiving both anti-OX40 and anti-PD-1 on days 5, 7, and 9, and sequential combination receiving anti-OX40 on days 5, 7, and 9, followed by anti-PD-1 on days 11, 13, and 15. The 4T1 tumor is known to be poorly immunogenic, and difficult to impact (365,366), but we were able to see a significant reduction in tumor growth with the sequential combination treatment (Figure 3.10A). Concurrent combination had no effect on the 4T1 tumors. When reversing the sequential combination, we again noted that only anti-OX40 first, followed by anti-PD-1 had a significant impact on tumor growth (Figure 3.10B). Additionally, the 4T1 tumor is known to be a highly metastatic model, capable of initiating lung metastases within 2-3 weeks (365). Thus we analyzed the metastatic burden in the lungs of treated mice at the time of their primary tumor-induced sacrifice. On average, sequential combination treated mice survived longer than concurrent combination treated mice, concurrent combination treatment resulted in a higher count of lung metastases at early time points (days 23 and 25) (Figure 3-10C). When fitted with linear regression trend lines, sequential combination treatment predicts a lower metastatic burden at later time points, suggesting this combination therapy to be more effective against preventing or reducing metastasis. Together these data confirm

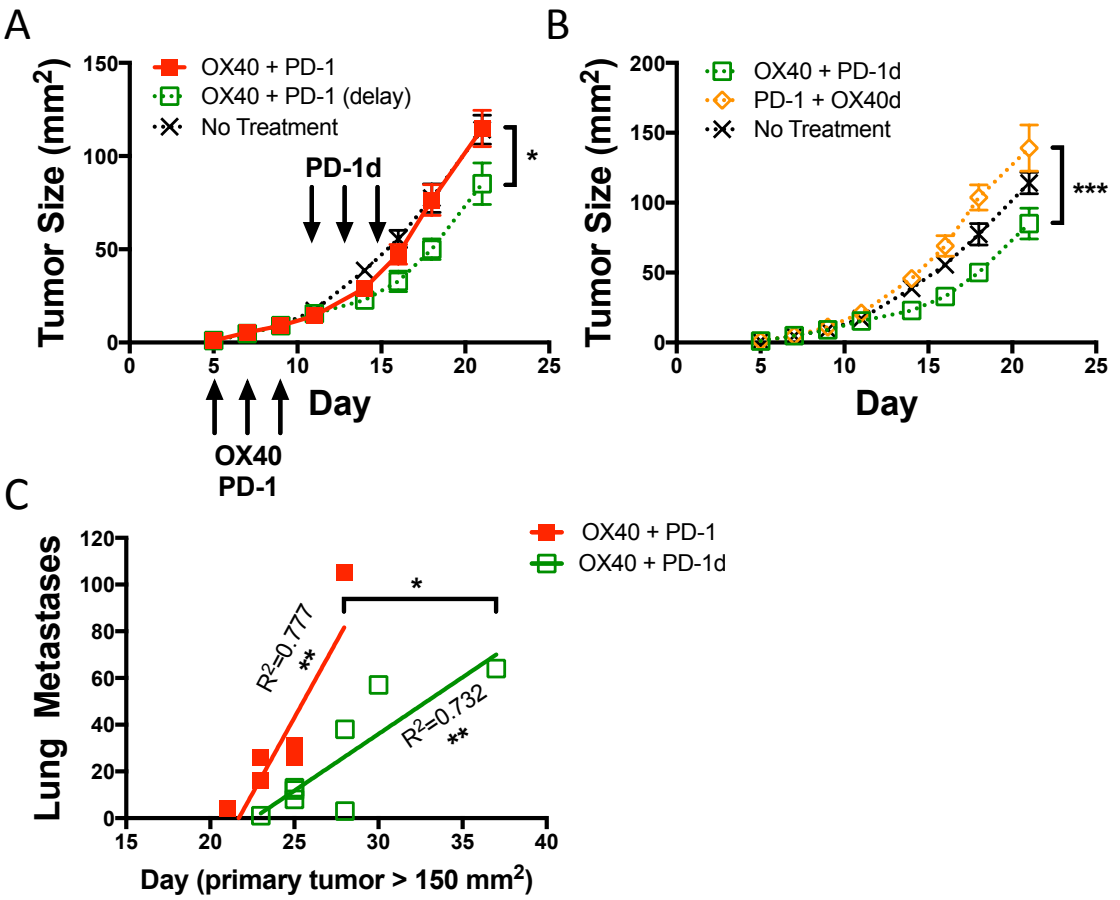

Figure 3-10: Sequential anti-OX40 and anti-PD-1 provides anti-tumor effect against primary and metastatic 4T1 tumors. Five-day 4T1 tumor-bearing mice were treated with either anti-OX40 and anti-PD-1 on days 5, 7, and 9 (concurrent) or anti-OX40 on days 5, 7, and 9 followed by anti-PD-1 on days 11, 13, and 15. (**A**) Mean tumor growth of treated tumors. n = 7, one representative of three independent experiments. (**B**) Mean tumor growth comparing sequential combination with a reversed treatment of anti-PD-1 on days 5, 7, and 9 followed by anti-OX40 on days 11, 13, and 15. n = 7. (**C**) Lung metastases at the time of primary tumor-related death. Mice were sacrificed when their primary tumor reached 150 mm^2 and lungs were counterstained and enumerated. Linear regression trend lines are shown with R^2 values and significance. Difference in slope was tested for significance and shown. n = 7, error bars represent SEM. One-way ANOVA with Tukey's multiple comparison test (A, B) and (C) were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

our findings in the transplantable MMTV-PyMT model, and demonstrate that sequential anti-OX40 plus anti-PD-1 is superior to concurrent combination in two unique preclinical models.

We have determined that untreated, transplanted MMTV-PyMT tumors contain a strong endogenous T cell infiltrate, with a majority of this infiltrate $CD4^+$ T cells (Figure 3-

7). Previous reports have noted that primary MMTV-PyMT tumors in the original spontaneous mice are not well infiltrated with CD8+ T cells, however an ErbB-2 peptide vaccine could dramatically increase CD8+ infiltrate and delay tumor growth (367). A recent publication also demonstrated that PD-1 blockade may not reinvigorate T cells to become effective memory T cells (186), proposing that vaccination may indeed benefit "hot" tumors. Thus, we sought to recruit more memory CD8+ T cells to the tumor in the hopes of further augmenting our sequential combination therapy by adding an autophagosome-enriched vaccine. Autophagosome-enriched vaccines have previously shown the ability to cross protect against differing MCA-induced sarcomas and are capable of being cross-presented to CD8+ T cells (264,265). Previous unpublished work from our lab suggests that autophagosome-enriched vaccines derived from allogeneic cell lines prime a stronger response than syngeneic ones. However vaccination with vaccine derived from the allogeneic C57MG tumor line administered in the tumor-draining LN at the initiation of sequential combination therapy with anti-OX40 and anti-PD-1 (day 7) had no further effect

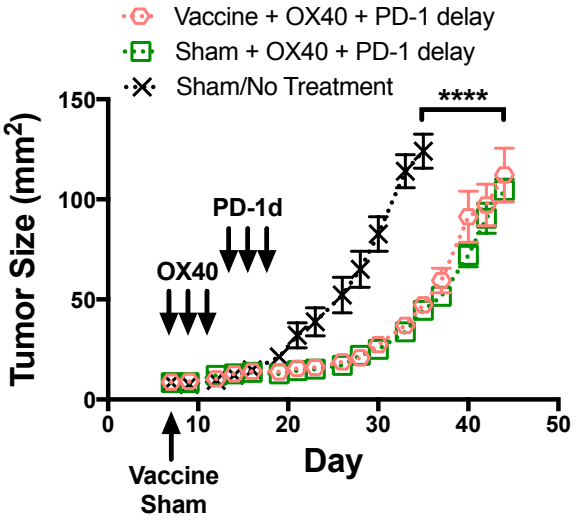

Figure 3-11: The addition of vaccination to sequential combination does impact therapeutic effect. Mean tumor growth of tumor-bearing mice treated with sequential combination of anti-OX40 plus anti-PD-1 with or without internodal vaccination of 10 μg C57MG-derived autophagosome-enriched vaccine. n = 7, one representative of two independent experiments., error bars represent SEM. One-way ANOVA with Tukey's multiple comparison test was performed for statistical significance. **** = $p < 0.0001$

on tumor growth delay (Figure 3-11). This suggests that "hot" tumors like MMTV-PyMT that benefit from PD-1 blockade (in combination with anti-OX40) may not further benefit from vaccination.

Despite possible differences in mechanism, targeting the PD-1 receptor or its ligand PD-L1 for immunotherapeutic intervention has shown similar results in the clinic (136,137). To investigate whether timing was critical for anti-OX40 combination with PD-L1 blockade, we compared a concurrent and sequential combination of anti-OX40 and anti-PD-L1. Anti-PD-L1 alone provided a short but significant delay to tumor progression, differentiating it from anti-PD-1 treatment (Figure 3-12A, Figure 3-2B). However in combination sequential anti-OX40 and anti-PD-L1 treatment delayed tumor growth for a longer period or time compared to concurrent combination, although this difference just failed to reach statistical significance ($p = 0.05$, Figure 3-12B). These data establish that sequential combination of anti-OX40 with PD-1 blockade provides superior therapy to concurrent combination in this preclinical mammary cancer model.

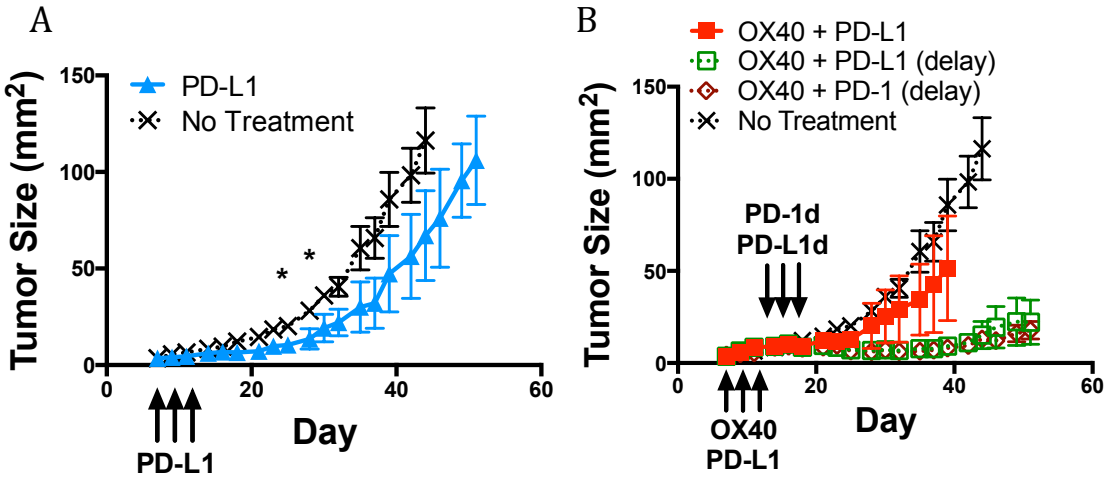

Figure 3-12: PD-L1 blockade provides an anti-tumor effect, but combined with anti-OX40, sequential combination is superior to concurrent combination. Mean tumor growth of MMTV-PyMT tumor-bearing mice treated with either (**A**) 200ug anti-PD-L1 on days 7, 9, and 11 or (**B**) a combination of anti-OX40 plus 200 µg of either concurrent (days 7, 9 and 11) anti-PD-L1 or delayed (days 13, 15, and 17) anti-PD-L1 or anti-PD-1. n = 5-6, one representative of three independent experiments is shown, error bars represent SEM. One-way ANOVA with Tukey's multiple comparison test were performed for statistical significance. * = $p < 0.05$

Sequential anti-OX40 and anti-PD-1 therapy maintains proliferating T cells and avoids acute cytokine induction and T cell exhaustion

The large spikes in multiple serum cytokines induced by concurrent anti-OX40 and anti-PD-1 led us to investigate cytokine levels following sequential combination therapy. We compared the levels of IFN-γ and TNF after completion of concurrent combination or anti-OX40 alone (day 13) and sequential combination (day 19) and again noted very high levels of both cytokines in concurrent combination animals on day 13 (Figure 3-13A, 3-13B). However, sequential combination treatment did not induce high levels of either cytokine at the earlier (anti-OX40 only) or later time points (Figure 3-13A, 3-13B). Additionally at day 19, we noted a significant decrease in the frequency of less differentiated intratumoral $CD4^+$ and $CD8^+$ T cells expressing BTLA (Figure 3-13C, 3-13D). Unlike 6 days earlier, when concurrent combination treatment induced increases in TIL proliferation (Figure 3-7G, 3-7H, 3-8D), at day 19 we noted a significant reduction in $Ki67^+$ proliferating $CD4^+FoxP3^-$ and $CD8^+$ T cells in the tumor with concurrent combination treatment (Figure 3-13E, 3-13F), congruent with an increase in exhausted and terminally differentiated T cells. Sequential combination however, resulted in higher frequencies of intratumoral $BTLA^+$ T cells and maintained T cell proliferation.

Additionally we compared the effects of treatment on the frequency of T cells expressing inhibitory receptors CTLA-4 and TIM-3 in the spleen and tumor at day 19 (two days after the final sequential anti-PD-1 dose). No treatment group resulted in significant changes to the frequency of $TIM\text{-}3^+$ $CD4^+$ cells in the tumor (Figure 3-14A). However, sequential combination maintained the frequency of TIM-3-expressing $CD8^+$ T cells generated by anti-OX40 monotherapy, avoiding the significant increase of this exhausted phenotype we noted with anti-PD-1 monotherapy or concurrent combination (Figure 3-14A). CTLA-4 expression was the opposite, with increased frequencies of $CTLA\text{-}4^+$ $CD8^+$

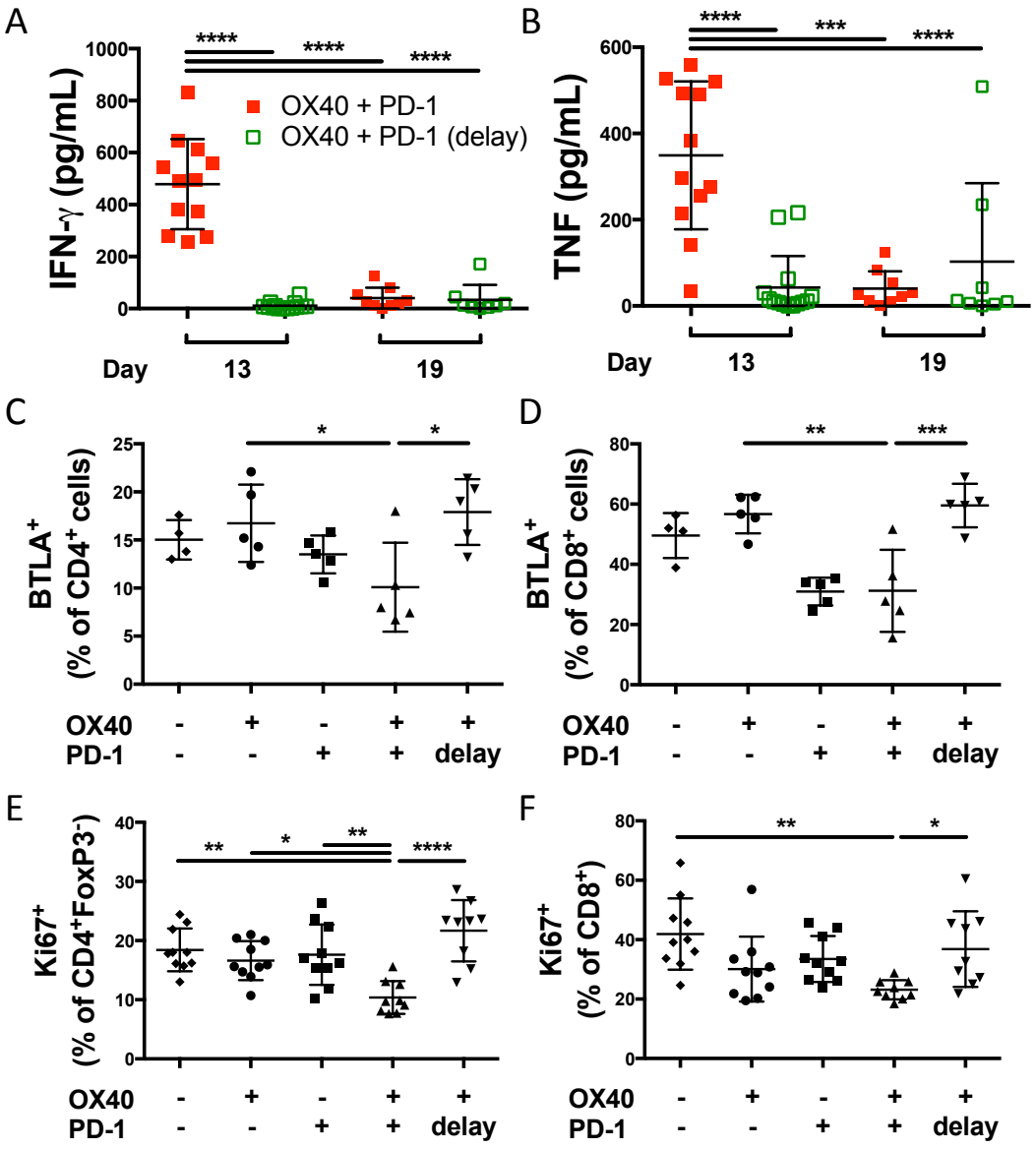

Figure 3-13: Anti-OX40 combined with delayed anti-PD-1 prevents acute serum cytokines increases and maintains less differentiated and proliferating intratumoral T cells. MMTV-PyMT tumor-bearing mice were treated as in Figure 3-9B. (**A,B**) Serum IFN-γ (**A**) and TNF (**B**) from day 13 and 19 animals treated with either concurrent anti-OX40 + anti-PD-1 or anti-OX40 + delayed anti-PD-1. n = 9-15, combination of two independent experiments, error bars represent SEM. (**C,D**) Frequency of BLTA$^+$ CD4$^+$ (**C**) and CD8$^+$ (**D**) cells from day 19 (two days after last delayed PD-1 treatment) tumors. n = 4-5. (**E,F**) Frequency of Ki67$^+$ proliferating conventional CD4$^+$FoxP3$^-$ (**E**) and CD8$^+$ (**F**) cells from day 19 tumors. n = 9-10, combination of two independent experiments. Error bars represent SEM. One-way ANOVA with Tukey's multiple comparison test were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

cells associating with the improved responses of anti-OX40 monotherapy and sequential combination (Figure 3-14A, 3-9C, 3-9D). Patterns of TIM-3 and CTLA-4 expression on CD8$^+$ T cells in the spleen were similar, but at much lower frequencies (Figure 3-14C). At this

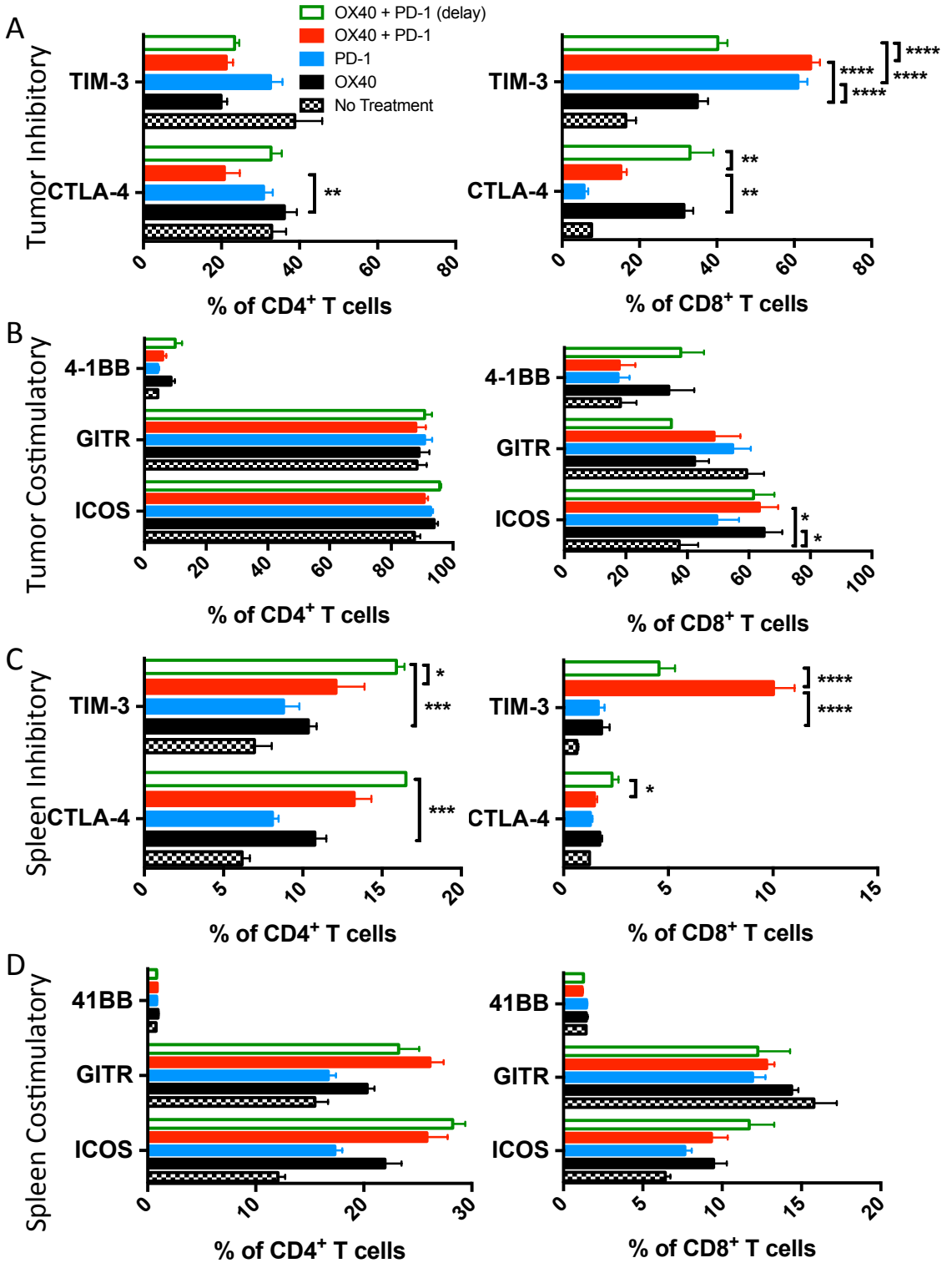

Figure 3-14: Anti-OX40 combined with delayed anti-PD-1 prevents increase in T cell inhibitory receptors. MMTV-PyMT tumor-bearing mice were treated as in Figure 3-9B. Flow cytometric quantification of surface expression of costimulatory and inhibitory receptors on CD4+ and CD8+ T cells from the tumor (**A,B**) and spleen (**C,D**) of MMTV-PyMT tumor-bearing mice treated with anti-OX40, anti-PD-1, or both, day 19 post tumor transplant (**A**) Frequency of TIM-3 and CTLA-4

inhibitory receptors expressed on intratumoral $CD4^+$ and $CD8^+$ T cells. (**B**) Frequency of 4-1BB, GITR, and ICOS costimulatory receptors expressed on intratumoral $CD4^+$ and $CD8^+$ T cells. (**C**) Frequency of TIM-3 and CTLA-4 inhibitory receptors expressed on splenic $CD4^+$ and $CD8^+$ T cells. (**D**) Frequency of 4-1BB, GITR, and ICOS costimulatory receptors expressed on splenic $CD4^+$ and $CD8^+$ T cells. n = 8-10, two independent experiments combined. Error bars represent SEM. One-way ANOVA with Tukey's multiple comparison tests were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$

later time point, the frequency of T cells expressing costimulatory molecules were not significantly altered, except for an increase in $4\text{-}1BB^+CD8^+$ T cells in the tumor with sequential combination treatment (Figure 3-14C, 3-14D). Combined these data demonstrate that concurrent combination treatment induces a strong, but short burst of T cell proliferation in the tumor, which coincides with acute cytokine secretion, an increase in $BTLA^-$ terminally differentiated and $TIM\text{-}3^+$ $CD8^+$ exhausted cells, and attenuated anti-tumor effect (Figure 3-2B, 3-2C). But administering anti-OX40 and anti-PD-1 in a sequential fashion maintains T cell proliferation in the tumor for a longer time frame (day 19), prevents acute cytokine secretion, and significantly reduces the TIL exhaustion and differentiation induced by concurrent combination treatment, providing a superior anti-tumor response (Figure 3-9C, 3-9D).

Given the increase in $TIM\text{-}3^+$ intratumoral and peripheral T cells after concurrent combination treatment, we next explored whether the attenuated effect of concurrent combination could be rescued or improved with additional checkpoint blockade targeting this inhibitory marker. We administered anti-TIM-3 antibodies with or without anti-CTLA-4 antibodies in sequence (beginning on day 13) after completion of concurrent anti-OX40 and anti-PD-1. As previously demonstrated (Figure 3-2B), anti-OX40 plus anti-PD-1 combination provided a significant tumor growth delay compared to untreated mice, but was not augmented with the addition of anti-TIM-3 or anti-TIM-3 combined with anti-CTLA-4 treatment (Figure 3-15). We hypothesize that despite increased TIM-3 expression on TIL, concurrent combination treated T cells may be incapable of being rescued due to

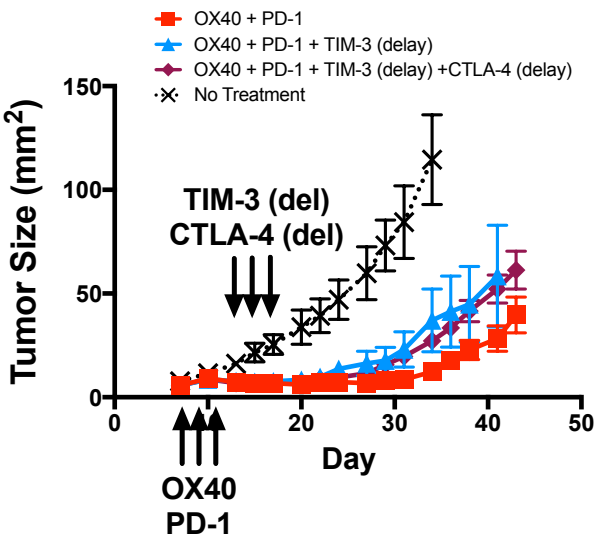

Figure 3-15: Delayed TIM-3 blockade does not rescue concurrent anti-OX40 plus anti-PD-1-treated animals. Mean tumor growth of MMTV-PyMT tumor-bearing mice treated with concurrent anti-OX40 plus anti-PD-1, followed by 250 µg. anti-TIM-3 with or without 100 µg. anti-CTLA-4 (delay, del) on days 13, 15, and 17. n = 6-7, one representative of two independent experiments is shown, error bars represent SEM.

exhaustion or dysfunction, as has recently been suggested by others (186,368). Alternatively, tumors may use other mechanisms of immune escape and suppression that is not impacted by additional checkpoint blockade.

Combination anti-OX40 plus anti-PD-1 increases tumor CD4⁺ T cell infiltrate, but only reduces proliferation of non-lymphocyte cells when given sequentially

We next asked if sequential combination treatment increased the recruitment of T cells into the tumors of treated mice. We utilized multispectral imaging to analyze T cell infiltrate on day 20 tumors treated with either concurrent or sequential combination of anti-OX40 and anti-PD-1 antibodies. Initially we noted an increase in $CD3^+$ T cell infiltrate in treated tumors regardless of the whether the combination was given concurrently or sequentially (Figure 3-16A, 3-16B). However, this increase seemed to be exclusively due to changes in $CD4^+$ cells, as $CD8^+$ cell numbers remained unchanged from untreated tumors (Figure 3-16C, 3-16D). The predominance of $CD4^+$ cells in the tumor, at this time point supported

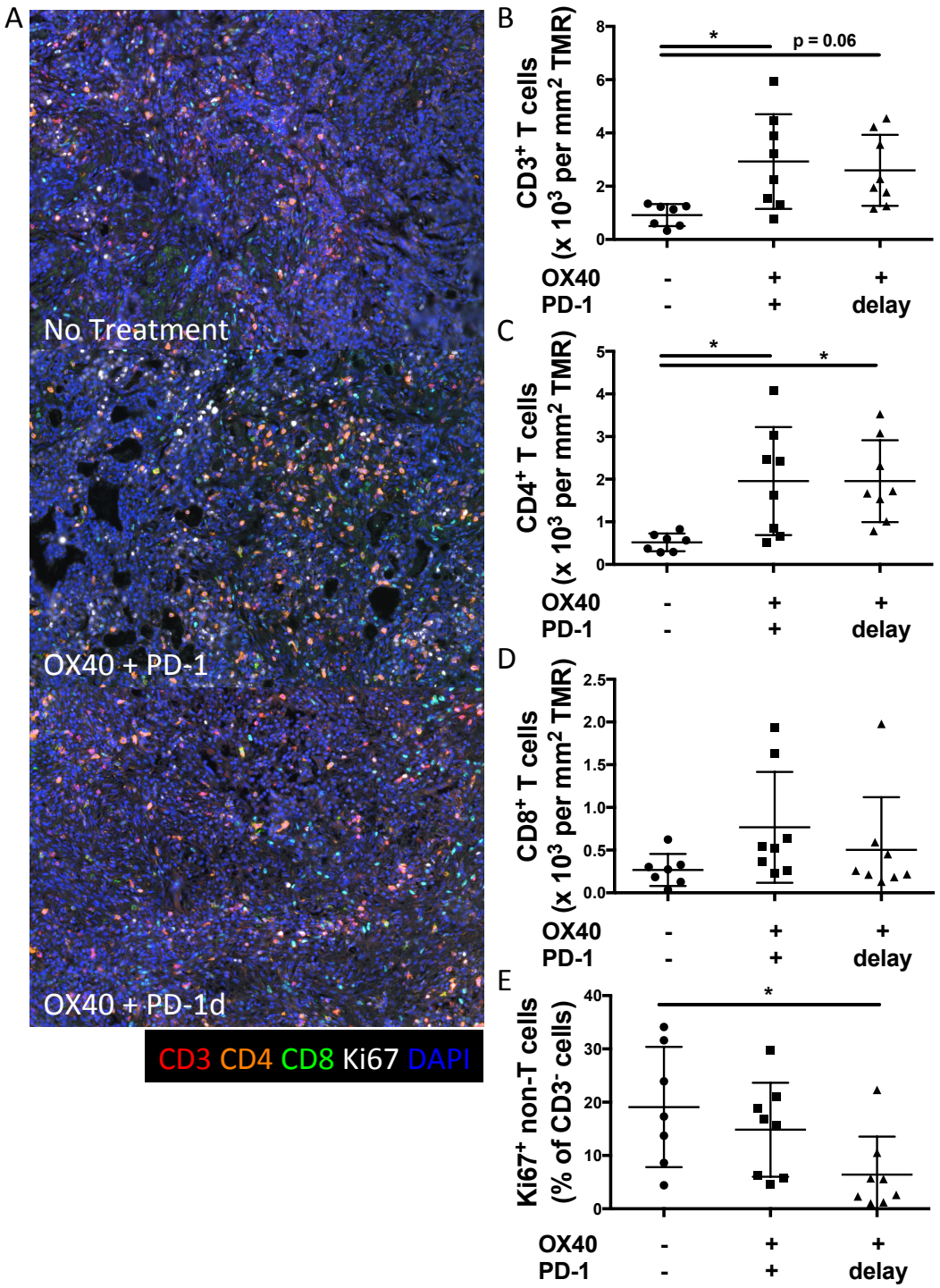

Figure 3-16: Combination treatment increases CD4⁺ T cell tumor infiltrate, but sequential combination decreases non-lymphocyte proliferation. . MMTV-PyMT tumor-bearing mice were treated with either concurrent (OX40 + PD-1) or sequential (OX40 + PD-1d) anti-OX40 plus anti-PD-1 as described in Figure 3-9B. Treated and untreated tumors were resected on day 20 and multispectral IHC was performed and analyzed. (**A**) Representative images of T cell "hot spot" fields. (**B-E**) Quantification of total $CD3^+$ (**B**), $CD3^+CD4^+$ (**C**), $CD3^+CD8^+$ (**D**), $Ki67^+DAPI^+CD3^-$ (**E**) cells per mm^2 of tumor. n = 7-8, two independent experiments combined, error bars represent SEM One-way

ANOVA with Tukey's multiple comparison tests were performed for statistical significance. * = $p < 0.05$

previous data indicating that the majority of T cells in treated and untreated tumors were $CD4^+$ on day 13 (Figure 3-7D). $CD4^+$ cells only outnumbered $CD8^+$ cells by a ratio of about 2:1, much less than the ratio we measured previously by flow cytometry at day 13 (~4-5:1). However this difference may be attributed to either the method of quantification (IHC versus flow cytometry) or difference in time points (day 13 versus day 20). Despite the lack of disparity in the ability to increase T cell infiltrate, sequential combination treatment significantly reduced the frequency of $Ki67^+$ proliferating non-lymphoid cells ($CD3^-$ cells, which includes both myeloid and tumor cells), while concurrent combination had no significant effect (Figure 3-16E). These data demonstrate that although sequencing combination therapy induced no gross difference in T cell infiltrate compared to concurrent combination at this time point, TIL in these mice were more effective at slowing tumor proliferation (and tumor growth, Figure 3-9C), which eventually leads to complete regression in some mice and improved survival (Figure 3-9D).

Anti-OX40 plus anti-PD-1 combination treatment targeting T cells alters intratumoral myeloid cells

Myeloid cells and more specifically TAMs play a major role in tumor development, progression, and metastasis in many human malignancies (369-371) and also in the murine MMTV-PyMT tumor (312,316). We next asked whether our T cell-targeted combination therapy impacted the myeloid compartment of treated tumors. In analysis of the $CD45^+CD3^-$ myeloid infiltrate in day 19 tumors, we noted no significant differences in the frequencies of $CD11b^+MHCII^-$, $CD11b^-MHCII^+$, or $CD11b^+MHCII^+$ cells in the $CD45^+CD3^-$ myeloid populations of tumors treated with either concurrent or sequential anti-OX40 plus anti-PD-1 combination treatment (Figure 3-17A-E). However, sequential combination and anti-

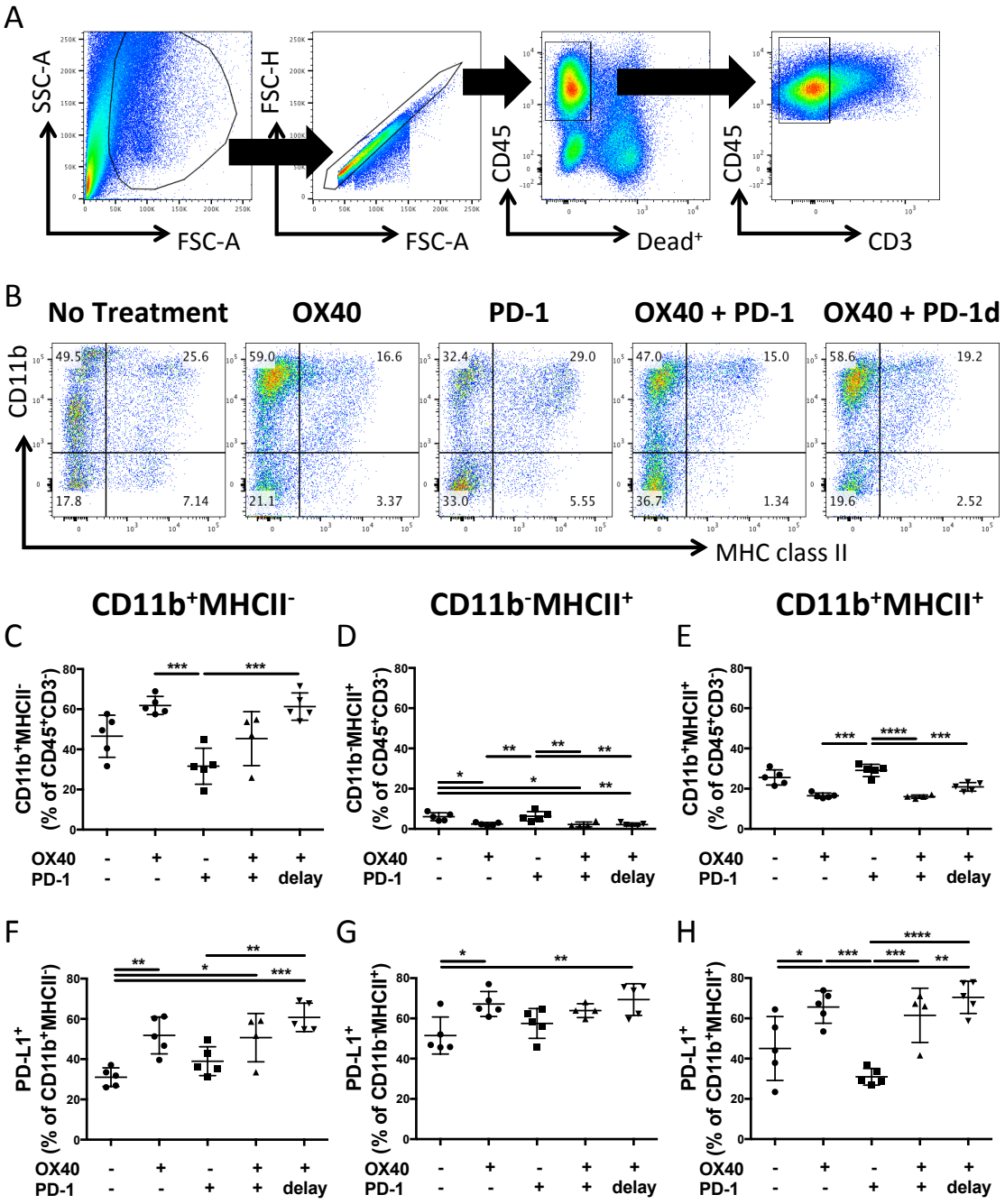

Figure 3-17: Anti-OX40 and anti-PD-1 combination treatment alters intratumoral myeloid populations and upregulates PD-L1. MMTV-PyMT tumor-bearing mice were treated with anti-OX40, anti-PD-1, or a concurrent or sequential combination. Tumors were resected on day 19 for analysis. (**A**) Flow cytometry gating strategy for phenotyping $CD45^+CD3^-$ myeloid cells. (**B**) Representative flow cytometry plots of CD11b and MHC class II expression on live, $CD45^+CD3^-$-gated cells. (**C-E**) Quantification of percentage of $CD11b^+MHCII^-$ (**C**), $CD11b^-MHCII^+$ (**D**), and $CD11b^+MHCII^+$ (**E**) cells. (**F-H**) Frequency of $PD\text{-}L1^+$ cells in the populations from C-E. n =4-5, one representative of two independent experiments. Error bars represent SEM. One-way ANOVA with Tukey's multiple comparison tests were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

OX40 monotherapy (both associated with a significant anti-tumor response (Figure 3-2B, 3-2C and Figure 3-9C, 3-9D)) induced an increase in the frequency of $CD11b^{+}MHCII^{-}$ cells compared to anti-PD-1 monotherapy (Figure 3-17C). No treatment significantly increased this population compared to untreated tumors however (Figure 3-17C). Significant decreases in both $CD11b^{+}MHCII^{-}$ and $CD11b^{+}MHCII^{+}$ populations also associated with response to treatment (Figure 3-17C, 3-9C). PD-L1 expression on both $CD11b^{+}MHCII^{-}$ and $CD11b^{+}MHCII^{+}$ cells was significantly augmented with either concurrent or sequential combination therapy and associated with anti-tumor response (Figure 3-17F, 3-17H). However, only sequential combination treated tumors had a higher frequency of $PD\text{-}L1^{+}$ cells than both untreated and PD-1 monotherapy (Figure 3-17F, 3-17H). This increase in PD-L1 expression on the sequentially treated group may be attributed to only receiving a dose of anti-PD-1 two days prior and/or an increase in local IFN-γ from an anti-tumor response. These data indicate that TAMs and other myeloid populations in the tumor can indeed be altered by T cell-targeted combination therapy, and suggest that PD-L1 expression on these cells may be used as a biomarker for response to therapy with T cell-targeted treatments.

Anti-tumor effect of OX40 costimulation combined with delayed PD-1 blockade requires both $CD4^{+}$ and $CD8^{+}$ T cells for optimal therapeutic effect

Given the contribution that $CD4^{+}$ T cells can have to T cell help and the high prevalence of $CD4^{+}FoxP3^{-}$ T cells in MMTV-PyMT tumors (Figure 3-7B, 3-7D), we sought to evaluate the prevalence and necessity of $CD4^{+}$ and $CD8^{+}$ T cells in the anti-tumor effect observed from sequential combination treatment. Phenotyping tumor infiltrating T cells in anti-OX40 plus delayed anti-PD-1-treated tumors on day 19, two days after the last anti-PD-1 dose, we noted conventional $CD4^{+}FoxP3^{-}$ T cells still made up a majority of the T cells in treated

tumors (Figure 3-18A). To assess the role of different T cell populations, either $CD4^+$ depleting, $CD8^+$ depleting, or Rat IgG non-depleting antibodies were injected on days 6 and 13 into MMTV-PyMT tumor-bearing mice before both anti-OX40 (beginning on day 7) and anti-PD-1 (beginning on day 13) were administered as described above (Figure 3-9B). Depletion resulted in greater than 90% reduction of the specific T cell population in the tumor (Figure 3-18A). $CD4^+$ T cell ablation completely eliminated the therapeutic efficacy provided by sequential anti-OX40 plus anti-PD-1, proving their necessity for this combination treatment (Figure 3-18C). $CD4^+$ T cell depletion also led to a significant increase in tumor infiltrating $CD8^+$ T cells compared to non-depleted tumors (Figure 3-18A, 3-18B), but despite this increase there was no therapeutic benefit from sequential combination treatment without $CD4^+$ T cells. $CD8^+$ T cells were also necessary for the observed therapeutic efficacy (Figure 3-18D). Although initially the therapeutic effect was unaltered, loss of tumor control became apparent at later time points (after day 30). Consequently, depletion of either $CD4^+$or $CD8^+$ T cells in combination treated mice reduced animal survival, although $CD8^+$ depleted mice had significantly longer ($p < 0.01$) survival than $CD4^+$ depleted or untreated animals (Figure 3-18E). These data strongly indicate that while conventional $CD4^+$ T cells play a major and necessary role in the immediate tumor control provided by sequential anti-OX40 plus anti-PD-1, $CD8^+$ T cells are also necessary for long-term anti-tumor effect.

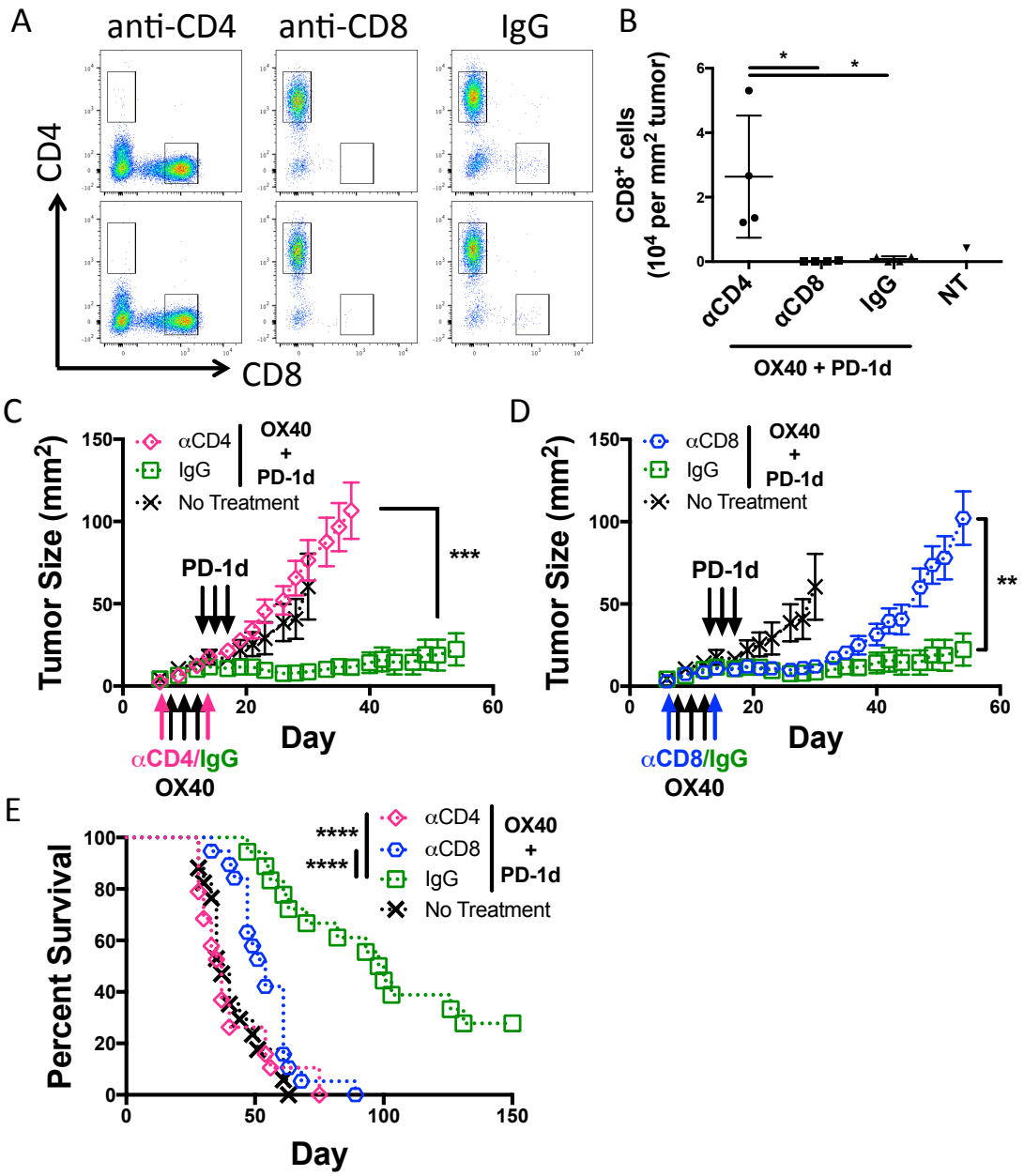

Figure 3-18: Anti-OX40 combined with delayed anti-PD-1 requires both CD4⁺ and CD8⁺ T cells for optimal therapeutic effect. MMTV-PyMT tumor-bearing mice were treated with anti-OX40 plus delayed anti-PD-1 (PD-1d), combined with 250 µg anti-CD4, 250 µg anti-CD8, or 250 µg rat IgG on days 6 and 13 (**A**) Representative plots of $CD45^+CD3^+$ T cells in the tumors of two CD4 or CD8 depleted or rat IgG-treated mice on day 20. (**B**) Quantification of total $CD8^+$ cells per mm^2 of tumor on day 20-21 determined via flow cytometry. n = 4, combination of two independent experiments. (**C-E**) Tumor growth of combination treated CD4-depleted (**C**) or CD8-depleted (**D**). n = 9-10, one representative of two independent experiments shown. Error bars represent SEM. (**E**) Survival of CD4 or CD8-depleted mice. n = 18-20, two independent experiments combined, error bars represent SEM. One-way ANOVA with Tukey's multiple comparison test (B, C, D) and Gehan-Breslow-Wilcoxon test (E) were performed for statistical significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

Discussion:

Antibodies targeting PD-1 and PD-L1 have recently been approved by the FDA for the treatment of multiple solid tumors. When combined with antibodies targeting CTLA-4, anti-PD-1 has shown substantial effects in clinical trials, with up to 50% of late stage melanoma patients experiencing tumor regression (212,213). While this combination treatment appears to be an improvement from monotherapies with either agent, a considerable portion of patients remain refractory to current combination immunotherapies and require other treatment options. Looking to the future it seems that combinations of immuno-oncology agents will offer improved results compared to monotherapies. While recent trials have focused on combined checkpoint blockade, agents that target costimulatory molecules such as OX40 may offer a novel approach to augment the effect of current immunotherapies targeting inhibitory receptors like PD-1. Treatments combining T cell-targeting antibodies with vaccines, adoptive T cell transfer, and targeted therapies are also being extensively explored preclinically (372-377). However, the designs of these types of studies are often empirical, and take a straightforward additive approach that may not be optimal considering the unique basic immunological mechanisms that influence combination treatment.

The questions of timing and sequence are important aspects of study design when complementing chemotherapy, radiotherapy, or targeted therapies, but as novel immunotherapies continue to demonstrate significant clinical impact it will be important to design treatment regimens that recognize and optimize compensatory, regulatory, and homeostatic immune mechanisms. While others have demonstrated that extended checkpoint blockade is beneficial with preclinical combination immunotherapies (295,378), to our knowledge ours is the first report to directly compare concurrent combination treatment with planned sequential treatment with the same agents. Given that a substantial

portion of patients does not respond to PD-1 blockade (in monotherapy or in combination) (136,212), our findings in a model system that is refractory to anti-PD-1 therapy suggest that the use of costimulatory stimulation may impact these patients. Anti-OX40 was able to drive more T cells into the tumor (Figure 3-7), and PD-1 blockade could then be combined in a sequential manner to further impact these T cells, preventing them from becoming exhausted. As clinical trials employing antibodies targeting other costimulatory molecules such as GITR and 4-1BB are currently being initiated, some in combination with established checkpoint blockade like PD-1/PD-L1 and CTLA-4 and novel inhibitory targets such as LAG-3 and TIM-3, understanding not just which T cell costimulatory or inhibitory molecules to stimulate or block but when to stimulate or block them is likely to be a critical component of an optimal therapeutic regimen. Our group highlighted this concept in a recent paper demonstrating the importance of timing when anti-CTLA-4 checkpoint blockade or anti-OX40 costimulation is combined with radiation (166).

In our studies concurrent anti-OX40 and anti-PD-1 attenuated anti-OX40-induced therapeutic efficacy and dramatically increased serum cytokine levels. A previous report noted reduced gene expression of BTLA in T cells treated with anti-OX40 and anti-PD-L1 (355). Similarly, we noted a significant decrease in peripheral and intratumoral T cells expressing BTLA after concurrent anti-OX40 plus anti-PD-1 treatment (Figure 3-6E, 3-6F, 3-13C, 3-13D), and others have suggested these $BTLA^-$ cells are less effective in the tumor, with reduced proliferative capacity and decreased responsiveness to costimulatory molecules (27). We also observed reduced proliferation in concurrent combination-treated T cells in the tumor, six days after the conclusion of treatment (Figure 3-13E, 3-13F), despite a significant increase in proliferation six days prior (Figure 3-7G, 3-7H). Also supported by our observation of less $BTLA^+$ T cells in the tumor at day 19 (Figure 3-13C, 3-13D), we reason that concurrent combination of anti-OX40 and anti-PD-1 impacts T cell

differentiation, indicated by loss of BTLA expression, and these terminally differentiated cells are less proliferative long term, making them less capable of mediating tumor destruction (Figure 3-19). Differentiation status of T cells is a key determinant of a T cell-mediated anti-tumor response, and increased success of adoptive T cell transfer has been linked with less differentiated cells that maintain higher proliferative capacity (379,380). In addition to a loss of BTLA, surface expression of other inhibitory receptors LAG-3, TIM-3, and CTLA-4 on T cells were also significantly

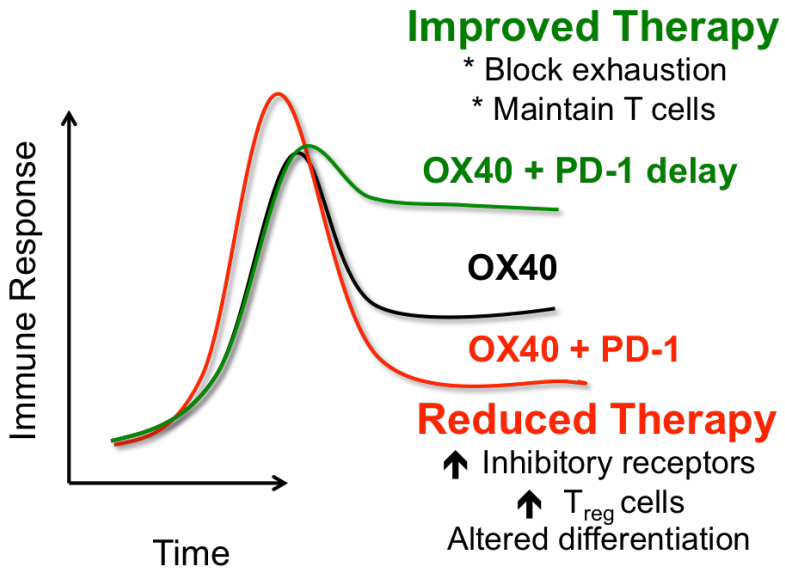

Figure 3-19: Proposed model for immune response to the combination of anti-OX40 and anti-PD-1 treatment.

amplified with concurrent combination treatment. This suggests that although TIL numbers from tumors treated with concurrent or sequential combination antibodies (Figure 3-16C, 3-16D) is not dramatically different, there is a difference in quality or functional capacity of these TIL. It is also important to note that T cells activated or primed with anti-OX40 of this improved quality were critical to the effectiveness of PD-1 blockade, as delayed anti-PD-1 had no effect on tumor growth compared to those treated with anti-OX40 (Figure 3-9F), despite untreated tumors having similar PD-1 expression at that time point (Figure 3-9A). As new IHC reagents become available and the amount of phenotypic markers that can be

identified in a single image increases, we hope to further elucidate differences in TIL populations using multispectral imaging. Nearest neighbor analyses can also be used to tease out the differences in TIL that make sequential therapy so much more effective than concurrent combination treatment.

We have demonstrated that sequential combination of anti-OX40 plus anti-PD-1 does not induce high cytokine levels or upregulate inhibitory receptors to the levels induced by concurrent combination. Given that effector cytokines can induce expression of the identified inhibitory molecules, this may likely be another mechanism to explain the attenuated effect of concurrent combination, which we hypothesize is a factor contributing to efficacy compared to concurrent combination (Figure 3-13A, 3-13B). With combination immunotherapies capable of inducing a strong cytokine response, the risk of "whack-a-mole", where one therapeutic target is neutralized but one or more other inhibitory receptor pops up, is a very real possibility, highlighted by our data with the concurrent combination of anti-OX40 and anti-PD-1. This effect is also seen clinically, where responses to anti-PD-L1 in patients with advanced cancers have been associated with increased expression of CTLA-4 (327). We also noted an increase in CTLA-4 expression on $CD8^+$ T cells in sequentially treated mice (Figure 3-14A, 3-14C), supporting this inhibitory receptor as a possible biomarker associated with positive response. However these upregulated inhibitory receptors also present possible targets for treatment, as well as possible biomarkers to monitor the success of combination treatments in generating an appropriate milieu to support a therapeutic anti-cancer immune response. In support of this concept, Koyama et al. demonstrated that tumor-bearing mice that progressed on anti-PD-1 upregulated TIM-3 on TIL (381). Consequently the anti-tumor effect of anti-PD-1 was greatly augmented by the sequential addition of an anti-TIM-3 antibody. However our data demonstrates that additional TIM-3 blockade may not be universally effective for those who

don't respond to anti-PD-1 therapy (Figure 3-15), and further research on the roles that PD-1 plays in both exhaustion and normal T cell function is needed.

While a tumor-specific T cell cytokine response has often been used as a biomarker for successful anti-tumor response, high levels of cytokines generated by immunotherapies are known to have negative and in some cases substantial consequences (382). Indeed mice treated with repeated doses of an agonist to OX40 or GITR showed a spike in IL-4 and severe toxicity (383) and 4T1-tumor bearing mice given repeated doses of anti-PD-1 or anti-PD-L1 resulted in fatal toxic effects (384). We observed major increases in serum cytokines in mice treated concurrently with the combination of anti-OX40 and anti-PD-1 and often these mice showed signs of toxicity such as ruffled fur and lethargy for roughly 2-4 days after treatment concluded. In some experiments mice in the combination treated groups even perished at the conclusion of treatment (approximately 15 days after tumor transplantation). Interestingly these experiments specifically used mice that were aged from 5-6 months. Experiments using younger mice aged 8-10 weeks showed signs of toxicity, however the effects were milder and all survived. Previous reports have shown substantial toxic events (including death) with treatment of an agonist to CD40 in combination with IL-2 in aged (> 16 months) populations of mice that did not occur in younger mice (385). Also, OX40 costimulation has reduced effects in older mice (386), suggesting that augmenting anti-OX40 with combination treatments may be important in elderly patients with cancer. Models utilizing humanized mice are currently being used to test for acute toxicity with monoclonal antibody treatment (387), however, given the variations inherent in humans as well as in preclinical models, it seems unlikely that the complexities of immune-associated toxic events can be determined in such models. Thus, determination of safety will require carefully executed phase I clinical trials of the combinations to be evaluated.

A prior report noted a synergistic anti-tumor effect from concurrent combination of anti-OX40 and anti-PD-1 (388). However the ID8 model of ovarian cancer used was impervious to anti-OX40 or anti-PD-1 monotherapy, and it is important to remember that in our transplanted MMTV-PyMT model the therapeutic effect of concurrent combination, although inferior to anti-OX40 alone, was not completely abrogated and did have a significant effect on tumor growth (Figure 3-2B). Thus we hypothesize that treatment of anti-OX40 combined with sequential anti-PD-1 may in fact augment the effects of concurrent combination in the ID8 model. While the synergistic combination effect in the ID8 model also required both $CD4^+$ and $CD8^+$ T cells, $CD4^+$ cells were more dispensable than $CD8^+$ cells, the opposite of our own findings. Disparities in tumor immunogenicity, tumor infiltrate, TAAs, and the mechanism of tumor regression between model systems may explain the differences between our work and theirs.

Depletion of constitutively CTLA-4-expressing regulatory T cells through ADCC has been suggested as a key mechanism for the anti-tumor effect of the anti-CTLA-4 antibody Ipilimumab (58,244). However, unlike CTLA-4, PD-1 is expressed on a broader range of T cells, including T_{reg} cells, but also many effector cells that may be necessary for an anti-tumor response. Thus depletion of $PD\text{-}1^+$ cells by PD-1-specific antibodies may actually hinder an anti-tumor response. In support of this, a recent report suggests that an anti-PD-1 blocking antibody that does not interact with Fcγ receptors and subsequently does not deplete $PD\text{-}1^+$ cells, is more effective than other clones which engage Fcγ receptor-expressing cells capable of ADCC induction (249). The G4 anti-PD-1 clone used in our work has a hamster IgG isotype, and previous work with this antibody has not demonstrated significant T_{reg} cell depletion in the liver (278) or brain tumors (389). Krempski et al. even identified a significant increase in intratumoral tumor-specific $CD8^+$ T cells after administration of G4 antibody (390). Additionally a previous publication using an anti-

CTLA-4 hamster IgG antibody also noted no depletion of T_{reg} cells, providing broader evidence that hamster IgG antibodies may not deplete (40). Using OT-1 cells in a non-tumor bearing vaccination model, we also saw no depletion of these OVA-specific $CD8^+$ cells after treating with G4 antibody (data not shown). Although we noted a significant decrease in T_{reg} cells in combination treated MMTV-PyMT tumors compared to those treated with anti-OX40 alone, there was no significant change between untreated and G4-treated tumors (Figure 3-8D). Additionally, the G4 antibody alone had no effect on the total number of $CD4^+FoxP3^-$ or $CD8^+$ cells compared to untreated tumors (Figure 3-7E, 3-7F). So while the possibility of T_{reg} cell depletion with the G4 antibody remains, it seems that it is limited to combination treatment, and not monotherapy.

A previous report utilizing T_{reg} cell ablation in this model system, noted a significant delay in tumor growth, but no additive benefit with PD-1 blockade (303). Another publication reported intratumoral T_{reg} cell depletion with monotherapy of the anti-OX40 clone OX86 (391) but our data demonstrated the opposite effect, with OX86 significantly increasing intratumoral T_{reg} cells (Figure 3-8D). While T_{reg} cells were actually increased in the spleen with combination treatment in our experiments (Figure 3-8C), it is possible that an increase in Fcγ receptor expression in the tumor microenvironment could lead to ADCC and specific depletion of T_{reg} cells there. TIL may express both OX40 and PD-1 and thus combination treatment would result in two different isotypes of antibodies bound to their surface. Crosslinking of one isotype's Fc with a specific Fcγ receptor on the surface of macrophages could decrease the proximity to other Fcγ receptors on the same cell, and increase the chances of ADCC and depletion. At the initiation of treatment (day 7), transplanted MMTV-PyMT tumors have scant TIL that express both OX40 and PD-1 (Figure 3-1), but expression of these molecules can be transient and double positive cells may exist

in higher numbers at later time points. This may explain the loss of T_{reg} cells in combination treated tumors compared to those treated with anti-OX40 alone.

$CD4^+$ T cells can provide cytokine support to "help" $CD8^+$ T cells, but have also demonstrated cytotoxic capabilities (392,393). Conflicting reports over the necessary contribution of $CD4^+$ and $CD8^+$ T cells for therapeutic benefit in the MMTV-PyMT model have been published, with some reporting $CD4^+$ T cells to be dispensable and in fact detrimental (312) and some suggesting that $CD8^+$ T cells are dispensable while conventional $CD4^+$ T cells are necessary (303). Our findings, showing a variance in the timing of necessary T cell populations provides a possible explanation for these discrepancies (Figure 3-18). While myeloid-targeted therapies such as anti-CSF1R may require $CD8^+$ T cells at early time points (316), some T cell-targeted therapies may not. Transient regulatory T cell ablation does not require $CD8^+$ T cells (303), but alone this therapy only slows tumor growth for a limited period of time before continued progression. Anti-OX40 combined with delayed anti-PD-1 provides a much longer period of stable disease, with some tumors reaching full regression. Given that OX40 is expressed almost exclusively on intratumoral $CD4^+$ T cells in this model, it shouldn't be surprising that an anti-OX40-generated effect would require $CD4^+$ T cells, even when the PD-1 pathway is also targeted. In support of this, when treated tumors were depleted of $CD4^+$ cells, even a significant increase in $CD8^+$ T cell tumor infiltration had no effect on tumor growth. Alternatively, CD8 depletion saw initial tumor growth delay, followed by rapid tumor progression, indicating that the early anti-tumor effect provided by sequential combination treatment is in fact independent of $CD8^+$ T cells, but these cells are necessary for a durable memory response. We hypothesize that since few intratumoral $CD8^+$ T cells express OX40 at the time of anti-OX40 treatment and the early anti-tumor effects are dependent only on $CD4^+$ T cells, the "help" provided by anti-OX40 stimulated $CD4^+$ T cells drives $CD8^+$ cells to

long term functionality and makes them necessary for long term tumor regression. Future experiments should determine how long $CD8^+$ T cells remain depleted in this model and by varying when depleting antibodies are given, establish the mechanisms by which $CD8^+$ T cells contribute to long-term tumor regression. Consistent with this hypothesis, anti-OX40 combined with delayed anti-PD-L1 (1 and 3 days post anti-OX40) treatment saw a boost in T cell function when antigen-specific $CD8^+$ T cells were transferred with $CD4^+$ help (355). Thus it seems clear that long-term therapeutic efficacy targeting OX40 and PD-1 in this model requires both $CD4^+$ and $CD8^+$ T cells, which corroborates a previous report utilizing anti-OX40 treatment alone in another tumor model (161).

Our results demonstrate that in some models the sequence and timing of antibody treatment targeting both costimulatory and inhibitory receptors is critical to success of the combined therapy. These data offer a strong rationale for delaying PD-1 or PD-L1 blockade or possibly other inhibitory receptor-targeted therapies such as CTLA-4, TIM-3, or LAG-3 until after costimulation has boosted the tumor-specific T cells to a state where checkpoints are inhibiting the anti-tumor response. Sequential treatment has the combined benefit of both optimizing the anti-tumor immune response as well as potentially minimizing possible toxicity from acute cytokine release. Given the complex nature of T cell costimulatory and inhibitory signaling, our data underscores the delicate balance that the immune system maintains and suggests a number of factors that ought to be explored in combination immunotherapy clinical trial designs.

CONCLUDING REMARKS

Immunotherapy is currently riding a wave of recent successes. From FDA approval for checkpoint inhibitors targeting the PD-1 and CTLA-4 receptors, to promising clinical trial results with CAR-transfected T cells and other methods of adoptive T cell transfer, clinical oncology has begun to embrace immunotherapy as a possible pillar of cancer care, joining radiation, chemotherapy, and surgery (targeted therapy is considered by some to also be a pillar). Hanahan and Weinberg's classic "Hallmarks of Cancer" (394) has even been updated to include evading the immune system (77). However, with this success, major new hurdles emerging revolve around what to do with patients who don't or won't (based on biomarker analysis) respond to checkpoint blockade. With these questions looming it appears that the field has plucked the low hanging fruit of blocking inhibitory receptors for "hot" tumor patients who already have primed a sufficient anti-tumor immune response ready to be unharnessed from inhibition. The next steps will be to focus on patients who do not respond to checkpoint blockade. For this reason we have tended to focus our preclinical work on utilizing murine model systems that are not strongly immunogenic and do not respond to single agent checkpoint blockade. Understanding the mechanisms of why some combination immunotherapies work in these models and others do not is incredibly valuable to shape clinical therapies for a wider variety of cancer patients in the future.

Many patients who do not respond to checkpoint blockade have "cold" tumors and will likely require a new anti-tumor immune response to be generated. Vaccination offers these patients the chance to prime a novel anti-tumor T cell response. The studies in chapter two demonstrate that the differing tumor immunogenicities of MCA-induced sarcomas MCA-304 and MCA-310 do not alter their protective effect when given as multiple vaccinations of a GM-CSF-secreting whole-cell tumor vaccine (Figure 2-4). Nor does the addition of GM-CSF alter the booster vaccinations efficacy with either tumor (Figure 2-4).

We initiated these experiments to address questions generated from previous work demonstrating reduced therapeutic efficacy with multiple vaccinations of poorly immunogenic GM-CSF-secreting melanoma D5-G6 (334). We do not have a strongly immunogenic melanoma to compare to the poorly immunogenic D5, and thus we postulated that using two MCA-induced tumors with opposing immunogenicities would be sufficient models to address our questions.

While the experiments in chapter two did not directly answer our initial question of whether efficacy of multiple vaccinations with D5-G6 attenuated single vaccine efficacy due to the addition of GM-CSF or the poor tumor immunogenicity of the D5 tumor, further examination by multispectral IHC of TIL generated from multiple vaccinations of the melanoma D5 or MCA-310 provide a plausible hypothesis for differences between the two tumor models. While multiple prophylactic vaccinations with MCA-310 or the GM-CSF-secreting MCA-310GM did not alter the number of T_{reg} cells in the tumor (Figure 2-7), GM-CSF-secreting D5-G6-vaccinated tumors had more T_{reg} cells than the parental D5 tumor (Figure 2-8). Given that our previous report demonstrated a large induction of T_{reg} cells with multiple vaccinations of D5-G6, this experiment confirms that T_{reg} cells are generated with multiple vaccinations of D5-G6, but importantly now demonstrates that this increase in peripheral T_{reg} cells correlates with an increase in the tumor. Combining these data with the disparities in survival we noted compared to the MCA-induces sarcomas, we believe that these models contrast in their use of T_{reg} cells as a tumor inhibitory mechanism. Thus our initial attempts to decouple the possible inhibitory effects of multiple vaccinations with GM-CSF from potential loss of efficacy due to the poor immunogenicity of the D5 tumor resulted in the realization that MCA-induced sarcomas do not accurately mimic the D5-G6-vaccinated tumor microenvironment. Thus they are not the correct model systems to address questions of whether multiple vaccinations of a GM-CSF-secreting strongly

immunogenic melanoma will attenuate its single vaccine efficacy. This emphasizes the value that multispectral imaging, with its ability to identify multiple phenotypes of T cells in the tumor, can have on evaluating the tumor microenvironment and potentially using this information to tailor personalized immunotherapy regimens.

Undoubtedly, the most impactful finding in chapter two, with the greatest translational implications, is the effect GM-CSF had on T cell tumor infiltrate in multiple vaccinated tumors. Multiple vaccinations with GM-CSF-secreting MCA-310GM significantly increased both overall T cell ($CD3^+$) and $CD8^+$ T cell infiltrates compared to multiple vaccinations without GM-CSF (Figure 2-7). Given the importance of TIL for both overall survival and response to both conventional therapy and immunotherapy (209,250,357), this provides further support for the continued use of GM-CSF as an adjuvant for cancer vaccination. By trafficking more T cells into the tumor, assuming that many of them are tumor-specific, vaccination increases the chance of response and induces a better tumor microenvironment to augment responses with other immunotherapies such as checkpoint blockade or costimulatory antibodies. Our studies have shown no indication that booster vaccinations will inhibit T cells primed with a first vaccination. Others have noted a synergistic effect with GM-CSF-secreting vaccines and agonist antibodies specific for OX40 (162). In support of this, unpublished work from our lab has also demonstrated a synergy with therapeutic vaccination and anti-OX40.

GM-CSF has critical effects on DCs (395), and therefore combining it with vaccination is believed to affect antigen presentation and the priming phase of a T cell response. Therefore the ability of GM-CSF to alter T cell trafficking to the tumor most likely occurs during T cell priming. Understanding the mechanisms of how this occurs, whether through alteration of chemokine receptors, the cytokine milieu, or T cell differentiation status, will improve the chances of successful cancer vaccination in the clinic moving

forward. This is critical not only for therapeutic vaccines, but also for prophylactic cancer vaccines. With improvements in peptide identification and bioinformatics, it is not implausible that in the near future prophylactic vaccination will be expanded to include the prevention of other cancer types besides human papilloma virus-induced cervical cancers, and the use of GM-CSF with these vaccines is an attractive adjuvant to bolster vaccine efficacy.

Unlike "cold" tumors that may require vaccination to prime a new anti-tumor response, the untreated transplanted MMTV-PyMT tumors we used in chapter three have a significant amount of TIL that can be activated without vaccination. However, given that these tumors do not respond to anti-PD-1 monotherapy (Figure 3-2), we believe that they are a reasonable preclinical model to represent breast cancer patients who also do not respond to anti-PD-1 (211). Despite being refractory to PD-1 checkpoint blockade, MMTV-PyMT tumor-bearing mice clearly respond to costimulation with anti-OX40 (Figure 3-2). Yet the effect of sequentially combining anti-OX40 and delayed dosing of anti-PD-1 far exceeded that of anti-OX40 alone (Figure 3-9). These results substantiate the potential of combination immunotherapies. However when anti-OX40 and anti-PD-1 were concurrently combined they showed a reduced anti-tumor effect compared to anti-OX40 monotherapy (Figure 3-2). Thus not all combinations of immunotherapies will be effective. The most important aspect of our findings is that combination therapies that appear to be ineffective can be drastically augmented with a change as simple as altering the timing of administration. This has substantial implications for the development of combination immunotherapies in the clinic, demonstrating a quick possible approach to remedy drug combinations that had previously shown little or no additive effect.

Changes to current methods of combining immunotherapies will require mechanistic studies to understand why combinations do or do not work together. The basic

science behind individual immunotherapy reagents is much more extensive than any reagents in combination, which is not surprising given the short amount of time since many combinations have been preclinically attempted. We demonstrated that the concurrent combination of anti-OX40 and anti-PD-1 dramatically increased the secretion of inflammatory cytokines (Figure 3-3), T cell inhibitory receptors (Figure 3-4, 3-5), spleen size (Figure 3-6), and the differentiation of T cells (Figure 3-6). Together this is evidence of a massive immune response not evident in groups treated with monotherapy or a sequentially administered combination, which correlates with a significantly attenuated anti-tumor effect (Figure 3-2). We postulate that this immune response was too broad and excessive, leading to both toxicity in the combination treated mice and the expansion of more non-specific T cells than tumor-specific T cells, which were not useful in the anti-tumor response. These data, along with other preclinical reports of cytokine release syndrome with repeated dosing of antibodies targeting T cell costimulatory or inhibitory receptors (383,384), highlight the delicate balance between function and dysfunction that the immune system maintains. Thus the field needs to remain cautious when using agents that target costimulatory molecules, especially in combination with other immunotherapies.

The theories of a relationship between the immune system and cancer William Coley and Paul Ehrlich first hypothesized over 100 years ago are finally beginning to be more broadly applied with the recent success of cancer immunotherapy. While checkpoint blockade may be immunotherapy's low hanging fruit that can impact a substantial, but minority of patients, much work still needs to be done to improve combination immunotherapies as the overall risk of cancer in the general population continues to rise. Through the work of both basic scientists and clinicians, great strides have been made in identifying additional suppressive mechanisms that negatively impact anti-cancer immune responses. Using biomarkers, the ability to measure a wide variety of compensatory

suppressive mechanisms after initial immunotherapy and personalize combination treatments to overcome this suppression will surely improve patient responses. Thus as a cancer researcher and more specifically a tumor immunologist, I remain optimistic that through improved combinations of both immunotherapeutic and conventional methods we will continue to not only improve overall survival rates, but see a majority of patients enjoy durable, complete responses. Unites States vice president Joe Biden recently suggested as much, saying: "Several cutting-edge areas of research and care—including cancer immunotherapy, genomics, and combination therapies—could be revolutionary." I agree with the vice president's statement and hope that my contributions to the field in this thesis will be transitioned from bench to bedside, becoming a small part of this revolution.

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