Modulating T cell development with implications for response to immune checkpoint blockade

By

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A DISSERTATION

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List of Abbreviations

ADT	Androgen deprivation therapy
AR	Androgen receptor
BCR	Biochemical recurrence
BTLA	B and T lymphocyte attenuator
CRPC	Castration-resistant prostate cancer
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DC	Dendritic cell
DHT	Dihydrotestosterone
DN	Double-negative
DP	Double-positive
HCT	Hematopoietic cell transplantation
HVEM	Herpes virus entry mediator
ICB	Immune checkpoint blockade
IFNγ	Interferon gamma
IL-2	Interleukin 2
LHRH	Luteinizing hormone-releasing hormone
mTEC	Medullary thymic epithelial cell
NKT	Natural killer T cell
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein 1 ligand 1
PD-L2	Programmed cell death protein 1 ligand 2
PSA	Prostate specific antigen
RTE	Recent thymic emigrant
SAg	Super antigen
SP	Single-positive
TCR	T cell receptor
TCR-Tg	T cell receptor transgenic
TEC	Thymic epithelial cell
TME	Tumor microenvironment
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cell
VISTA	V-domain Ig suppressor of T-cell activation

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Abstract

Immune checkpoint blockade (ICB) has revolutionized the ways in which we treat some cancers, producing durable responses. However, many patients fail to respond to ICB. It is theorized that patients require tumor-specific T cells to respond effectively to ICB, but aged individuals have compromised T cell diversity partially due to reduced thymic output. The thymus is responsible for producing naïve T cells, which have the potential to respond to a wide variety of immunologic insults, such as tumors, while ensuring that these cells do not recognize self-proteins and cause autoimmunity. However, the thymus involutes with age, in part because of post-pubertal sex steroid hormones. Prostate cancer is one of the leading causes of cancer-related deaths in males worldwide and is known to be driven by sex steroid hormones, specifically androgens. New therapeutics to treat prostate cancer patients are urgently needed, but the application of ICB has been largely ineffective in this disease. The current standard of care for treating prostate cancer patients involves androgen deprivation therapy (ADT). A surprising side-effect of ADT is regeneration of the thymus and an increase in thymic output. Whether these newly generated T cells can participate in the antitumor immune response remains an open question, but it is known that an increase in TCR diversity is associated with better response to ICB. Therefore, ADT-induced thymic regeneration should increase the likelihood of response to ICB in prostate cancer patients. Interestingly, these immune checkpoint molecules are first expressed during T cell development in the thymus and the application of ICB during thymic regeneration may alter the kinds of T cells produced in this context. The goal of this dissertation is to determine the role of a common target of ICB, programmed cell death protein 1 (PD-1), in regulating T cell development in the

thymus. Answering these outstanding questions will lead us to better understand how ADT and ICB may be rationally combined to produce durable antitumor responses in patients with prostate cancer.

To better define the role of the commonly targeted protein in ICB, PD-1, in regulating T cell development, we defined the expression of PD-1 on thymocyte populations spanning the developmental trajectory as well as the expression of the ligands, PD-L1 and PD-L2 on thymic epithelial cells (TEC). We find that PD-1 limits the development of regulatory T cells (Tregs) and their progenitor populations specifically as other cell populations remain unchanged in PD-1 deficient animals. Because Treg development is known to be driven by strong signaling through the TCR as well as through CD28 costimulation, we sought to examine these signaling pathways. Using protein analysis as well as single-cell RNA sequencing, we determine that measures of TCR signal strength are reduced in PD-$1^{-/-}$ Treg progenitor populations. Surprisingly, CD28 expression is subtly increased in PD-1 deficient thymocytes but responsiveness to CD28 signaling was not altered. Instead, we demonstrate that PD-1 deficient thymocytes produce more IL-2, a niche limiting cytokine known to drive Treg induction in the thymus. This increase in IL-2 could account for the observed increase in Tregs in PD-$1^{-/-}$ animals. Collectively, these data suggest that inhibition of PD-1 leads to an increase in thymically-produced Tregs. This may be a mechanism of resistance to ICB in prostate cancer patients who are undergoing ADT-induced thymic regeneration and are treated with PD-1 blocking antibodies.

Chapter 1 : Introduction

Prostate Cancer

Incidence and mortality

Prostate cancer is the second most frequent cancer in males and accounts for about 7.3% of newly diagnosed cancers globally and 27% of newly diagnosed cancers in the United States (1, 2). Because prostate cancer risk is associated with aging, rates are threefold higher in countries with higher life expectancies (2). As a result, prostate cancer is one of the leading causes of cancer-related deaths in males worldwide.

In the United States of America, approximately 80% of people with prostate cancer are diagnosed with disease confined to the prostate, 15% with locoregional metastases, and 5% with distant metastases. Life expectancy for people with localized disease can be as high as 99% over five years, especially when diagnosed at an early stage (1). Early diagnoses have increased in recent years due to prostate specific antigen (PSA) screening, which has increased the detection of clinically indolent tumors that are progressing slowly and can be effectively treated. Those diagnosed with late-stage disease, such as distant metastases, have a much lower overall survival of 30% at five years (1).

Treatments

Androgen receptor (AR) is the most studied oncogene in prostate cancer and, as a result, is the target of most therapeutics (Fig. 1.1). In the normal prostate epithelium, AR binds to androgens, such as dihydrotestosterone (DHT) or testosterone, and translocates to the nucleus where it can bind to genes with androgen response elements to alter the transcriptional profile of the cell. AR regulates genes important for normal prostate function; however, in the diseased state, AR primarily promotes cell growth, which drives tumorigenesis (3).

For localized disease, patients may undergo active surveillance; otherwise, these patients receive surgical or radiotherapeutic interventions, with or without androgen deprivation therapy (ADT). Therapeutic decisions for localized disease are based upon estimates for a biochemical recurrence (BCR). BCR is estimated using a variety of measures including baseline prostate specific antigen (PSA), Gleason score or International Society of Urologic Pathologists (ISUP) grade, and stage. This stratifies patients into low-risk (BCR estimated at >25%), intermediate-risk (BCR estimated at 25-50%), and high-risk (BCR estimated at >50%) (4).

For those with metastatic disease, the standard treatment includes ADT until disease progression, followed by docetaxel plus prednisone with continuation of ADT (5). Conventional ADT includes suppression of gonadal androgen production to castrate levels via luteinizing hormone-releasing hormone (LHRH) agonists (e.g. leuprorelin) or antagonists (e.g. degarelix). Despite castration-level serums of testosterone, many patients progress due upregulation of AR (6) in addition to the

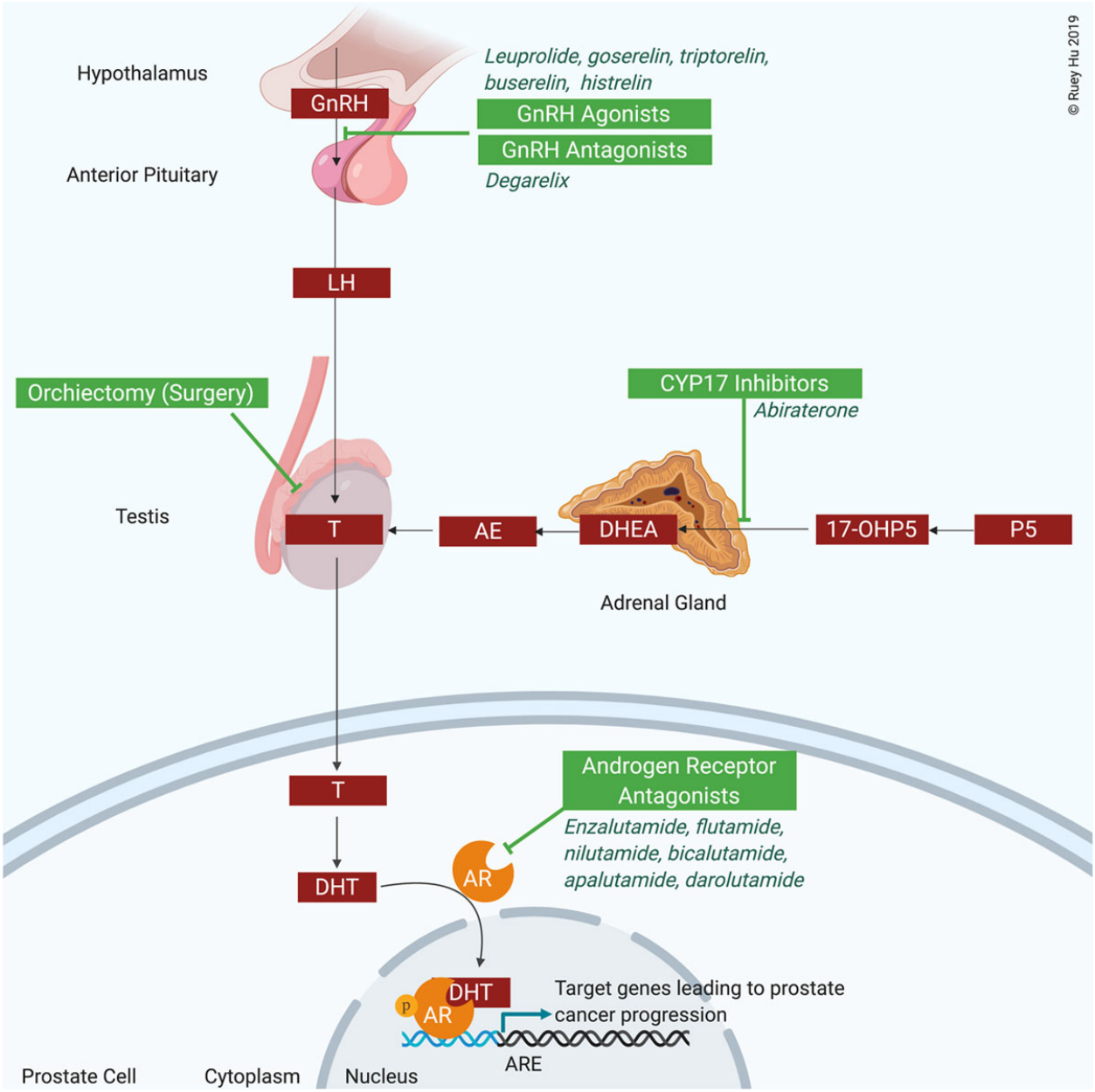

Figure 1.1 Targets for ADT

Figure 1.1: Cartoon schematic (with permission from ATVB(7)) detailing the targets for androgen deprivation therapy (ADT) in prostate cancer.

presence of the androgen DHT in the tissue, potentially due to the acquired ability to convert precursor steroids to DHT (8-10). This led to the development of CYP17A inhibitors (e.g. abiraterone acetate), which prevent this enzyme from catalyzing DHT production from glucocorticoids and cholesterol, displaying clinical efficacy (11, 12). Additionally, androgen receptor signaling inhibitors have been developed (e.g. enzalutamide) to further inhibit the effect of AR (13). These therapeutics work by blocking binding of AR to androgens, AR translocation the nucleus, and AR's ability to bind to DNA (14). Together, these therapies all induce prostate cancer cell death and clinical remission as indicated by a decrease in PSA level and/or tumor regression.

Even though ADT is initially quite effective, prostate cancer will eventually stop responding to androgen suppression and will progress to castration-resistant prostate cancer (CRPC) over a period of 18-20 months (15). CRPC is defined by a rise in PSA or evidence of disease progression via imaging despite castrate levels of testosterone in the serum. CRPC prognosis is poor with survival varying from 9 and 30 months (16). Therefore, it is critical that we develop novel therapeutic strategies to treat individuals with CRPC.

Contribution of the Thymus to Antitumor Immunity

T cell development

The thymus provides a unique environment that facilitates the development of T cells, which are lymphoid cells that make up an arm of the adaptive immune

system. T cells offer protection by engaging with antigen-presenting cells, which display antigens bound either to major-histocompatibility complexes (MHC) I or MHC II. Antigens displayed on MHC I are recognized by CD8 T cells, also known as cytotoxic T cells, while antigens presented on MHC II are recognized by CD4 T cells, otherwise known as helper T cells. T cells engage with MHC molecules via their T cell receptor (TCR), which results in the activation of the cells to undergo proliferation and differentiation into a variety of memory and effector subtypes (17).

The key maturational events during the process of T cell development in the thymus include: (i) entry of lymphoid progenitor cells from the bone marrow, (ii) commitment to the T cell lineage via differentiation into a T cell precursor, (iii) formation of the TCR, (iv) positive selection, and (v) negative selection (Fig. 1.2). Developing thymocytes can be classified by their expression of the coreceptors, CD4 and CD8, leading to three distinct stages of maturation: double-negative (DN), double-positive (DP), and single-positive (SP). The DN stage can be further divided based upon expression of CD25 and CD44 cell surface expression to delineate DN1 – 4 subpopulations.

Current data supports the affinity model of thymic selection (18-20). Briefly, lymphoid progenitors enter the thymus at the corticomedullary junction and commit to the T cell lineage to generate DN1 thymocytes (CD25-CD44+). DN1 thymocytes migrate towards the subcapsular cortical region and differentiate into

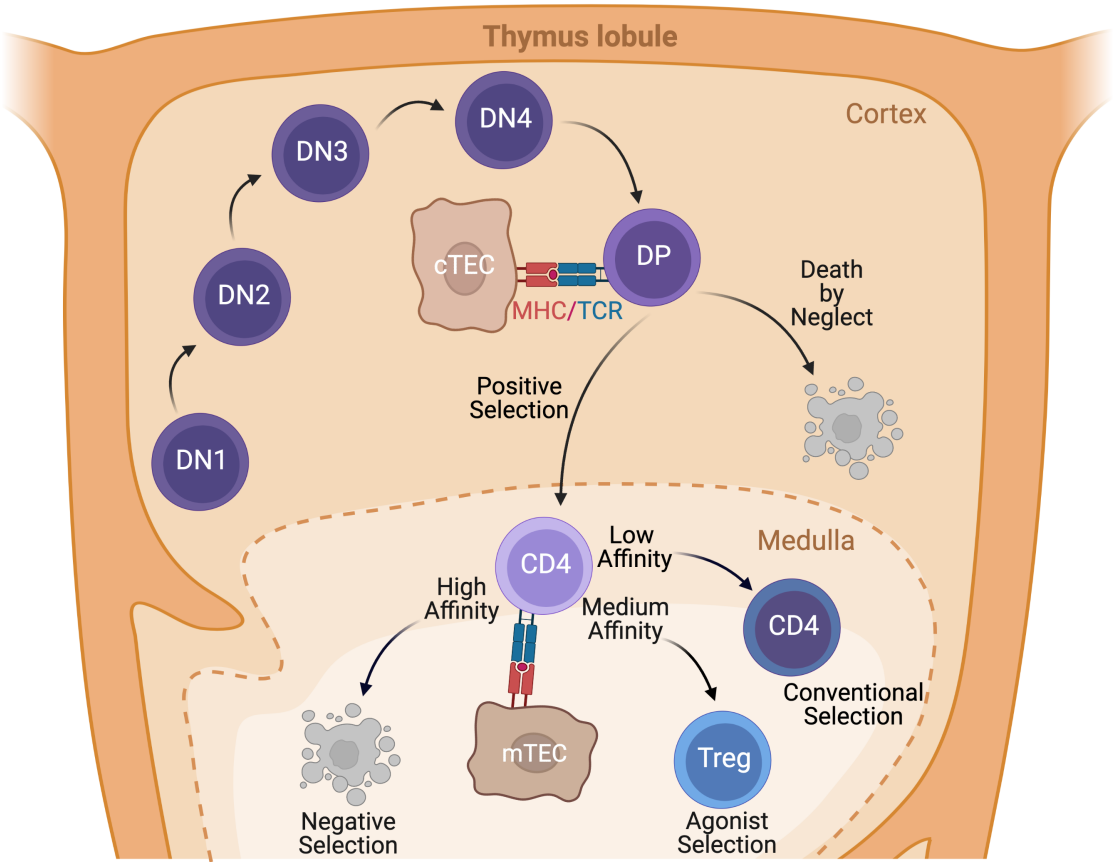

Figure 1.2 T cell development

Figure 1.2: Cartoon schematic demonstrating the developmental steps of T cells in the thymus (created with BioRender.com).

DN2 thymocytes (CD25+CD44+) and then to DN3 thymocytes (CD25+CD44-), which corresponds to TCRβ chain rearrangement to generate the pre-TCR. Signaling through the pre-TCR allows for rearrangement of the TCRα chain, resulting in the formation of the full TCR. Upon expression of the TCR, both co-receptors, CD4 and CD8, are upregulated to generate DP thymocytes. Interactions of thymocytes at the DP stage with MHC-peptide complexes expressed by thymic epithelial cells (TECs) or dendritic cells (DCs) induces TCR signaling, the strength of which determines the fate of the developing thymocyte. A lack of signaling through the TCR results in death by neglect, but intermediate signals facilitate positive selection, supporting the survival and further maturation of a thymocyte. During positive selection, the appropriate co-receptor, CD4 or CD8, is upregulated to generate SP thymocytes. These newly generated SP thymocytes migrate into the medulla where they are queried for reactivity to self-antigens through their interaction with specialized antigen-presenting cells that present tissue restricted antigens: if the developing SP thymocyte receives strong signaling through its TCR, this indicates self-reactivity, and the thymocyte is deleted through the process of negative selection. Only those cells that are not self-reactive complete the maturation process.

A small subset of developing thymocytes that receive a strong TCR signal will undergo the process of agonist selection whereby these cells are directed into specialized cell lineages including invariant natural killer T cells (iNKT), regulatory T cells (Tregs), or CD8αα T cells (19). The location of agonist selection varies

and, therefore, the cells that are required for the selection of these specialized lineages also vary. Thymocytes that will go on to become iNKT cells are thought to be selected off of DP thymocytes in the cortex that express lipid antigens on an MHC-I-like receptor called CD1d (21). In contrast, thymocytes that are directed into the Treg lineage are thought to be selected off of the specialized thymic epithelial cells in the medulla that express tissue restricted antigens (21). The location of CD8$\alpha\alpha$ T cell selection remains under investigation. There is evidence of two precursor populations, one which develops in the cortex and displays evidence of agonist selection and the other which develops in the medulla but does not appear to be agonist selected (22).

After the highly regulated process of T cell development, only about 1% of thymocytes will mature and emigrate to the periphery (23).

Thymic involution

The thymus undergoes involution with age such that thymic epithelial cells (TECs) are replaced with adipose tissue and thymic architecture becomes compromised, resulting in decreased thymic output (24-27). One potential cause of age-related thymic decline is the presence of sex hormones that begins with puberty (28, 29). In particular, AR is known to be expressed in TEC (30), which makes these cells susceptible to the effects of androgens (31, 32). For this reason, androgens are thought to be an important contributor to thymic involution; however, the connection between the increased levels of androgens following puberty onset

and the initiation of thymic involution is still debated. In addition to age-related thymic decline, the thymus is sensitive to acute stressors such as glucocorticoids, infections, radiation, and cytoablative therapies (33, 34).

Age-related thymic involution leads to a decrease in the T cell repertoire due to fewer naïve T cells being exported from the thymus into the periphery (35). The decline in thymic output results in homeostatic proliferation of existing memory T cells that yields expansion of T cell clones, many of which are virus-specific (36-39). For example, cytomegalovirus-specific clones are thought to constitute nearly 6% of the CD4 T cell repertoire and up to 25% of the CD8 T cell repertoire in the elderly (36, 39). In individuals over the age of 75, it has been reported that less than 1% of the naïve T cell repertoire diversity is preserved (37). This dramatic decline in diversity of the T cell repertoire impairs the ability of aged individuals to respond to immunologic challenges, including responding to tumor-specific antigens (40, 41). This decline in TCR repertoire diversity could partially explain why cancer incidence is higher in the elderly since elderly individuals may have fewer tumor-specific T cells that can mount an effective anti-tumor immune response (42, 43). Indeed, it has been reported in several different cancers that patients exhibited a decreased TCR repertoire diversity compared with healthy controls and, further, those patients with a lower TCR repertoire diversity had a poorer prognosis (41, 44-47).

Additionally, thymic involution may be associated with a relative increase in the production of immunosuppressive Tregs (48). In the thymus, this is thought to occur because there are fewer TECs, which weakens the interactions between TECs and developing thymocytes. This leads to a decrease in TCR signal strength such that CD4+ thymocytes that might have otherwise been deleted through negative selection are now being diverted into the Treg lineage (49). This means that cells that should have been deleted through negative selection are surviving and developing into Tregs, modifying the Treg repertoire to include self-antigens that might be co-expressed on tumors allowing for recruitment of these Treg into the tumor (50). It has been well-established that the presence of Treg in the tumor leads to an immunosuppressive microenvironment and facilitates tumor progression due to immune escape (51, 52). However, other studies have found that thymic Treg generation is impaired with aging (53, 54). These data can also be explained by reduced interactions of developing thymocytes with mTECs, which would decrease the potential encounter with tissue restricted antigens that drive Treg development (54). A portion of the Tregs present in the thymus are in fact recirculating and are thought to regulate new Treg development by limiting IL-2 generation, a niche limiting cytokine critical for Treg development (53). Therefore, the fact that Tregs seem to accumulate in secondary lymphoid organs with age might be a result of an increase in peripherally induced Tregs (iTregs) rather than thymically derived Tregs, an area of investigation still underway (55, 56).

Thymic regeneration

Incredibly, the thymus has the capacity to regenerate. There are therapeutic strategies that are being employed to try to harness the thymic regenerative capacity to increase thymic output in elderly individuals (57). These strategies could conceivably be employed to improve response to infections, vaccinations, and improve antitumor immunity.

There are many different strategies that are being employed to stimulate thymic regeneration (57-59) (Fig. 1.3). One major class of treatments include exogenous addition of growth factors including keratinocyte growth factor (KGF, also known as fibroblast growth factor 7), growth hormone (also known as insulin-like growth factor 1), and epidermal growth factor. These treatments tend to target TEC proliferation and maturation, which results in an increased thymic cellularity, restored thymic architecture, and greater thymic output. Several trials (NCT01233921, NCT03042585, and NCT02356159) are exploring the effects of KGF on the prevention of graft versus host disease and on immune reconstitution following hematopoietic cell transplantation (HCT) in hematologic cancers.

Another major class of treatments include administration of exogenous cytokines. Some cytokines, such as interleukin-22 (IL-22) and receptor activator of NF-κB ligand (RANKL), are also thought to target TEC like the growth factors described above; however, other cytokines such as IL-7, IL-12, and IL-21 are thought to target developing thymocytes to increase their proliferation and maturation.

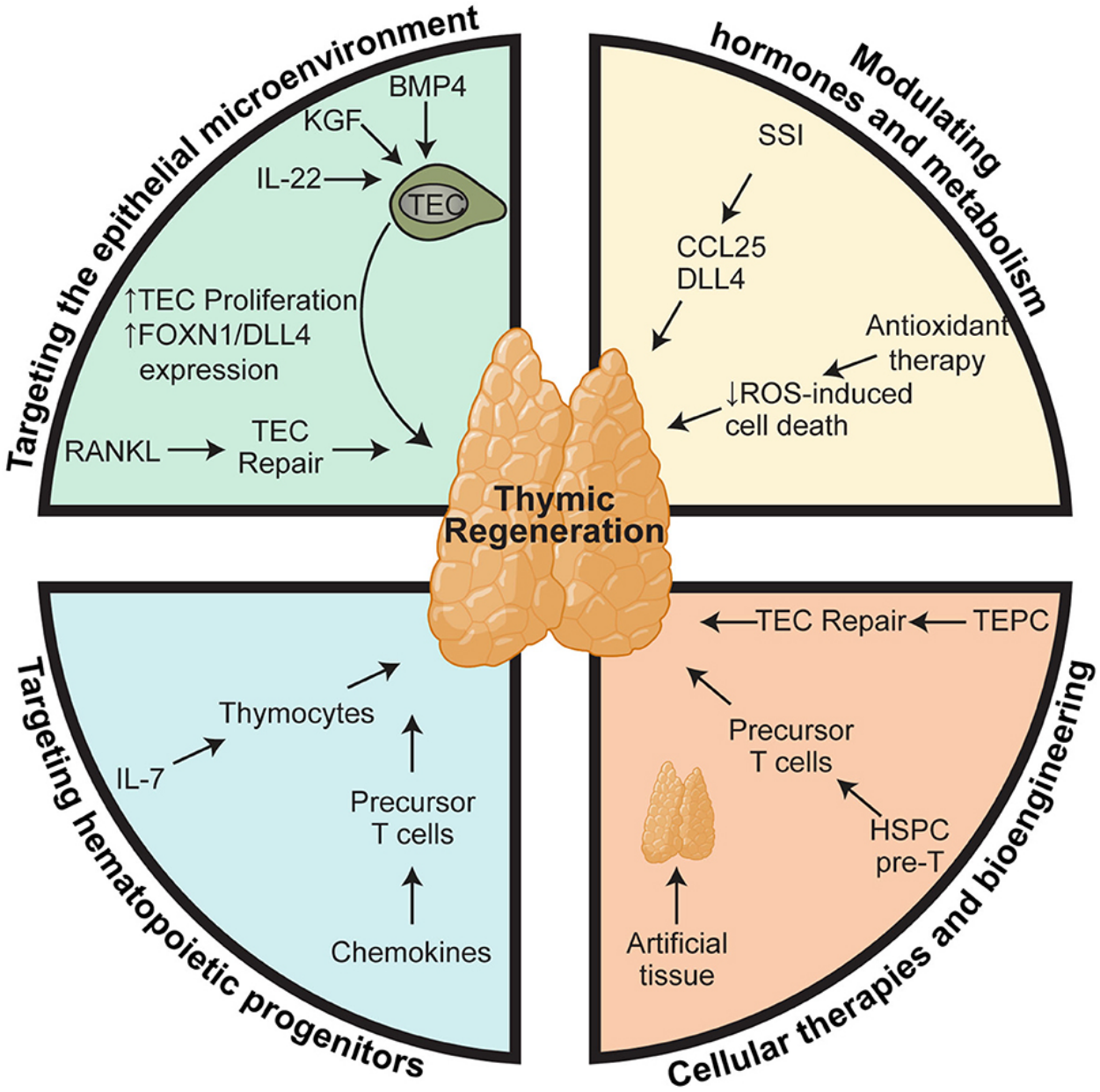

Figure 1.3 Methods for inducing thymic regeneration

Figure 1.3: Cartoon schematic (with permission from Front. Immunol. (34)) describing the current methods for inducing thymic regeneration.

Treatment with IL-7 is in clinical trials (NCT00477321, NCT01190111, NCT01241643, NCT00839436, and NCT00684008) to promote enhanced T cell recovery in patients with human immunodeficiency virus (HIV) or patients that have undergone HCT.

Another major strategy for inducing thymic regeneration includes sex steroid ablation, which is the most relevant for the work presented herein. Specifically, the negative effects of androgens on the thymus can be reversed through ADT, which is achieved surgically through bilateral orchiectomy or chemically through treatment with LHRH agonists or antagonists. Although the mechanism is debated, it has been well-established that ADT results in thymic regeneration in animal models as well as in prostate cancer patients (26, 60-62). ADT-induced thymic enlargement is attributed to the proliferation of TEC through the upregulation of chemokine (C-C motif) ligand 25 (CCL25) in mTEC (63) and delta-like 4 (DLL4) in cTEC (64). This results in increased thymic cellularity, restored thymic architecture, and enhanced thymopoiesis (26, 60-62). Many compounds have been developed to block androgens transiently and reversibly for the treatment of prostate cancer, as previously discussed. One of these, an LHRH agonist, was used prior to HCT and reports demonstrated that there were significantly increased numbers of total lymphocytes, especially naïve CD4+ T cells, with a concomitant increase in TCR repertoire diversity (65). There are two trials that are on-going that aim to assess the use of an LHRH agonist in improving immune recovery following HCT (NCT01746849 and NCT01338987). Together, this evidence highlights the importance of sex steroids, especially androgens, as

a mechanism for inducing thymic regeneration. These therapies could be utilized to increase antitumor responses in the aged as well.

Recent thymic emigrants

Recent thymic emigrants (RTE) are a population of T cells that have recently completed their development in the thymus and emigrated into the periphery. RTE were first described in the 1980's through intrathymic injection of fluorescein isothiocyanate (FITC) followed by identification of FITC+ peripheral T cells 18-24 hours later using flow cytometry (66, 67). This technique allowed for the analysis of the newest RTE in comparison to more mature peripheral T cells, which led to the identification of cell surface proteins that could distinguish RTE to allow for the study of RTEs in the absence of other methods of tagging these cells (68). Since these early days, a newer tool for studying T cell development was developed in 2004 and is now widely used: RAG2-GFP transgenic mice, which have GFP expression driven by the RAG2 promoter that is active during TCR rearrangement and marks T cells that have completed V-D-J recombination of their TCR as GFP+ (69). This model further defined these cells as a phenotypically and functionally distinct T cell subset.

Current research suggests that CD8+ RTE demonstrate diminished proliferation and generation of effector cytokines when stimulated *in vitro* (69) and are skewed towards short-lived effector cells with *in vivo* infection models (70). Despite this skewing, antigen-specific CD8+ RTEs generate lower amounts of effector

cytokines, which remains true even after 60 days when RTEs should have transitioned into the mature T cell compartment (70).

CD4+ RTE stimulated *in vitro* also proliferate less, produce fewer cytokines, and do not upregulate activation markers such as the high-affinity IL-2 receptor (CD25) compared to their mature counterparts (69). Further, CD4+ RTE express lower levels of master transcription factors associated with lineage differentiation and produce less of the corresponding cytokines when polarized to T helper (Th) 1, Th17, or induced Tregs. Interestingly, these cells are not dysfunctional, but are rather skewed towards the Th2 lineage as evidenced by both *in vitro* and *in vivo* studies where these cells actually produce more Th2-associated effector cytokines compared to mature T cells (71).

RTEs mature in secondary lymphoid organs after they egress from the thymus. Specifically, evidence suggests that maturation occurs in the spleen or lymph nodes and that this maturation is associated with phenotypic changes (alteration of cell surface proteins) and functional changes (increased production of effector cytokines) (72). This process occurs over 2 – 3 weeks and is characterized by the maturation of the bulk population rather than selective outgrowth and survival of a small population of already mature RTE (72). The factors driving this maturation process are still under investigation (73). It remains unclear what the purpose of having RTE undergo further maturation in the periphery might be; however, one theory is that RTE might be more prone to tolerance and anergy as they are being

exposed to extrathymic antigens for the first time, to which they may not be tolerized (74). Some of these are self-peptides not efficiently presented in the thymus (75), antigens derived from commensal bacteria (76), or food antigens (77). This is evidenced by the phenotype of RTE (TCR^{hi} and $CD28^{lo}$), which could allow for signaling through the TCR without sufficient costimulation through CD28, resulting in anergic T cells (69). Also in line with this hypothesis, CD4+ RTE are more likely to become Treg (78) and are more sensitive to Treg suppression compared to more mature CD4+ T cells (79).

RTE contribute to the maintenance of the naïve T cell repertoire throughout life as evidenced by the fact that thymectomy in mice results in demonstrable holes in the T cell repertoire (80). Similarly, humans that have undergone thymectomy secondary to cardiac surgery show signs of premature immuno-aging, including lower naïve T cell counts and expansion of oligoclonal memory T cells, both of which would normally be seen in individuals much older than the patients described (81). There is further evidence that RTE are still important in maintaining the T cell pool even in aged individuals where thymic output has declined significantly but is still measurable (82, 83).

Inhibitory Proteins as Cancer Therapeutics

T cell exhaustion

T cell exhaustion is a phenomenon first described during chronic viral infections, such as LCMV Clone 13 or Docile, where CD8 T cells are continuously exposed

to antigens and terminally differentiate into a dysfunctional state (84-86). This is in comparison to acute infections, such as LCMV Armstrong, which are cleared and preferentially generate terminal effector T cells. This dysfunctional state (Fig. 1.4) is characterized by the loss of proliferative capacity, a decrease in the production of effector molecules (granzyme B, perforin), a defect in secretion of effector cytokines (IL-2, INFγ, TNFα), and a loss of cytotoxic potential(87). Exhausted T cells can be identified by their expression of multiple inhibitory receptors such as PD-1, TIM-3, LAG-3, CTLA-4, and TIGIT (88), with the number of inhibitory receptors expressed indicative of the severity of dysfunction (89). These inhibitory receptors will be discussed further in subsequent sections.

Differentiation to a terminal exhaustion lineage versus the terminal effector lineage is determined by antigen exposure type (chronic versus acute) and coincides with large changes in the transcriptional (90) and epigenetic (91) landscapes. Transcription factors downstream of TCR signaling (BATF, IRF4, NFATc1, and TOX) promote terminal exhaustion, whereas other transcription factors such as TCF1 promote stemness and maintain a population referred to as progenitor exhausted T cells (92-98). TCF1 promotes stemness in CD8+ T cells by inhibiting expression of effector-associated genes such as *Prdm1* (Blimp1),

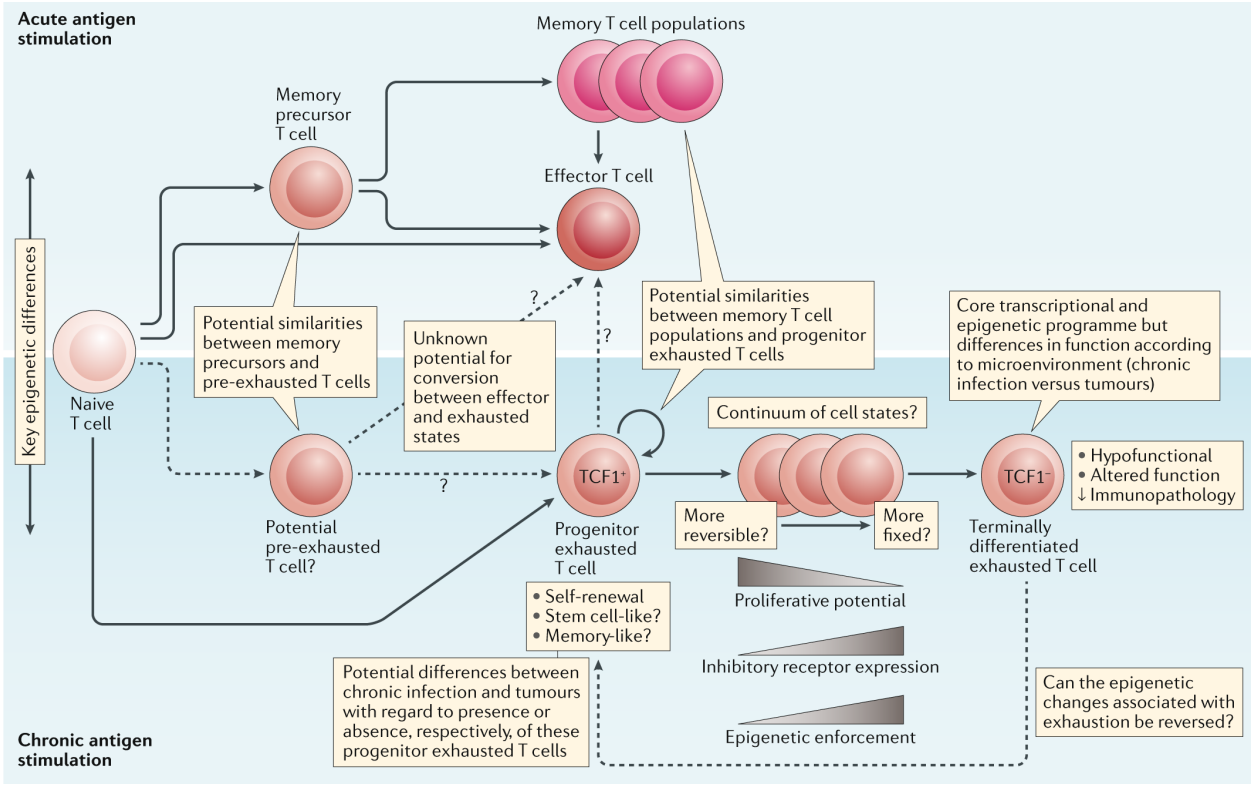

Figure 1.4 CD8 T cell differentiation to terminal exhaustion

Figure 1.4: Cartoon schematic (with permission from Nat Rev Immunol(99)) detailing the development of terminally exhausted T cells compared to effector and memory T cell subsets during chronic versus acute antigen stimulation.

Runx3 (RUNX3), *Id2* (ID2), and *Tbx21* (Tbet) and favoring central memory through promotion of *Eomes* (EOMES), *Bcl6* (BCL6), and *Bcl2* (BCL2) expression (100). Research demonstrates that TCF1 is a fate-decision transcription factor that maintains memory programs and promotes differentiation away from effector programs towards exhaustion via the TCF1-EOMES-BCL2 axis (96). Therefore, T cells differentiate down the exhaustion pathway with a distinct progenitor population before becoming terminally exhausted. It has been reported that this progenitor population is responsible for the response to immune checkpoint blockade, which is why this population is of interest clinically (101).

Immune checkpoint molecules

Cancer creates an environment of chronic antigen exposure, so it is unsurprising that tumors also generate terminally exhausted T cells like those described in the context of chronic infection (102). Immune checkpoint molecules were described by James Allison and Tasuku Honjo, who were awarded the 2018 Nobel Prize in Physiology and Medicine. Work from their labs demonstrated that T cells rapidly upregulate several inhibitory receptors upon activation, which are now referred to as checkpoint molecules, that serve to temper T cell function and that blocking these receptors could enhance antitumor immunity (103-107) (Figure 1.5). These inhibitory receptors are thought to be an evolutionary adaptation that prevents autoimmunity in the context of chronic antigen exposure; however, as previously mentioned, the expression of multiple checkpoint molecules leads to more severe T cell dysfunction and characterizes the state of T cell exhaustion in the context of

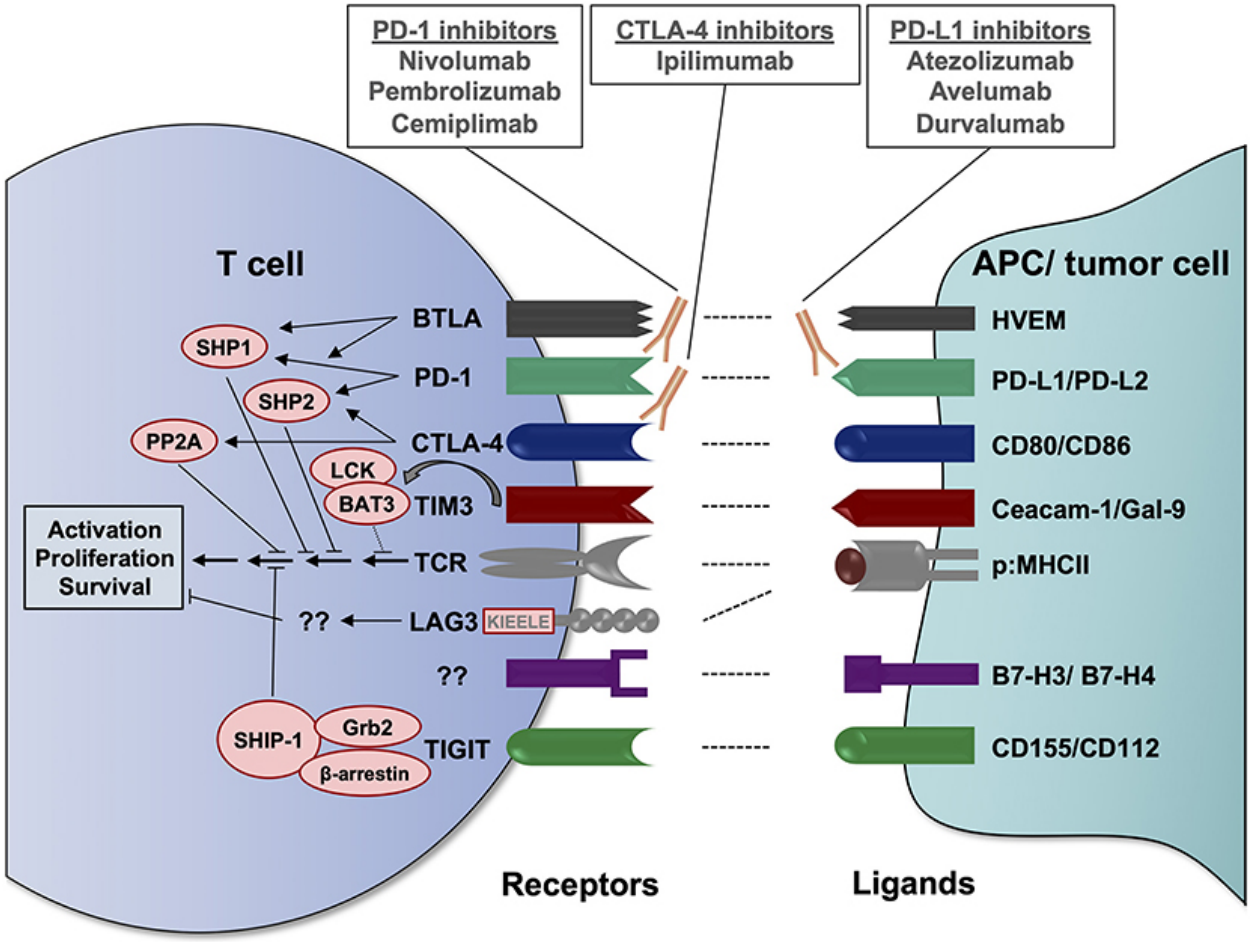

Figure 1.5 Immune checkpoint molecules

Figure 1.5: Cartoon schematic (with permission from Front. Immunol. (108)) representing checkpoint molecules and their signaling mechanisms.

chronic infections and tumors.

CTLA-4, the first immune checkpoint molecules to be clinically targeted, is a CD28 homolog that has a higher binding affinity for CD80/CD86 (also known as B7.1/B7.2) (109, 110). It is thought that CTLA-4 can prevent the stimulatory signal provided by CD28 through either competitive binding (111) or transendocytosis of ligands (112), while also delivering inhibitory signals that actively counter CD28/TCR signaling through the activation of the protein phosphatases, SHP2 and PP2A (113-115). The importance of CTLA-4 as a checkpoint molecule is evidenced by the lethal systemic autoimmunity of CTLA-4 deficient mice (116, 117). While CTLA-4 is expressed by activated CD8+ effector T cells, its major role seems to be through its impact on two subsets of CD4+ T cells: downregulation of helper T cell activity and enhancement of Treg immunosuppressive activity (118, 119). Therefore, blockade of CTLA-4 is thought to enhance effector CD4+ T cell activity and inhibit Treg-dependent immunosuppression within the tumor. Preclinical studies demonstrated the efficacy of CTLA-4 antibody blockade in immunogenic tumors, which suggests that if there is an endogenous antitumor response, anti-CTLA-4 can enhance that response to induce tumor regression (103). These preclinical findings led to the production and testing of CTLA-4 antibodies, which produced clinical responses in ~10% of melanoma patients and led to the approval of ipilimumab by the US Food and Drug Administration for the treatment of advanced melanoma (120-122).

PD-1 is a CD28 homolog that contains inhibitory motifs in its cytoplasmic tail, an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM), which recruit SHP1 and SHP2 upon engagement with PD-1's ligands, PD-L1 and PD-L2 (104, 123, 124). Because ITIMs are found in other inhibitory receptors, it was originally thought to play the dominant role in the inhibitory function of PD-1; however, studies have indicated that it is the ITSM motif that is essential for PD-1's inhibitory activity (125). Activation of PD-1 leads to inhibition of signals downstream of the TCR, particularly PI3K/Akt activation (126). A more recent study has demonstrated that CD28 costimulation may be the main target of PD-1 inhibition (127), but this has been contested by other work (128). The importance of PD-1 as a checkpoint molecule is evidenced by the strain-specific lethal autoimmunity of PD-1 deficient mice: C57BL/6 PD-$1^{-/-}$ mice develop a lupus-like phenotype (129), while BALB/c PD-$1^{-/-}$ mice develop a cardiomyopathy (130). PD-1 is induced on peripheral T and B cells upon activation through TCR or BCR signaling or by common γ chain cytokines(131, 132); however, its main effect in the context of cancer seems to be suppressing cytotoxicity of CD8+ T cells as a means of immune evasion by PD-L1-expressing tumor cells (107, 133, 134). Studies demonstrated the efficacy of PD-1 blockade across tumor types, but particularly in the setting of metastatic melanoma (135), which led the US Food and Drug Administration to approve both pembrolizumab and nivolumab as first-line therapies (136, 137).

The role of the thymus in responding to immune checkpoint blockade

For ICB to be effective, there must be T cells that are able to recognize tumor-associated antigens present in the patient. In line with this thinking, there are reports describing that the tumor mutation load is correlated with response to immunotherapy, which is thought to occur because tumors with a higher mutational load produce more immunogenic tumor-associated antigens (138, 139). However, it should be noted that the hypothesis that increased mutational burden improves the response to ICB is now debated (140) and appears inconsistent among different tumor types, especially those with lower tumor mutational burden such as prostate cancer (141). Despite this controversy, it still stands to reason that a broad TCR repertoire would increase the chance of tumor-associated antigen recognition in response to treatment with ICB, leading to better clinical outcomes. Indeed, there is evidence that this is true in the most common ICB approaches, anti-CTLA-4 and anti-PD-1/PD-L1 therapies.

Anti-CTLA-4 therapy generally broadens the blood TCR repertoire (142, 143) and leads to the expansion of both tumor-specific (144) and bystander T cell clones (145) in the tumor microenvironment (TME). Two studies have demonstrated that increased peripheral TCR diversity in the blood was associated with better clinical outcomes in melanoma patients treated with anti-CTLA-4 (146, 147). However, at least one study found that the pre-treatment TCR clonality within the tumor of metastatic melanoma patients did not predict response to anti-CTLA-4 (148), with another study demonstrating that high pre-therapy TCR clonality in the blood was

actually associated with poorer responses (149). This may be because TCR skewing indicates an increase in T cell clones responding to an antigen, such as a tumor-associated antigen, which is inadequate to control the tumor and thus might indicate a lack of ability of these cells to respond to anti-CTLA-4 therapy.

Some research indicates that higher baseline TCR diversity does correlate with better disease control in those receiving anti-PD-1 therapy in melanoma (147), gastrointestinal cancers (150), and classical Hodgkin lymphoma (151). However, other research demonstrates that it is rather increased TCR clonality that is associated with a better response to anti-PD-1 treatment (149). Melanoma patients receiving PD-1 therapy showed increased TCR clonality, which was 10x higher in responders compared to non-responders. This increase in TCR clonality coincided with a reduction of TCR diversity of intra-tumoral lymphocytes, presumably due to clonal expansion of tumor-reactive clones (148). Similar results were found in metastatic urothelial cancer (152) and renal cell carcinoma (153). This suggests that patients with increased TCR clonality after treatment have increased tumor-specific T cells that have expanded and are being harnessed for antitumor immunity.

However, TCR diversity may not be a specific enough marker for determining response to checkpoint blockade. Instead, researchers might need to look at markers that distinguish tumor-specific T cells, such as PD-1+ cells. One such study examined CD8+ PD1+ cells and found that melanoma patients with higher

pre-treatment TCR diversity in this cell subset did show longer progression free survival when treated with anti-PD-1 (154). Similarly, higher pre-treatment TCR diversity in CD8+ PD1+ T cells was also reported in non-small cell lung carcinoma patients that had longer progression free survival and overall survival in response to anti-PD1 therapy (155).

Overall, it seems that blood TCR diversity at baseline and increased TCR clonality following treatment is associated with response to ICB in some, though not all, studies. Factors that may explain the discrepancies described include tumor- or site-specific factors, intra-tumoral mutation burden, previous treatments the patients have undergone, and the method used to evaluate TCR diversity. Therefore, it is reasonable to say that a broader TCR repertoire before ICB is preferable and that improving TCR repertoire diversity would be beneficial for increasing response rates, such as through inducing thymic regeneration as previously described.

Immune checkpoint blockade in prostate cancer

Prostate cancer offers a unique context for ICB because these patients are treated with ADT, which induces thymic regeneration and an increase in thymic output. Newly generated T cells available from thymic regeneration could be tumor-specific and drive a favorable response to ICB. ICB has largely failed in the context of prostate cancer, but there are some studies that have showed promise

in a subset of patients. These studies have been reviewed elsewhere (156) but will be briefly described here.

Several trials are being conducted in hormone-sensitive prostate cancer (NCT03879122, NCT03951831, NCT04477512, NCT03795207, NCT04126070), but only one trial has published its findings. In this single-arm, single-institution pilot, the authors combined cryotherapy to the prostate gland with pembrolizumab and ADT in oligometastatic hormone-sensitive prostate cancer patients. Primary endpoint was 1 year PSA < 0.6 ng/mL and 42% of the twelve patients treated met this endpoint, but this regimen infrequently led to sustained disease control following testosterone recovery (157).

Several trials have sought to apply anti-CTLA-4 therapy to mCRPC patients. In one phase III randomized trial of 400 mCRPC patients that were chemotherapy naïve and had no visceral metastases, no survival benefit was seen in patients treated with ipilimumab as a single agent compared to those treated with placebo; however, there was an increase in median free survival and PSA response rate in the ipilimumab-treated patients suggesting some antitumor activity in this patient population (158). Another phase III trial examined 799 mCRPC patients with at least one bone metastasis who were progressing after chemotherapy (docetaxel). These patients were randomly assigned to either receive bone-directed radiotherapy with ipilimumab or with placebo. Primary analysis didn't show any difference in overall survival (159); however, 2.4 years of follow-up

demonstrated a survival benefit in the ipilimumab cohort, suggesting some efficacy (160).

Other trials have used anti-PD-1 or anti-PD-L1 therapies in mCRPC patients. A phase 1b clinical trial sought to examine the efficacy of pembrolizumab in patients with advanced solid tumors with PD-L1 > 1%, including a cohort of mCRPC patients. There was some preliminary evidence of antitumor activity with a modest overall response rate of 17.4%, with 8 out of those 23 patients having stable disease (161). In a phase II study included patients with mCRPC that were treated with pembrolizumab: 133 patients had PD-L1 positive disease, 66 patients had PD-L1 negative disease, and 59 patients had bone predominant disease regardless of PD-L1 status. Pembrolizumab monotherapy demonstrated antitumor activity only in those with bone predominant disease, but not the other two groups (162). One small phase I trial examined atezolizumab monotherapy in 35 mCRPC patients and demonstrated some antitumor effect, but the 50% PSA response rate was only 8.6% (163).

Many more trials have sought to apply some type of combination therapy to patients with mCRPC. A phase III clinical trial of 759 mCRPC patients who were progressing on abiraterone were treated with enzalutamide alone or enzalutamide plus atezolizumab. This trial did not meet the primary endpoint for overall survival, but biomarker analysis did demonstrate that progression free survival was better in patients that were treated with the combination therapy if the patients had

higher PD-L1 expression or greater CD8 T cell infiltration, suggesting efficacy in a subset of patients (164). Two trials investigated the combination of nivolumab with ipilimumab. The first trial compared mCRPC patients that were chemotherapy naïve to those who had progressed after chemotherapy and found a higher objective response rate, median progression free survival, and median overall survival in the chemotherapy-naïve patients compared to those who progressed after chemotherapy; however, there were grades 3 and 4 level toxicities reported in 42-53% of patients which brings into question the safety of this combination (165). The second trial examined the use of nivolumab with ipilimumab with or without enzalutamide in AR-V7-expressing mCRPC patients and found no difference in efficacy (166). In a phase II single-arm study, patients with mCRPC progressing on enzalutamide were treated with pembrolizumab. This small study of 28 patients did show antitumor effects with 18% (5 of 28 patients) demonstrating a PSA decline of $\geq$ 50% and, of patients with measurable disease at baseline, 25% (3 of 12) achieved an objective response (167). A study from the Moran Lab sought to elucidate differences between responders and non-responders and found a T cell-intrinsic effect of androgen blockade, which primed the T cells to respond to PD-1 therapy in responders (168). These promising results led to an ongoing phase III trial (NCT03834493) to further understand how this combination therapy may be applied in prostate cancer. In addition to this ongoing trial, there are many more seeking to examine different combination therapies in mCRPC.

The Role of Inhibitory Proteins in T Cell Development

The role of inhibitory proteins like checkpoint molecules has been extensively studied in the periphery (169, 170). However, these inhibitory molecules and their ligands are first expressed in the thymus. Early expression of checkpoint molecules during T cell development might inform T cell lineage fate decisions before these cells ever arrive in the periphery. This section will discuss the evidence for checkpoint molecules in regulating T cell development.

Cytotoxic T-lymphocyte associated protein 4 (CTLA-4)

Adapted from DOI: https://doi.org/10.1016/j.trecan.2023.04.007(171)

Thymic epithelial cells (172, 173), DCs (172-174), and B cells (175, 176) express receptors B7-1 and B7-2, which can bind to CD28 to provide stimulatory signals or to CTLA-4 to provide inhibitory signals. B7-1/B7-2-expressing cells have been demonstrated to be important in facilitating negative selection (172-174, 177-179). CTLA-4 can be detected on the cell surface of a small subset of DP and SP thymocytes, with intracellular stores being identified in some DN thymocytes; however, CTLA-4 can be upregulated on all developing thymocytes upon TCR ligation (180, 181). These data demonstrate that CTLA-4 is expressed early in T cell development when inhibitory signals could modify TCR signal strength and inform cell fate decisions (Fig. 1.6). This section will highlight the current findings about the potential role of CTLA-4 in regulating thymocyte development.

The role of CTLA-4 in negative selection remains controversial. No major defects in T cell selection were observed in CTLA-4-deficient animals (182) and studies

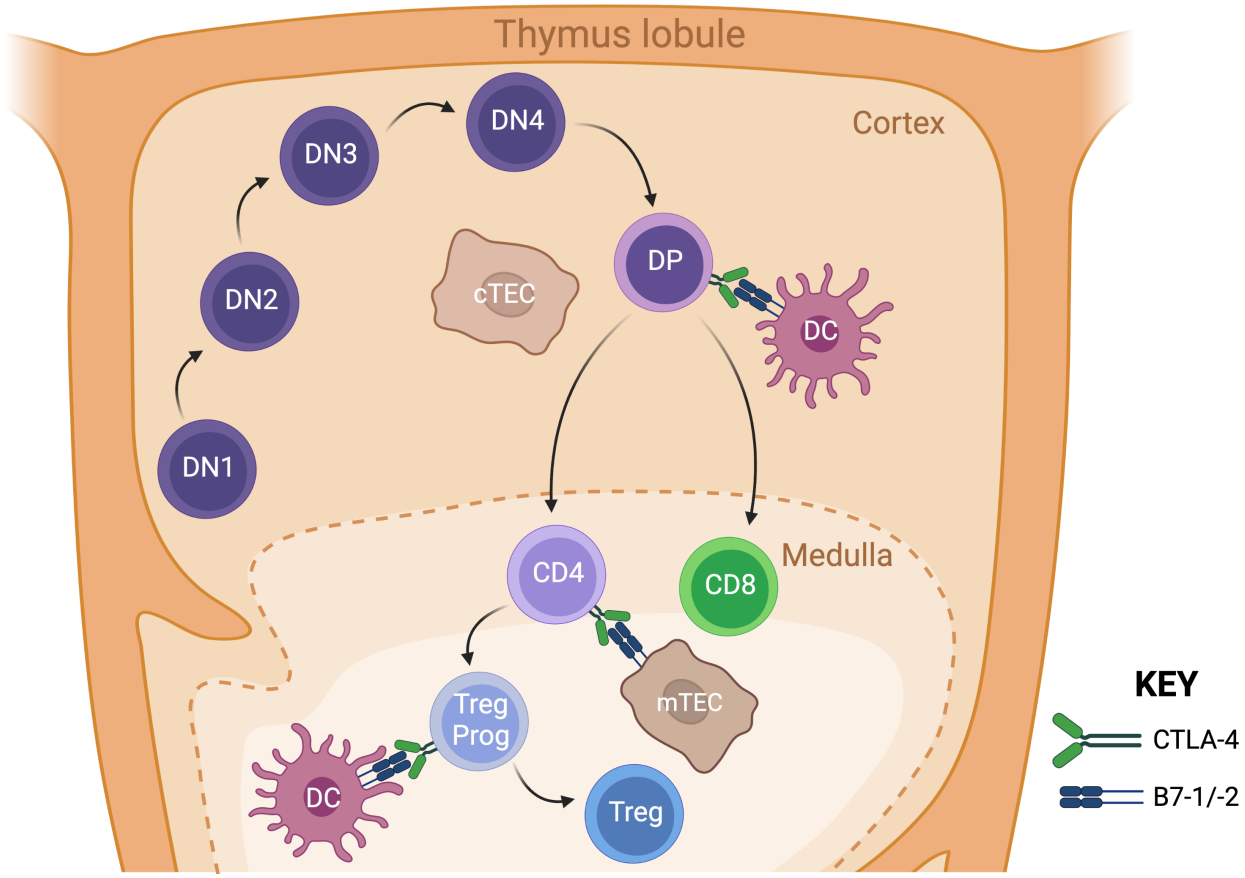

Figure 1.6 Expression of CTLA-4 and its ligands during T cell development

Figure 1.6: Cartoon schematic demonstrating the presence of CTLA-4 and its ligands during the developmental steps of T cells in the thymus (created with BioRender.com).

employing different TCR transgenic (TCR-Tg) mice on a CTLA-4-deficient background found no defect in negative selection (183-185).

However, CTLA-4 may modulate negative selection by interfering with the binding of the costimulatory molecule, CD28, thus inhibiting negative selection. Several studies have suggested such a role for CTLA-4 (180, 181, 186, 187). Blocking CTLA-4 during deletion reversed B7-dependent inhibition of cell death, suggesting that CTLA-4 inhibits negative selection (180, 181). Using a super antigen (SAg) model of negative selection in BALB/c mice, the authors demonstrate impaired deletion in B7-1/B7-2 double knockout animals (186). In a series of elegant experiments, they explored the contribution of CD28 alone or with CTLA-4 on the negative selection of SAg-reactive thymocytes. Ultimately, they concluded that differences in CD28 and CTLA-4 signaling in negative selection of antigen-reactive thymocytes may reflect that this deletion can occur during the transition of a DP to a SP thymocyte (188). In another study that uses an overexpression model of CTLA-4, Takahashi and colleagues demonstrate that the deletion of endogenous SAg-specific T cells was impaired, which was reversed when the authors overexpressed nonfunctional CTLA-4 (187). Together, these data provide evidence that CTLA-4 signaling during negative selection inhibits TCR derived apoptotic stimuli in both induced and natural models of clonal deletion and inhibition of CTLA-4 could divert T cell development.

Aside from these studies, there is additional evidence that CTLA-4 augments negative selection. Kwon and colleagues demonstrate that the addition of soluble CTLA-4 blocking antibody inhibited antigen-induced cell death by inhibiting TCR-mediated activation of DP thymocytes. Additionally, CTLA-4 competes with CD28 for ligand, which would reduce signaling through costimulatory molecules to further decrease DP activation (189). Further experiments utilizing CTLA-4 blockade indicated that signaling through CTLA-4 enhances activation of DP thymocytes. These data provide evidence that CTLA-4 may regulate negative selection; however, these models do not mimic naturally induced negative selection so the phenomena described could be a result of artificial model systems.

CTLA-4 has been reported to be important in agonist selection, especially in the development of Tregs. Verhagen and colleagues observed that CTLA-4 knockout animals had about a 6-fold increase in thymic Tregs, which is consistent with an increase in CD28 signaling known to drive Treg development (190). Notably, B7-1, B7-2, CD28, and CTLA-4 are differentially expressed within the thymus consistent with migration during maturation. CD28 is highly expressed on DP thymocytes, but is downregulated to intermediate expression on SP thymocytes (191). CTLA-4 is expressed by only a subset of DP and SP thymocytes (191). Notably, the B7-1 and B7-2 ligands are restricted to the medulla (172, 173). This is of importance because negative selection and some agonist selection (e.g. Tregs) occur in the medulla, suggesting that CTLA-4 is poised to impact these

developmental lineages due to expression of its ligands in this region of the tissue. As Tregs are known to be selected for in the thymic medulla where tissue restricted antigens are expressed, the authors were surprised to find FoxP3+CD4+ T cells (Tregs) in the cortex of CTLA-4 knockout animals. These FoxP3+ cells were DP thymocytes that had expressed FoxP3 prematurely. The authors demonstrate that blocking CD28 eliminated aberrant expression of FoxP3, suggesting that CTLA-4 regulates the CD28-dependent expression of FoxP3. Similar observations were made in an inducible model of CTLA-4 deficiency (192) suggesting that without regulation of costimulation, cells are aberrantly diverted into the Treg lineage. This highlights the importance of both costimulation and coinhibition in directing lineage fate decisions. Together these data indicate that CTLA-4 may be one mechanism that prevents aberrant induction of Tregs in the cortex so that they develop in the correct context of the medulla.

Programmed cell death protein 1 (PD-1)

Adapted from DOI: https://doi.org/10.1016/j.trecan.2023.04.007(171)

PD-1 is a CD28 homolog that contains inhibitory motifs in its cytoplasmic tail, which recruit SHP-1 and SHP-2 upon engagement with PD-L1 and PD-L2 (104, 123, 124). Interestingly, PD-1 is first expressed on DN thymocytes undergoing TCRβ selection, one of the earliest signaling events to instruct thymocyte fate. It is unknown whether PD-1 influences these early lineage fate decisions (193, 194). Upon TCR ligation, the tyrosine residues in the immunoreceptor tyrosine-based

inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM) on the cytoplasmic tail of PD-1 are phosphorylated. This phosphorylation recruits SHP-2, and to a lesser extent SHP-1, dephosphorylating signaling molecules downstream of TCR and CD28 and inhibiting PI3K/Akt and the Ras/MEK/Erk pathways (126, 195-197). Therefore, PD-1 is expressed early in T cell development where inhibition of TCR could alter lineage fate decisions (Fig.1.7). This section will highlight the current findings about the potential role of PD-1 in regulating thymocyte development.

Despite the early expression of PD-1 in the thymus, the comprehensive reviews of PD-1 signaling, and its effects are derived primarily from experiments performed in peripheral T cells (169, 198). From these studies, we know that PD-1 signaling decreases T cell proliferation and cytokine production in the periphery. Whether PD-1 signaling has similar effects during T cell development remains undetermined. The implications of a reduction in either proliferation or cytokine production could profoundly alter thymocyte development by altering the thymic microenvironment and thus T cell fate.

The ligands for PD-1, PD-L1 and PD-L2, share 38% homology and recent data indicate that they have similar binding affinities, but have different mechanisms of binding (199, 200). Furthermore, PD-L1 and PD-L2 differ in their expression patterns within the thymus. PD-L1 has been identified on a subset of DN and SP

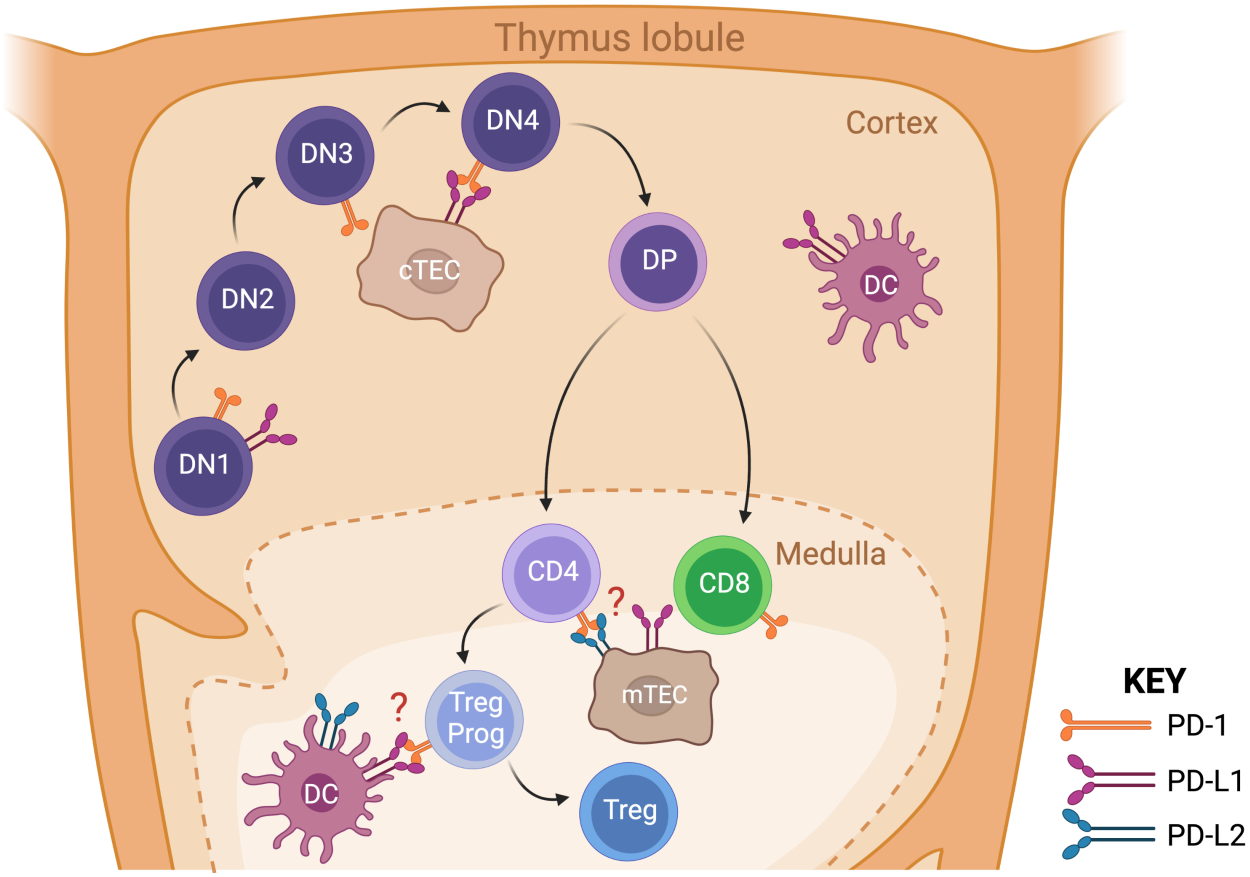

Figure 1.7 Expression of PD-1 and its ligands during T cell development

Figure 1.7: Cartoon schematic demonstrating the presence of PD-1 and its ligands during the developmental steps of T cells in the thymus (created with BioRender.com).

thymocytes (201), thymic epithelial cells (202), and dendritic cells (202-204) in both the cortex and medulla. In contrast, PD-L2 expression is more restricted: it is only expressed in the medulla by dendritic cells, macrophages, and medullary thymic epithelial cells (202, 203). This pattern of expression is of interest because developmental stages occur in different regions of the thymus: positive selection and the selection of iNKT cells are thought to occur in the cortex, while negative selection and selection of other agonist selected cell populations, such as Tregs, are thought to occur in the medulla. Differential expression of PD-1's ligands could regulate the impact PD-1 signaling has on cell fate depending on the location, and the developmental stage, of the thymocyte.

To date, most studies about PD-1 shaping T cell responses have focused on effector or exhausted T cells. However, there is evidence that PD-1 plays a role in regulating thymocyte development and therefore could be a critical regulator of the composition of the functional T cell repertoire. A potential role for PD-1 in positive selection was demonstrated using a TCR-Tg model in which transgenic CD8 T cells are potentially autoreactive (129). In crossing these mice to PD-1 knockout animals, the authors observed a decrease in DP thymocytes with a concomitant increase in CD4 and CD8 SP thymocytes, indicating that PD-1 regulates positive selection in this animal model. In a follow-up study, the authors mimicked β selection *in vivo* and found that PD-1 specifically inhibits TCRβ selection (194). Using a PD-1 overexpression model, Keir and colleagues found evidence of a reduction in positive selection due to an increase in DP thymocytes

(205). This group demonstrated that TCR crosslinking induced PD-1 expression on a subset of CD69+ DP thymocytes, a population of cells that had successfully completed positive selection. Concurrent crosslinking of TCR and PD-1 inhibited this upregulation of CD69. Interestingly, they demonstrated that PD-1's inhibition can overcome the increase in TCR-driven thymocyte maturation by costimulation with either CD2 or CD28 in *in vitro* thymocyte selection assays. The authors determined that it was specifically PD-1's interaction with PD-L1 that inhibited positive selection by examining PD-L1 and PD-L2 deficient animals that overexpressed PD-1. Together, these data indicate that PD-1 signaling plays a role in regulating positive selection.

Given the induction of PD-1 expression on mature T cells after strong TCR engagement with cognate pMHC, it is plausible that negatively selecting signals in the thymus also induce PD-1. However, PD-1's role in negative selection remains controversial. Several groups found that *Pcdc1* was induced during negative selection (206-209). Schmitz and colleagues utilized N15 H-2^b mice stimulated with the VSV8 peptide to induce negative selection and found that expression of *Pcdc1* was increased during negative selection (207). Another group using the HY model found that PD-1 was upregulated in HY male thymocytes (negatively selected) compared with female thymocytes (not negatively selected) (206). Similar results have been reported by other groups (208, 209). In contrast to these findings, other groups have demonstrated that PD-1 is not required for negative selection in various TCR-Tg models (129, 210).

These data demonstrate that PD-1, while not necessary for negative selection, is upregulated during the process.

There is limited evidence that PD-1 may impact agonist selection; however, its role has not been well-studied among different agonist selected cell types. Ellestad and colleagues transplanted PD-1 knockout hematopoietic stem cells into Rag2 knockout animals and found that PD-1 deficient cells were more likely to become Tregs compared to wildtype cells (211). Although this data is compelling, it should be noted that Rag2 knockout animals have altered thymic architecture including impaired TEC maturation, especially mTEC that are critical for Treg development (212). This makes using Rag2 knockout animals as a model for agonist selection complicated. In another study, the authors examined PD-1 knockout animals as well as mixed bone marrow chimeras and determined that the absence of PD-1 increased thymic Treg development (213). To ensure that the increase in Tregs was due to an increase in thymic selection, the authors utilized a novel Treg-derived TCR-Tg (MJ23) model, which was previously found to be specific for a prostate self-antigen that is also a tumor-associated antigen, and facilitated the development of Tregs in the thymus (214). When the authors generated mixed bone marrow chimeras using PD-1 deficient and PD-1 sufficient MJ23 cells engrafted at a low precursor frequency, they found that MJ23 PD-1 knockout cells were more likely to become Tregs in the thymus (213). Together, these results indicate that PD-1 may play a role in regulating agonist selection of Tregs; however, the mechanism of this regulation remains to be determined. To

this point, it is possible that PD-1 could be directly inhibiting signaling through the TCR to limit Treg development. Perhaps more attractive is the hypothesis that PD-1 is inhibiting CD28 costimulation, which is known to be important for Treg development. Further work is required to tease apart these hypotheses. Additionally, more research is needed to determine if PD-1 is regulating other agonist selected cell populations, including iNKT cells and CD8αα T cells.

When considering the published data, it is plausible that PD-1 blockade alters the repertoire of T cells being selected for with important implications for tumor biology. Indeed, evidence suggests that there may be an increase in immunosuppressive Tregs resulting from PD-1 blockade. As discussed, elderly populations at the highest risk of cancer already have a relative increase in the production of Tregs; therefore, adding PD-1 blockade would further increase Treg generation. Some of these Tregs will likely be responsive to self-antigens that are expressed on tumors, allowing for greater recruitment of these Tregs into the tumor and the facilitation of an even more immunosuppressive microenvironment, leading to therapeutic resistance.

Other inhibitory proteins

Adapted from DOI: https://doi.org/10.1016/j.trecan.2023.04.007(171)

The emerging identification of novel T cell inhibitory receptors and their contribution to positive, negative, or agonist selection has not been well studied. However, we know that some of these molecules are expressed during T cell

development and at times when they could conceivably impact cell fate decisions (Fig. 1.8).

B and T lymphocyte attenuator (BTLA) is a type I transmembrane receptor that belongs to the Ig-superfamily. It is like PD-1 in that it has ITIM and ITSM motifs, but engagement of BTLA leads to the preferential recruitment of SHP-1, which mediates the inhibitory effects of this receptor (197, 215). The only known ligand is the Herpes virus entry mediator (HVEM), but its expression in the thymus remains unreported (216). Han and colleagues found that BTLA was upregulated on thymocytes upon positive selection (217). TCRα chain-deficient animals, whose thymocytes fail to undergo positive selection, did not have any BTLA positive thymocytes. Conversely, most of the thymocytes that were positively selected for on the AND TCR-Tg background expressed BTLA. The authors found that BTLA was upregulated along with CD69 and TCR during β selection, consistent with BTLA being upregulated during positive selection with weak TCR signals. In the absence of BTLA, thymocyte development was reported to be normal, although the data was not shown. Another group also reported normal thymocyte development in BTLA deficient animals (215); however, the authors did not examine agonist selected cell populations. A more recent report found that BTLA deficient animals had an increase in thymic γδ T cells (218), suggesting that BTLA may impact agonist selected cell populations. To rule out an impact of BTLA in thymocyte development, future studies should consider examining negative and agonist selection.

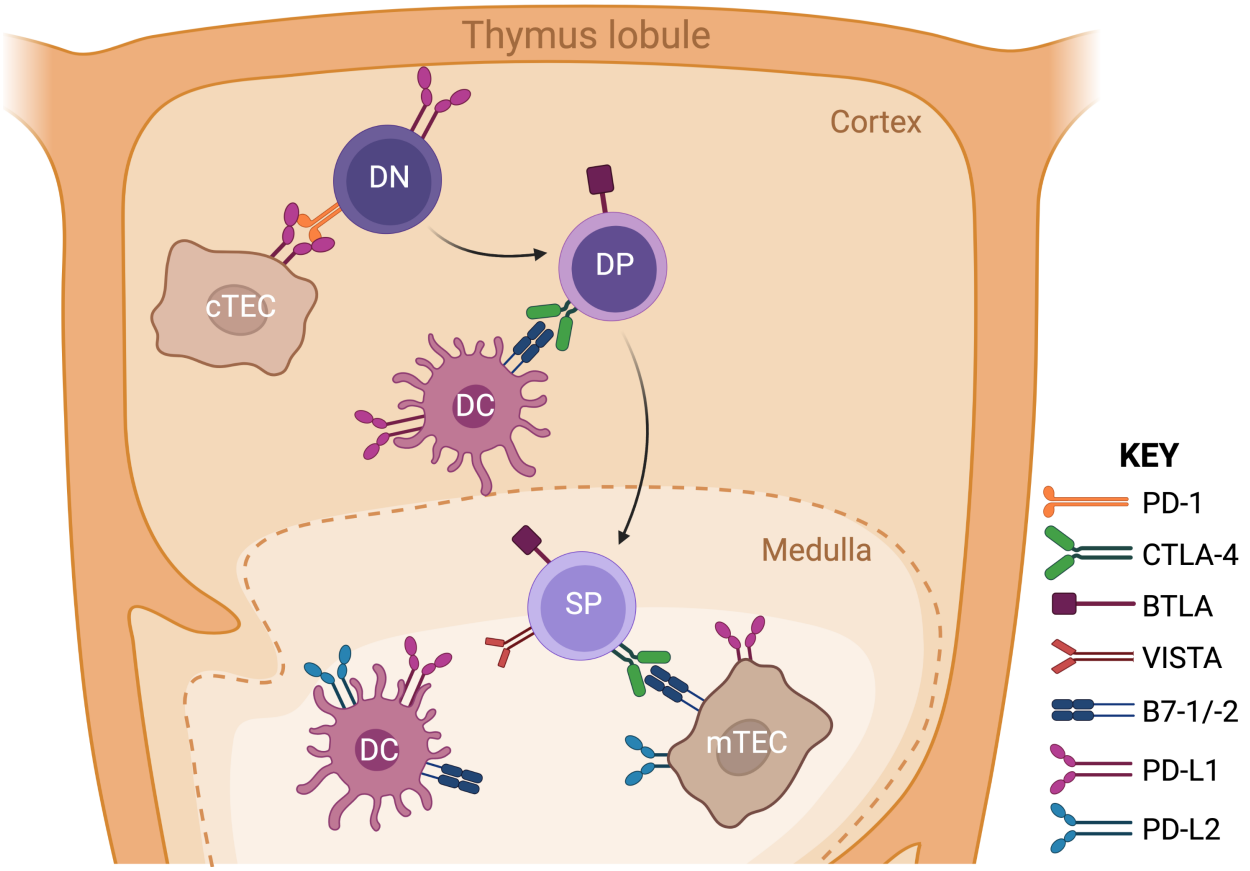

Figure 1.8 Expression of inhibitory receptors and their ligands during T cell development

Figure 1.8: Cartoon schematic demonstrating the presence of inhibitory receptors and their ligands during the developmental steps of T cells in the thymus (created with BioRender.com).

V-domain Ig suppressor of T-cell activation (VISTA) is an immune checkpoint receptor that promotes the suppression of T cell activation, proliferation, and cytokine production (219). VISTA is distinct from PD-1 and CTLA-4 in that it may act as both a ligand and a receptor when regulating immune responses. Within the thymus, VISTA expression was observed on CD4 and CD8 SP thymocytes (219). VISTA deficient mice have normal numbers and frequencies of DN, DP, CD4 SP, and CD8 SP thymocytes; however, the authors did not examine agonist selected cell populations so the impact on these cells remains unclear (220). ElTanbouly and colleagues used single-cell RNA sequencing to determine if there were any differences in the TCR repertoire of VISTA deficient animals and their wildtype littermates by examining frequency of autoreactive TCRs. The authors found no differences in the frequency of autoreactive TCRs between VISTA deficient and wildtype animals. Therefore, gross thymocyte development appears normal in the absence of VISTA, but the impact of VISTA on T cell development may be more nuanced and warrants further investigation.

Chapter 2 : PD-1 limits thymic IL-2 production and regulatory T cell development

Adapted from manuscript in preparation:

Caruso B., Weeder B.R., Thompson R.F. & Moran A.E. PD-1 restrains thymic IL-2 production and regulatory T cell development. *Submitted to J Immunology.*

Contributions: A.E.M. conceived the study and designed all the experiments with B.C. B.C. performed all the experiments and analyzed the data. Single-cell data analysis was performed by B.R.W. with guidance from R.F.T. For all other experiments, B.C. and A.E.M. interpreted the data. B.C., B.R.W., and A.E.M. wrote the manuscript.

Abstract

Inhibitory proteins, such as programmed cell death protein 1 (PD-1), have been extensively studied in peripheral T cell responses to foreign, self, and neoantigens. Notably, these proteins are first expressed during T cell development in the thymus. Reports suggest that PD-1 limits regulatory T cell (Treg) development, but the mechanism by which PD-1 exerts this function remains unknown. The present study expands the evaluation of PD-1 and its ligands in the thymus, demonstrating that some of the highest expressers of PD-1 and PD-L1 are agonist selected cells. Surprisingly, we reveal a selective role for PD-1 in regulating the developmental niche only for Tregs as other agonist selected cell populations, such as natural killer T cells, remain unchanged. We also ruled out PD-1 as a regulator of proliferation or cell death of agonist selected Tregs and further demonstrated that PD-1 deficient Tregs have reduced TCR signaling. Unexpectedly, the data suggests that PD-1 deficient thymocytes produce elevated levels

of IL-2, a Treg niche limiting cytokine. Collectively, these data suggest a novel role for PD-1 in regulating IL-2 production and the concurrent agonist selection of thymic Tregs. This observation has implications for the use of checkpoint blockade in the context of cancer and infection.

Introduction

The thymus is critical in establishing and maintaining a diverse peripheral T cell pool capable of mounting an effective response against a variety of immunologic insults. This diversity is largely due to rearrangement of germline T cell receptor (TCR) genes, which generate T cells that have a broad range of specificities. Thymocytes interact with ligands presented on self-MHC molecules in the cortex and those with productive TCRs will receive survival signals necessary for continued maturation in the process of positive selection. Developing thymocytes are then queried for self-reactivity in the medulla and those that receive strong TCR signals in response to self-ligands are deleted in the process of negative selection. A portion of cells that demonstrate some reactivity to self-ligands are diverted into alternative lineages in the process of agonist selection to produce natural killer T cells (NKT), $\gamma\delta$ T cells, CD8$\alpha\alpha$ T cells, or regulatory T cells (Tregs) (19).

Co-stimulatory and co-inhibitory molecules affect cell fate decisions in the thymus by enhancing or dampening signaling through the TCR. The most well-characterized pathway involves CD80/CD86 (B7-1/B7-2) ligands that bind to and activate CD28 and CTLA-4. Signaling through CD28 serves to enhance TCR signaling while CTLA-4 dampens TCR signaling (221). The B7 family has expanded to include programmed cell

death protein 1 (PD-1) (104), which is a CD28 homolog containing inhibitory motifs in its cytoplasmic tail that recruit Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2 upon engagement with its ligands (123), PD-L1 (222) and PD-L2 (124). This leads to dephosphorylation of signaling molecules downstream of TCR (128) and CD28 (127). The importance of the PD-1-inhibitory signal is demonstrated by the phenotype of PD-1-deficient (PD-$1^{-/-}$) mice: C57BL/6 PD-$1^{-/-}$ mice display features of lupus (129) and BALB/c PD-$1^{-/-}$ mice develop a cardiomyopathy (130). Together, these observations establish a role for PD-1 in regulating tolerance, however, it's notable that loss of this inhibitory protein does not result in more wide-spread autoimmunity.

Because co-stimulation and co-inhibition are known to modify TCR signaling, it stands to reason that these pathways may also regulate thymocyte selection. Studies have shown that PD-1 is expressed by developing thymocytes (193). Further, PD-L1 is expressed throughout the thymus while PD-L2 is limited to medullary stromal cells (202). Notably, the expression of both PD-L1 and PD-L2 by medullary stromal cells overlaps with Aire+ medullary thymic epithelial cells that mediate negative and/or agonist selection (223, 224). Some of the first studies to implicate PD-1 in T cell development were derived from PD-$1^{-/-}$ TCR transgenic mice which revealed a role for PD-1 in facilitating TCRβ-selection and inhibiting positive selection (225, 226). Further studies have suggested that PD-$1^{-/-}$ cells are more likely to become Tregs in the thymus (211, 213), pointing to a role in the regulation of agonist selection. Despite these observations, the mechanism(s) by which PD-1 contributes to agonist selection remains unclear.

Herein we define the expression of PD-1 and its ligands, PD-L1 and PD-L2, during thymocyte development. We demonstrate that PD-1 specifically restrains thymically derived Tregs as other agonist selected T cell populations remain unchanged. Interestingly, PD-1$^{-/-}$ thymocytes produce more IL-2, a Treg niche limiting cytokine, which could be responsible for the increase selection of Tregs in PD-1 deficient animals. Our findings highlight the importance of understanding the role of PD-1 in regulating T cell development with implications for the blockade of this pathway in the context of cancer and infections.

Materials and Methods

Animals

C57BL/6 (stock #000664), C57Bl/6;CD90.1 (also referred to as Thy1.1, stock #000406), and C57BL/6;CD45.1 (also referred to as B6.SJL, stock #002014) were purchased from the Jackson Laboratory. PD-L1$^{-/-}$ mice and PD-1$^{-/-}$ mice were a gift from A.H. Sharpe (Harvard Medical School, Boston, MA). Nur77-GFP mice were previously described (227) and purchased from the Jackson Laboratory. All animals were maintained under specific pathogen-free conditions in the Oregon Health & Science University (Portland, OR) animal facility.

Thymic epithelial cells (TEC), thymocyte, and lymphocyte isolation

TEC were isolated and enriched as previously described (228). For thymocyte isolation, thymi were harvested and disrupted with a 1-cc syringe plunger through a 70 μm nylon cell strainer (BD Biosciences) and filtered to obtain a single cell suspension using PBS

containing 2% FBS and 0.01% sodium azide. Spleens were harvested and processed to obtain single cell suspensions using the frosted ends of microscope slides in PBS containing 2% FBS and 0.01% sodium azide. Spleens were incubated with ammonium chloride potassium lysing buffer (Lonza) for 3 minutes at room temperature to lyse red blood cells. Cells were rinsed with PBS containing 2% FBS and 0.01% sodium azide.

Flow cytometry

For flow cytometry analysis, thymocytes and splenocytes were incubated for 20 minutes at 4°C with LIVE/DEAD Fixable Aqua (ThermoFisher) and mouse BD Fc block (BD Pharmingen) in PBS. When indicated, cells were incubated for 20 minutes at room temperature with PBS-57-loaded CD1d tetramer (NIH tetramer core). Cells were then incubated for 20 minutes at 4°C with the following extracellular antibodies: CD5 (53-7.3), CD44 (IM7), TCRβ (H57-597), CD8b (H35-17.2), PD1 (J43), CD8a (53-6.7), CD4 (RM4-5 or GK1.5), CD24 (M1/69), CD25 (PC61.5), CD28 (37.51), CD45.1 (A20), CD69 (H1.2F3), CD73 (TY/11.8), PD-L1 (10F.9G2), PD-L2 (TY25), Thy1.2 (53-2.1), Thy1.1 (HIS51), Qa2 (695H1-9-9). TECs were incubated for 20 minutes at 4°C with LIVE/DEAD Fixable Aqua (ThermoFisher) and the following extracellular antibodies: UEA1 (biotin), Ly-51 (6C3), CD45 (30-F11), MHCII (M5/114.15.2), Epcam (G8.8), PD-L1 (10F.9G2), PD-L2 (TY25). Intracellular protein FoxP3 (FJK-16s) and Ki67 (SolA15) were detected using the FoxP3 Transcription Factor Concentrate and Diluent (eBioscience). Intracellular protein IL-2 (JES6-5H4) was detected with the BD Fixation/Permeabilization kit (BD Biosciences). All antibodies and viability dyes were purchased from BioLegend, BD

Biosciences, eBioscience or Invitrogen. Data were collected with a Fortessa flow cytometer (BD Biosciences) and analyzed using Flowjo software (Tree Star).

BrdU labelling

Animals were injected with 200 μg of BrdU (Sigma) intraperitoneally. After 24 hours, thymi were harvested and processed to single cell suspension as described. For detection of BrdU incorporation, thymocytes were fixed with BD Fixation/Permeabilization buffer (BD Biosciences) for 20 minutes at room temperature followed by incubation with BD Cytoperm Permeabilization Buffer Plus (BD Biosciences) for 10 minutes on ice. Cells were then re-fixed with BD Fixation/Permeabilization buffer (BD Biosciences) for 5 minutes at room temperature. Thymocytes were treated with 30 μg DNase I for 1.5 hours at 37°C to expose BrdU epitopes. Flow cytometric staining was then performed with BrdU (Bu20a).

Bone marrow chimera generation

Femur and tibia bones were harvested from animals and a 27-gauge needle was used to flush out the bone marrow. The bone marrow was filtered through a 70 μm nylon cell strainer (BD Biosciences) using a 1-cc syringe plunger and rinsed with PBS containing 2% FBS. Mature CD8+ T cells were depleted using CD8a-PE (53-6.7) and the EasySep PE-positive selection kit (Stemcell Technologies). Animals received 500 rads x-ray followed by 450 rads x-ray four hours later to lethally irradiate the animals in an CIX2 X-Ray Cabinet (Xstrahl). Mice were then anesthetized using isoflurane and reconstituted

with $1x10^6$ – $5x10^6$ cells injected retro-orbitally. Animals were maintained on antibiotic drinking water with 2 mg/mL of neomysin sulfate (Gibco) for two weeks.

In vitro activation and intracellular cytokine staining

Thymocytes were plated at $1x10^6$ cells/well in 96-well plates and stimulated for 8 hours with PMA (80 nM) and ionomycin (1.3 μM) in the presence of brefeldin A (BFA). Cells were then stained for surface markers, fixed and permeabilized using the BD Fixation/Permeabilization kit (BD Biosciences), and stained for intracellular cytokines.

Phospho-STAT5 identification by flow cytometry

Thymocytes were stained with LIVE/DEAD Fixable Aqua (ThermoFisher) at 4°C for 20 minutes. Cells were then rested in pre-warmed serum-free RPMI for 30 minutes at 37°C to allow dephosphorylation of STAT5 from *in vivo* signaling. Cells were stimulated with pre-warmed IL-2 in RPMI (1 ng/mL, 0.25 ng/mL, 0.05 ng/mL, 0 ng/mL) for 30 minutes in a 37°C water bath. At the end of the incubation, cells were immediately placed on ice and ice-cold fixative from the FoxP3 Transcription Factor Concentrate and Diluent kit (eBioscience) was added. Cells were allowed to fix and permeabilize for 20 minutes on ice. Cells were then stained for FoxP3 and re-fixed with 4% paraformaldehyde on ice for 30 minutes followed by re-permeabilization with prechilled 90% methanol. Then, the samples were stained with pSTAT5 (pY694) (BD Bioscience) or isotype control at room temperature for 40 minutes followed by cell surface markers at room temperature for 20 minutes.

Phospho-AKT identification by flow cytometry

Thymocytes were rested at 37°C for 20 minutes to allow for dephosphorylation of AKT. Cells were then stained with LIVE/DEAD Fixable Aqua (ThermoFisher) at 4°C for 20 minutes. Cells were washed and incubated with 5 μg/mL anti-CD3-biotin (Invitrogen) and 5 μg/mL anti-CD28-biotin (BD Bioscience) along with extracellular markers on ice for 15 minutes. To stimulate the cells, they were incubated with 20 μg/mL of streptavidin (ThermoFisher) for the indicated timepoints (0, 2, 5, or 10 minutes) at 37°C before being fixed with Phosflow Lyse/Fix Buffer (BD Bioscience) for 10 minutes at 37°C. Cells were washed and resuspended with prechilled Phosflow Perm Buffer III (BD Bioscience) dropwise and incubated on ice for 1 hour. Cells were washed twice before being stained with either isotype control (Cell Signaling) or pAKT (pS473) (Cell Signaling) overnight at 4°C.

Cell sorting for single-cell RNA sequencing

Thymi from four WT or PD-1$^{-/-}$ mice were pooled. Thymocytes were obtained as previously described. CD8a+ cells were depleted using CD8a-biotin (53-6.7) and rapid spheres (Stemcell Technologies). This was followed by another depletion of CD8b+ cells using CD8b-biotin (H35-17.2) to adequately enrich for CD4+ thymocytes. Cells were stained with LIVE/DEAD Fixable Aqua (ThermoFisher) followed by extracellular staining for CD24 (M1/69), CD25 (PC61.5), CD73 (TY/11.8), CD4 (RM4-5), and CD8a (53-6.7). CD25- and CD25+ populations were then sorted (supplemental Fig. 5A). Cell sorts were performed on an Aria (BD Biosciences) with an 85-micron nozzle.

Sample sequencing and alignment

Sorted cells underwent single-cell RNA-seq library preparation using the 10X Genomics Chromium platform. Single-cell capturing and cDNA library generation were performed using the Next GEM Single Cell 5' Reagent Kit v2 (10X Genomics) according to the manufacturer's instructions. Sequencing was performed following 10x Genomics instructions using NovaSeq 6000 (Illumina) at the Massively Parallel Sequencing Shared Resource (MPSSR) at Oregon Health & Science University. Raw sequencing reads were aligned to the mouse reference genome GRCm38 and quantified using CellRanger (10x Genomics, v6.2.1).

Single cell processing and normalization

Unless otherwise specified, all single cell analysis was performed using R v.4.2.2 and Seurat v.4.3.0 (229). All Seurat objects were merged, and cells were filtered to only include those with greater than 500 features and less than 25% of reads aligning to mitochondrial genes. Cell cycle scoring was performed for each cell by taking mouse analogues of the provided S and G2M gene lists from Seurat as input into the CellCycleScoring function. Read counts were normalized using the NormalizeData and ScaleData wrappers from Seurat with S and G2M scores regressed. The FindVariableFeatures function was used to identify the 2,000 most variable genes for downstream analysis.

Single cell clustering

Principal component (PC) analysis was performed using the 2,000 most variable features and the first 10 components were determined to capture the most dataset variance based on the elbow plot. Clustering was then performed after calculating nearest neighbors using the 10 selected PC's as input to the FindNeighbors function. Clustering was performed using the Louvain algorithm with a clustering resolution of 0.5. This resolution was selected by minimizing the average silhouette width per cluster across all clusters identified using the FindClusters function with resolutions ranging from 0.2 to 0.7. Cluster stability across neighboring resolutions was visually assessed using the clustree package(230) (Fig. 2.9B). Positive differentially expressed markers for each of the 14 identified clusters were determined using Seurat's FindAllMarkers function and manual cell type annotation was performed based on the top markers for each cluster as well as canonical markers defined in literature (Fig. 2.10B, C).

Gene set enrichment

Gene set enrichment analysis (GSEA) was performed to compare WT vs. PD-1$^{-/-}$ enrichment, as well as to independently compare enrichment in WT vs PD-1$^{-/-}$ semi-mature Tregs specifically (cluster 0 vs. cluster 1). In each instance, differentially expressed genes between the two groups were identified using the Seurat FindMarkers function with a minimum log-2 fold threshold of 0.2. Differentially expressed gene lists were ordered by log-2 fold change and GSEA was performed using the msigdb R package from the Molecular Signatures Database (231). When identifying enriched gene sets, all Hallmark gene sets were considered, as well as 16 immune specific gene sets from the Reactome database.

Statistical analysis

Statistical analysis was performed using either unpaired two-tailed Student *t* test or multiple unpaired T tests using the Holm-Šídák method of correction for multiple comparisons in GraphPad Prism 9 (GraphPad Software). Data are presented as mean with error bars representing standard error of the mean (SEM). Statistical tests and P values are specified in the respective figure legends, and P values < 0.05 were considered significant. Biological replicates (individual animals) for each experiment are indicated in the figure legends. All experiments were repeated as indicated in the figure legends.

Study Approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University (Portland, OR).

Results

PD-1 and its ligands, PD-L1 and PD-L2, are expressed in the thymus

It has previously been established that PD-1 and its ligands are expressed in the thymus (193, 202), but the exact kinetics of PD-1 expression throughout thymocyte development remain undefined. To confirm thymic expression of PD-L1 and PD-L2, thymi from 6-week-old mice were harvested and enriched for thymic epithelial cells to examine the expression of PD-L1 and PD-L2 in cortical ($UEA1^{lo}$, $Ly51^{hi}$) and medullary ($UEA1^{hi}$, $Ly51^{lo}$) thymic epithelial cells using flow cytometry (Fig. 2.1A). PD-L1 was expressed equally on

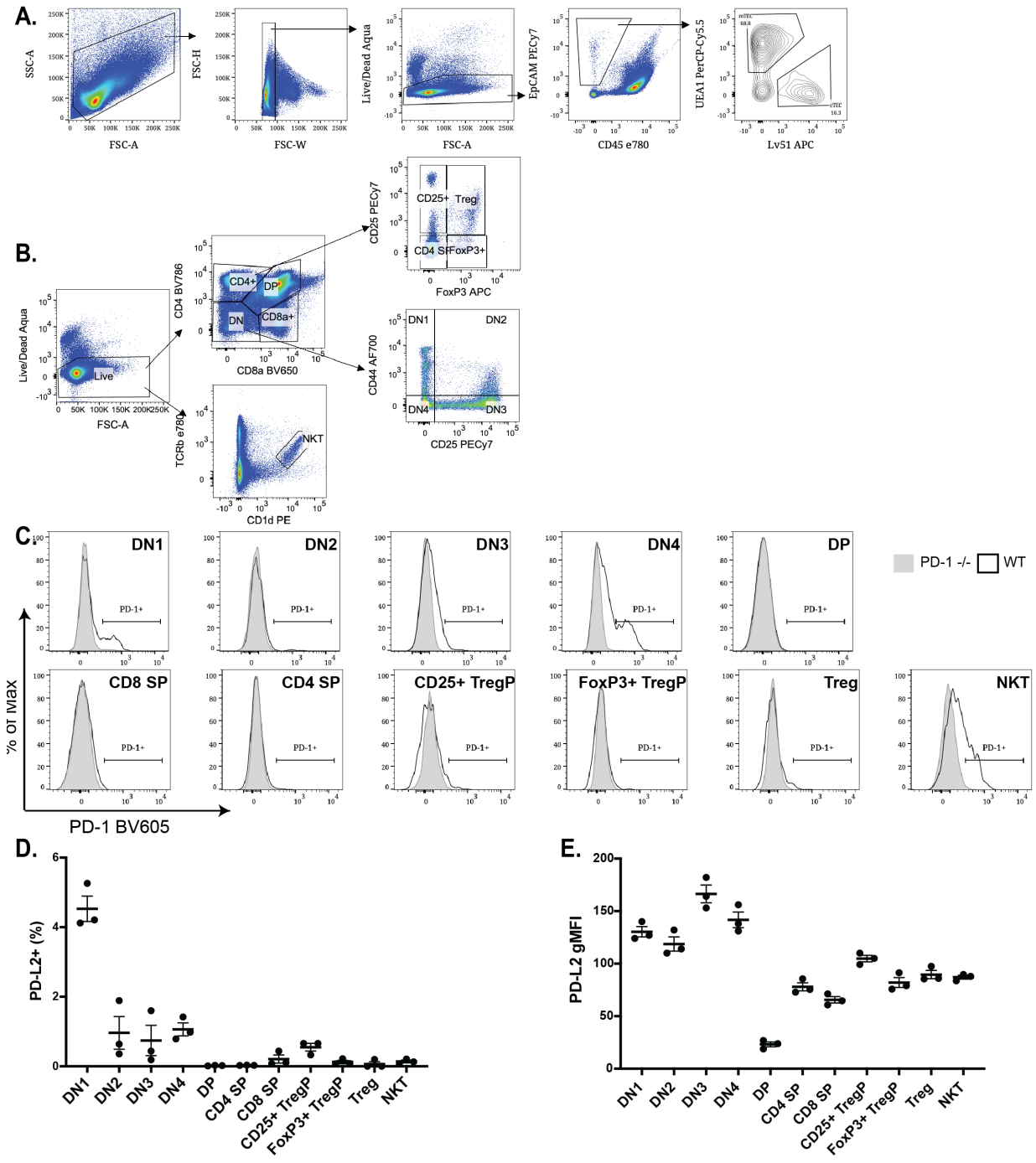

Figure 2.1 PD-1, but not PD-L2, are expressed by thymocytes

Figure 2.1: PD-1, but not PD-L2, are expressed by thymocytes. A) Gating strategy for identification of cTEC and mTEC. **B)** Gating strategy for identification of thymocyte populations. **C)** Histograms depicting PD-1 expression in thymi from 3-week-old WT mice. **D)** Quantification of PD-1 gMFI. Data are representative of 3 independent experiments. **E)** Quantification of PD-L2 gMFI in WT thymocytes. Data are representative of 2 independent experiments.

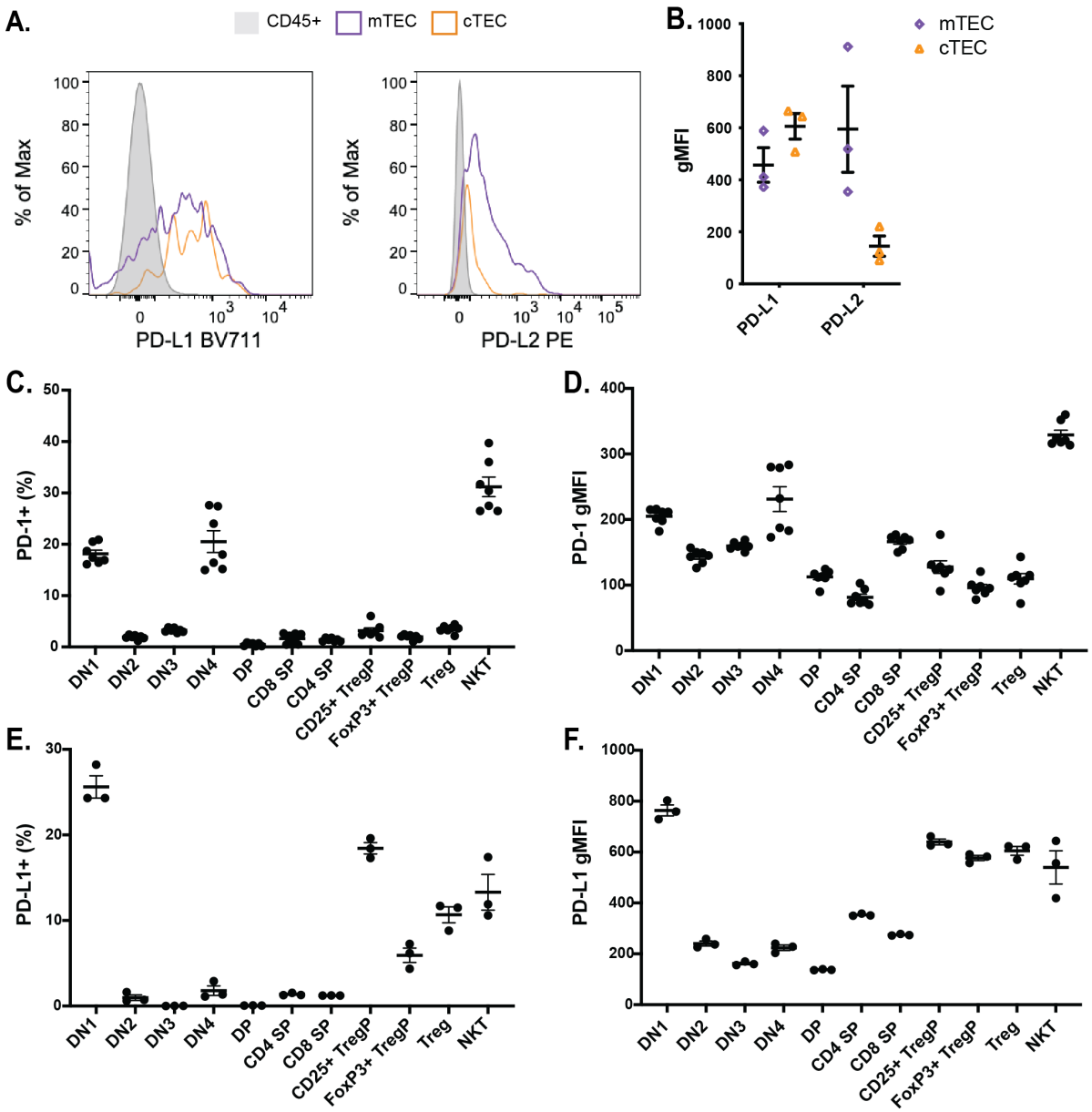

Figure 2.2 PD-1 and its ligands, PD-L1 and PD-L2, are expressed in the thymus

Figure 2.2: PD-1 and its ligands, PD-L1 and PD-L2, are expressed in the thymus. A) Thymi were harvested from 6-week-old WT mice (n = 3) and depleted of CD45+ thymocytes by panning to enrich for thymic epithelial cells. Staining for PD-L1 and PD-L2 in medullary thymic epithelial cells (mTEC) and cortical thymic epithelial cells (cTEC) and **B)** gMFI of PD-L1 and PD-L2 in mTEC and cTEC. Data representative of 3 independent experiments. **C)** Thymi were harvested from 3-week-old WT mice. Frequency of PD-1+ cells and **D)** gMFI of PD-1 from 2 combined experiments (n = 7). **E)** Frequency of PD-L1 and **F)** gMFI of PD-L1 across thymocyte development (n = 3). Data are representative of 3 independent experiments.

both cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC), while PD-L2 was expressed only in a subset of mTEC (Fig. 2.2A, B). Next, we examined PD-1 expression across early thymocyte development (Fig 2.1B) and found that a subset of DN1 and DN4 highly expressed PD-1 (Fig. 2.1C, Fig. 2.2C, D). In post-positive selection thymocyte subsets, we observed natural killer T (NKT) cells were the highest expressers of PD-1 (Fig. 2.1C, Fig 2.2C, D). Interestingly, we found that thymocytes also expressed PD-L1, with agonist selected cell populations such as regulatory T cells (Treg) and NKT cells (Fig. 2.2E, F) having some of the highest expression. Notably, thymocytes did not express PD-L2 (Fig. 2.1D, E). These data demonstrate that PD-1 and its ligands are expressed throughout thymocyte development and could play a role in regulating the development of T cells.

PD-1 restrains thymic Treg development

Given that PD-1 and its ligands are expressed in the thymus, PD-1 signaling could regulate thymocyte maturation. Indeed, previous studies have suggested that PD-1 inhibits β selection (225, 226), but PD-1's role in regulating other stages of thymocyte development has not been well characterized. To elucidate the role of PD-1 in thymocyte development, we examined the thymus of 3-week-old PD-$1^{-/-}$ animals. We found that there were no differences in the early double negative (DN1 – 4) stages of thymocyte development (Fig. 2.3A – C) in PD-1 deficient animals. In contrast, PD-$1^{-/-}$ animals had a significant increase in Tregs, along with CD25+ (CD25+ TregP) and FoxP3+ (FoxP3+ TregP) Treg progenitors. However, not all agonist selected T cell populations were expanded as NKT cell frequencies remained unchanged (Fig. 2.3D – F). To ensure that

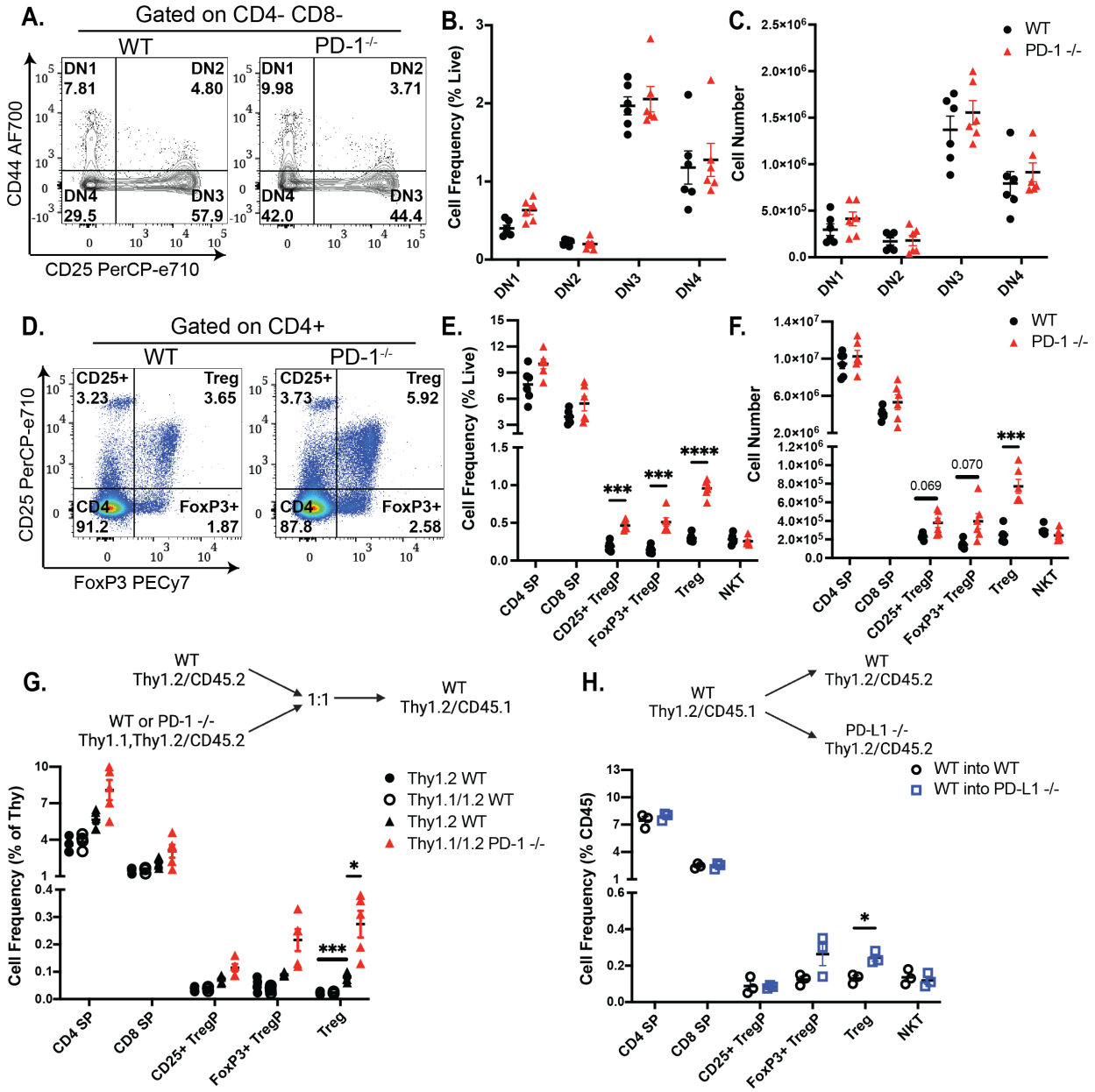

Figure 2.3 PD-1 signaling restrains thymic Treg development

Figure 2.3: PD-1 signaling restrains thymic Treg development. Thymi were harvested from 3-week-old WT or PD-1$^{-/-}$ mice (n = 6). **A)** Identification of double negative thymocyte populations and quantification of DN populations as **B)** % live and **C)** cell number. **D)** Identification of regulatory T cells and their progenitor populations using expression of CD25 and FoxP3 to delineate CD4 SP (CD25- FoxP3-), CD25+ TregP (CD25+ FoxP3-), FoxP3+ TregP (CD25- FoxP3+) and Treg (CD25+ FoxP3+) and quantification of thymocyte populations as **E)** % live and **F)** cell number. Data are combined from two experiments. **G)** Schematic demonstrating the generation of mixed bone marrow chimeras. Thymi were harvested six weeks after reconstitution (n = 5). The frequency of thymocyte populations is presented as % congenic marker (Thy). Data are representative of 2 independent experiments. **F)** Schematic demonstrating the generation of bone marrow chimeras. Thymi were harvested six weeks after reconstitution (n = 3). The frequency of thymocyte populations is presented as % congenic marker (CD45). Data are representative of 3 independent experiments. Multiple unpaired T tests using the Holm-Šídák correction for multiple comparisons. * P<0.05, *** P<0.001, **** P<0.0001

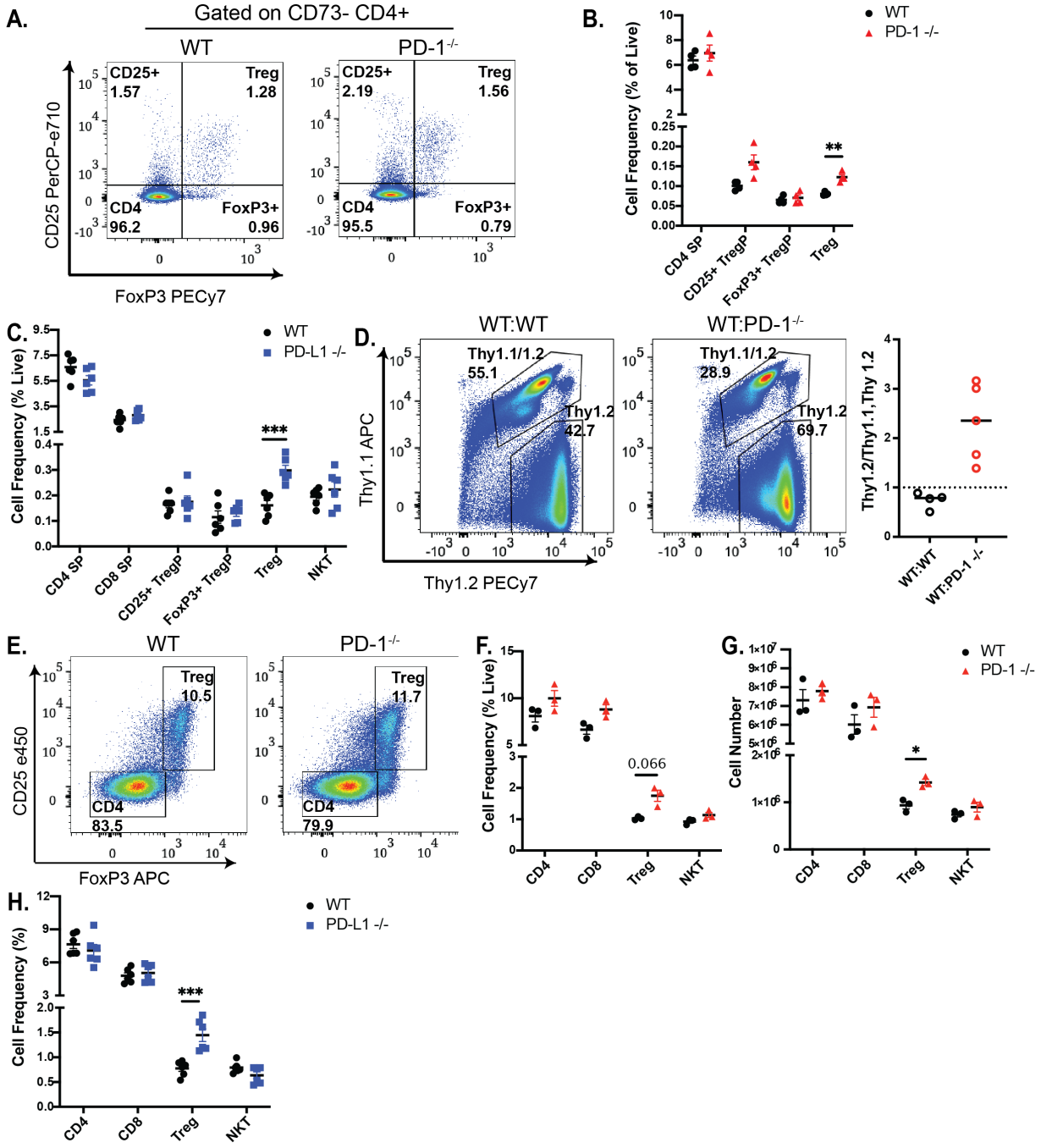

Figure 2.4 PD-1 signaling restrains Treg development in the thymus

Figure 2.4: PD-1 signaling restrains Treg development in the thymus. **A)** Flow plots depicting the gating of CD73- CD4+ thymocytes and **B)** frequency of Tregs and their progenitor populations (n = 3). Data are representative of 2 independent experiments. **C)** Frequency of thymocyte populations from 3-week-old WT or PD-L1$^{-/-}$ mice (n = 6). Data are combined from 2 experiments. **D)** Mixed bone marrow chimeras were generated at a ratio of 50:50 (n = 5). Chimerism of WT:WT and WT:PD-1$^{-/-}$. Data are representative of 3 independent experiments. **E)** Flow plots of splenocytes from 3-week-old WT or PD-1$^{-/-}$ mice (n = 3). **F)** Frequency and **G)** cell number of splenocyte populations. Data are representative of 3 independent experiments. **H)** Splenocytes from 3-week-old WT or PD-L1$^{-/-}$ mice (n = 6). Data are combined from 2 experiments. Multiple unpaired T tests using the Holm-Šídák correction for multiple comparisons. * P<0.05, ** P<0.02, *** P<0.001

these Tregs were not recirculating, we examined CD73- cells and quantified Tregs and their progenitors. We found that the significant increase in Tregs remained when CD73- cells were examined (Fig. 2.4A, B).

We next explored whether PD-1 restrains Treg development in a cell-intrinsic manner. To answer this question, mixed bone marrow chimeras were generated at a ratio of 1:1 (WT:PD-1$^{-/-}$ or WT:WT) (Fig. 2.4D). Consistent with our observations in PD-1$^{-/-}$ mice, there was a significant increase in Tregs in the PD-1$^{-/-}$ donor T cells compared to WT cells. Notably, we also observed an increase in Tregs in WT donor T cells in the WT:PD-1$^{-/-}$ mixed bone marrow chimeras compared to controls (Fig. 2.3G).

To understand the contribution of PD-L1 in supporting the phenotype we observe, we examined PD-L1$^{-/-}$ animals and also observed an increase in Tregs, but not NKT cells (Fig. 2.4C), further suggesting that PD-1 signaling is specifically regulating Treg development. Because some thymocyte populations expressed high levels of PD-L1, we speculated that PD-L1 on the stromal cells could be contributing to Treg development. Thus, we generated bone marrow chimeras of WT bone marrow into either WT or PD-L1$^{-/-}$ mice. We observed an increase in Tregs specifically in the PD-L1$^{-/-}$ animals (Fig. 2.3H), suggesting that PD-L1 on the stromal cells contributes to Treg development.

Given the increase in thymic Tregs in PD-1$^{-/-}$ animals, we also evaluated peripheral tissues for frequency of Tregs. We found that there was an increase in Tregs in both PD-1 deficient (Fig. 2.4 E – G) and PD-L1 deficient (Fig. 2.4 H) animals.

PD-1 deficient thymocytes are more mature

Given the increase in Tregs in PD-1$^{-/-}$ thymi, we postulated that perhaps in the absence of this co-inhibitory signal, thymocytes mature at an accelerated rate. To explore this, thymi from 5-week-old WT and PD-1$^{-/-}$ animals were profiled with markers that can distinguish immature from mature thymocytes, HSA (also known as CD24) and Qa2 (232). Interestingly, there were fewer immature thymocytes (HSA^{hi}Qa2^{lo}) and more mature (HSA^{lo}Qa2^{hi}) CD4 SP and CD25+ TregP thymocytes in PD-1$^{-/-}$ animals (Fig. 2.5A, B).

Because these thymocytes appear to be more mature, we hypothesized that the PD-1 deficient cells may be more proliferative leading to an increase in Tregs. Importantly, we saw no difference in cell proliferation as measured by Ki67 (Fig. 2.5C, Fig. 2.6A). We also evaluated proliferation by injecting 3-week-old WT and PD-1$^{-/-}$ animals with 200 μg BrdU intraperitoneally and harvested thymi 24 hours later. We saw no difference in the frequency of BrdU+ cells between WT and PD-1$^{-/-}$ animals (Fig. 2.5D, Fig. 2.6B). Taken together, these data suggests that the increased abundance of Tregs is not due to a proliferative advantage.

An alternative hypothesis for the increase in Tregs is that negative selection of these cells is compromised in PD-1$^{-/-}$ animals. We examined cell death through staining thymocytes from 3-week-old WT and PD-1$^{-/-}$ animals for cleaved caspase-3. We observed no differences in cleaved caspase-3 staining in WT and PD-1$^{-/-}$ animals (Fig. 2.5E, Fig.

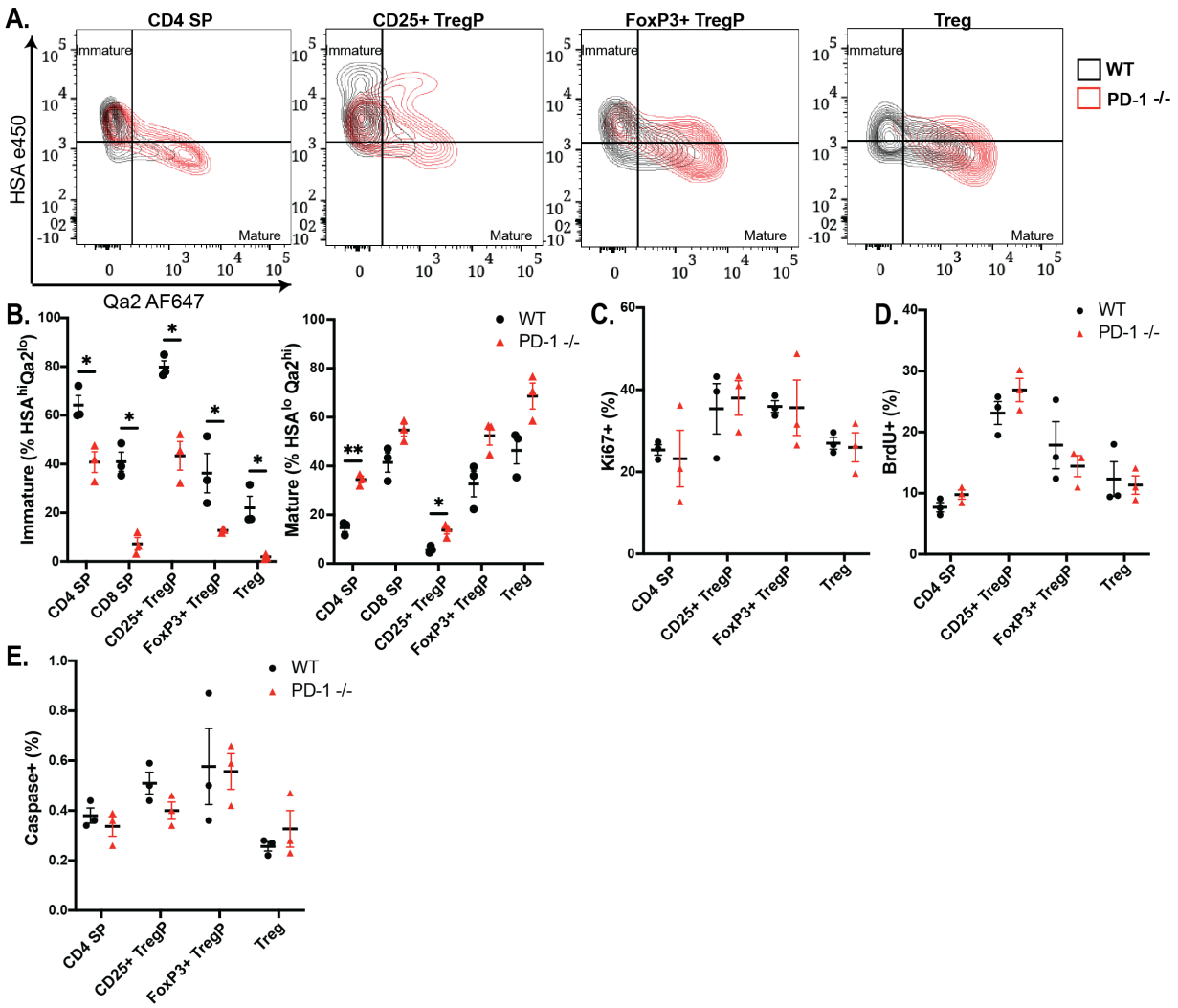

Figure 2.5 PD-1 deficiency leads to an increase in thymocyte maturation with no difference in cell proliferation or cell death

Figure 2.5: PD-1 deficiency leads to an increase in thymocyte maturation with no difference in cell proliferation or cell death. Thymi were harvested from 5-week-old WT or PD-1$^{-/-}$ mice (n = 3). **A)** Flow plots for the expression of HSA and Qa2. **B)** Quantification of immature cells (%HSA^{hi}Qa2^{lo}) and mature cells (%HSA^{lo}Qa2^{hi}). **C)** 3-week-old WT or PD-1$^{-/-}$ mice (n = 3) were injected with 200 μg of BrdU I.P. Thymi were harvested 24 hours later and stained for proliferation markers and cleaved caspase-3 to mark dying cells. Quantification of Ki67+ cells (%). **D)** Quantification of BrdU+ cells (%). **E)** Quantification of cleaved caspase 3+ cells (%). Data are representative of 3 independent experiments. Multiple unpaired T tests using the Holm-Šídák correction for multiple comparisons. * P<0.05

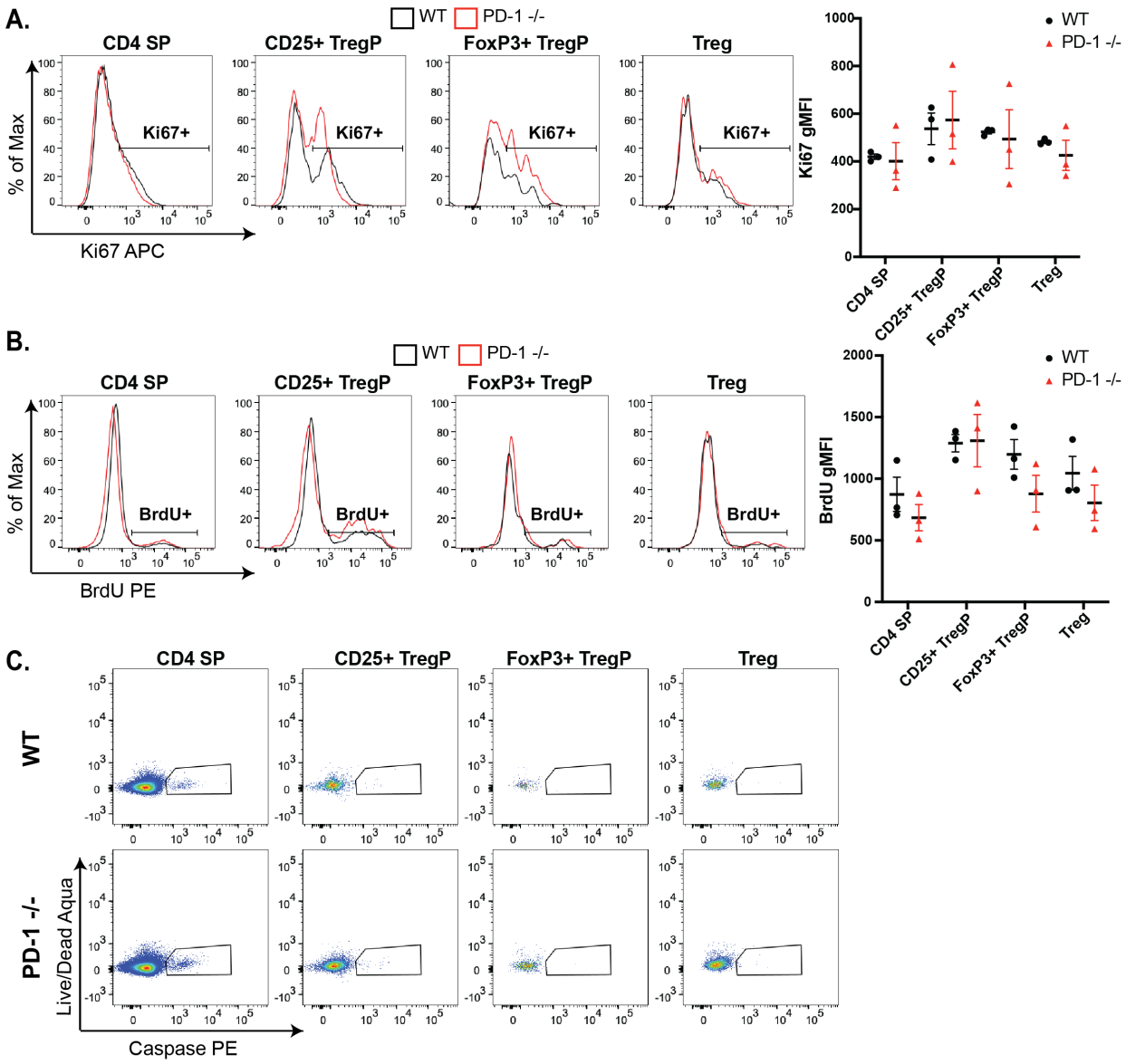

Figure 2.6 PD-1 deficiency does not alter cell proliferation or cell death

Figure 2.6: PD-1 deficiency does not alter cell proliferation or cell death. A) Histograms depicting Ki67 from 3-week-old WT or PD-1$^{-/-}$ mice (n = 3) and Ki67 gMFI. **C)** 200 μg of BrdU was injected i.p. into 3-week-old WT or PD-1$^{-/-}$ mice (n = 3) and thymi were harvested 24 hours later. Histograms depicting BrdU and BrdU gMFI. **D)** Thymi from 3-week-old WT or PD-1$^{-/-}$ mice (n = 3) were harvested and stained for cleaved caspase-3. Flow plots depicting staining viability dye and cleaved caspase 3. Data are representative of three independent experiments.

2.6C), suggesting that the increase in Tregs in PD-1 deficient thymocytes was not due to diminished cell death.

PD-1 deficiency alters TCR signal strength

Given that PD-1 can temper TCR signaling (128), we sought to determine whether PD-1 was restraining Treg development through modulation of TCR signaling. It has been demonstrated that Nur77 expression correlates with TCR signal strength such that higher levels of Nur77 indicate stronger signaling through the TCR (227). To assess Nur77 expression when PD-1 signaling is disrupted, we reconstituted WT or PD-L1 -/- mice with bone marrow from Nur77-GFP reporter animals. We evaluated thymi 6-8 weeks after reconstitution and noted a decrease in Nur77-GFP expression in FoxP3+ TregP and Treg in PD-L1$^{-/-}$ mice (Fig. 2.7A, B) compared to WT. When we specifically looked at Nur77-GFP+ cells and examined the gMFI of the GFP+ cells, we found a significant decrease in overall Nur77GFP expression in CD4 SP, CD25+ TregP, FoxP3+ TregP, and Treg of the PD-L1$^{-/-}$ compared to WT mice (Fig. 2.8A).

Because Nur77 expression was decreased when PD-1 signaling was disrupted, we hypothesized that thymocytes with lower affinity TCRs were being selected into the Treg lineage in the absence of PD-1 signaling. To test this hypothesis, we examined expression of CD5, which is known to correlate with TCR signal strength (233-235). We determined that overall CD5 expression was reduced on FoxP3+ TregP (Fig. 2.7C, D). Because changes in CD5 expression are often subtle, we gated the top 50% of CD5-expressing cells as CD5^{hi} and the bottom 50% as CD5^{lo} based upon the CD4 SP

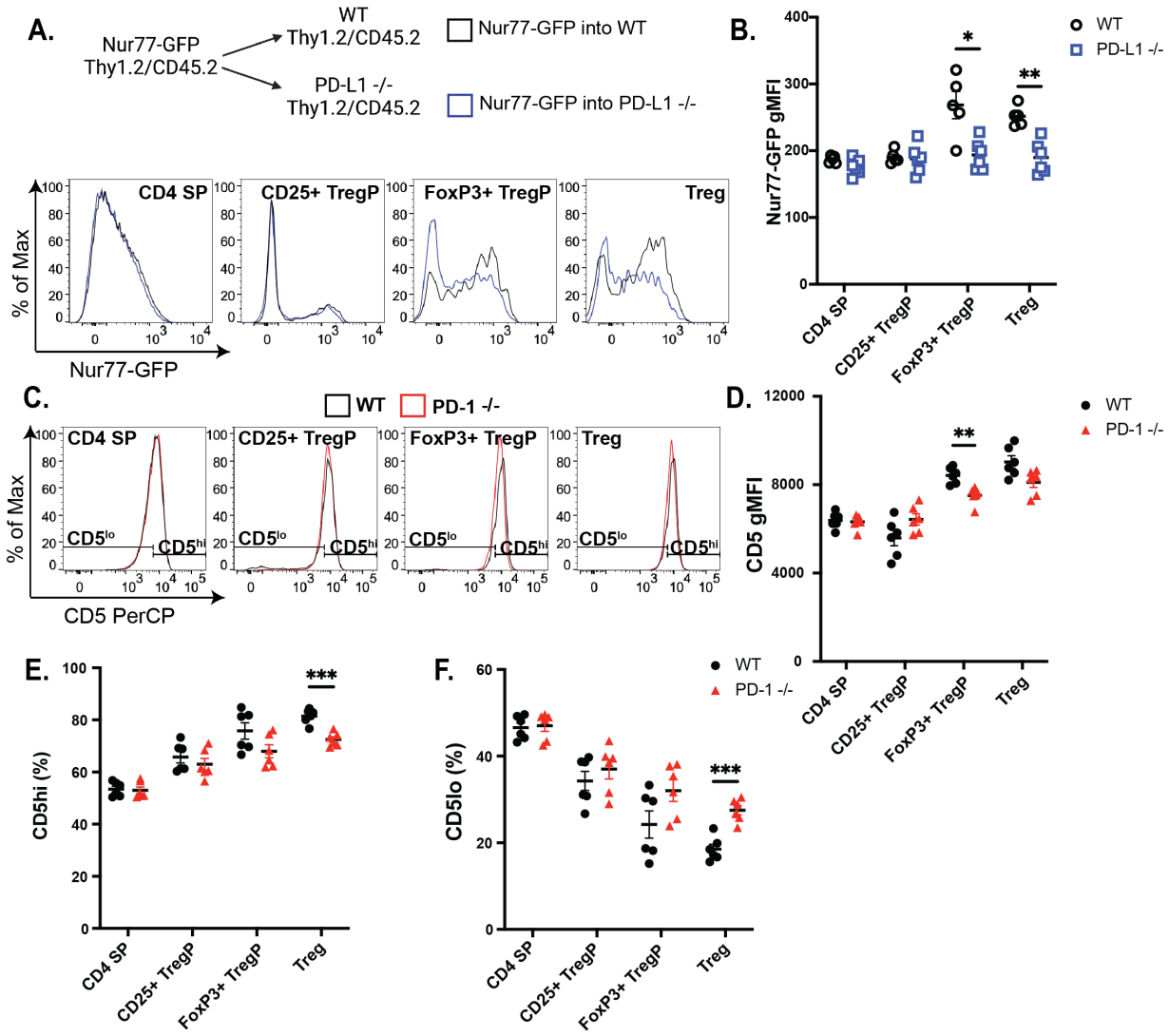

Figure 2.7 PD-1 deficiency alters TCR signal strength

Figure 2.7: PD-1 deficiency alters TCR signal strength. A) Schematic of bone marrow chimera generation. Thymi were harvested after six weeks of reconstitution (n = 5). Histograms depicting Nur77-GFP expression and **B)** gMFI of Nur77-GFP. Data are representative of 2 independent experiments. **C)** Thymi were harvested from 3-week-old WT or PD-1$^{-/-}$ mice (n = 6). Histograms depicting CD5 expression. CD4 SP thymocytes were gated so that the top 50% of cells were CD5^{hi} and the bottom 50% of cells were CD5^{lo} and then gates were applied to all populations of interest. Representative of three independent experiments. **D)** CD5 gMFI and **E)** frequency of CD5^{hi} and **F)** CD5^{lo} cells. Data are combined from 2 independent experiments. Multiple unpaired T tests using the Holm-Šídák correction for multiple comparisons. * *P*<0.05, ** *P*<0.01, *** P<0.001

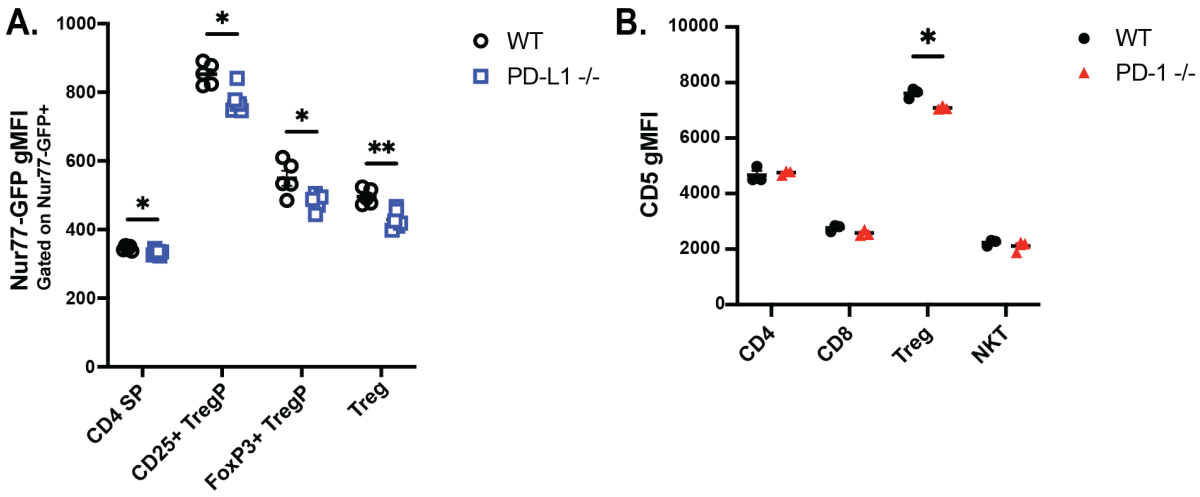

Figure 2.8 PD-1 knockout alters measures of TCR signal strength

Figure 2.8: PD-1 knockout alters measures of TCR signal strength. A) Nur77-GFP gMFI in populations of interest from bone marrow chimeras at 6 weeks post-reconstitution (n = 5). **B)** CD5 gMFI in splenocytes from 3-week-old WT and PD-1$^{-/-}$ (n = 3). Multiple unpaired T tests using the Holm-Šídák correction for multiple comparisons. * *P*<0.05, ** P<0.01

population and applied these gates to all populations of interest (Fig. 2.7C). Using this gating strategy, we found that there was a decrease in the $CD5^{hi}$ Treg population (Fig. 2.7E) with a concomitant increase in the $CD5^{lo}$ Treg population (Fig. 2.7F). CD5 levels are thought to be set in the thymus and then persist in the periphery, so we examined CD5 expression in splenocytes and found a decrease in Treg CD5 gMFI (Fig. 2.8B). These data suggested that PD-1 may limit Treg development by regulating T cell receptor signal strength.

Single-cell RNA sequencing confirms a decrease in TCR signal strength and an increase in maturity markers in PD-$1^{-/-}$ thymocytes

To understand how PD-1 is regulating Treg development, we sorted CD4+CD25- thymocytes which will contain conventionally selected CD4s (termed CD4) and CD4+CD25+ which will contain Tregs and the CD25+ TregP (termed Treg+P) from 3-week-old WT and PD-$1^{-/-}$ thymi (Fig. 2.9A). Because there are relatively limited numbers of Tregs and their progenitors in the thymus, we performed single-cell RNA sequencing (scRNAseq) using the 10X Genomics platform. We captured 28,302 cells total and 24,655 cells passed quality control and were taken on for further analysis. Fourteen unique clusters were identified (Fig. 2.10A – C, Fig. 2.9B) with one large cluster comprised mostly of TregP and a second comprised of CD4 and Treg (Fig. 2.9C). To begin understanding differences between cells sorted from WT or from PD-$1^{-/-}$ animals, we performed a pseudo-bulk analysis comparing WT to PD-$1^{-/-}$ cells followed by gene set enrichment analysis (GSEA). This analysis suggested that TCR signaling was significantly

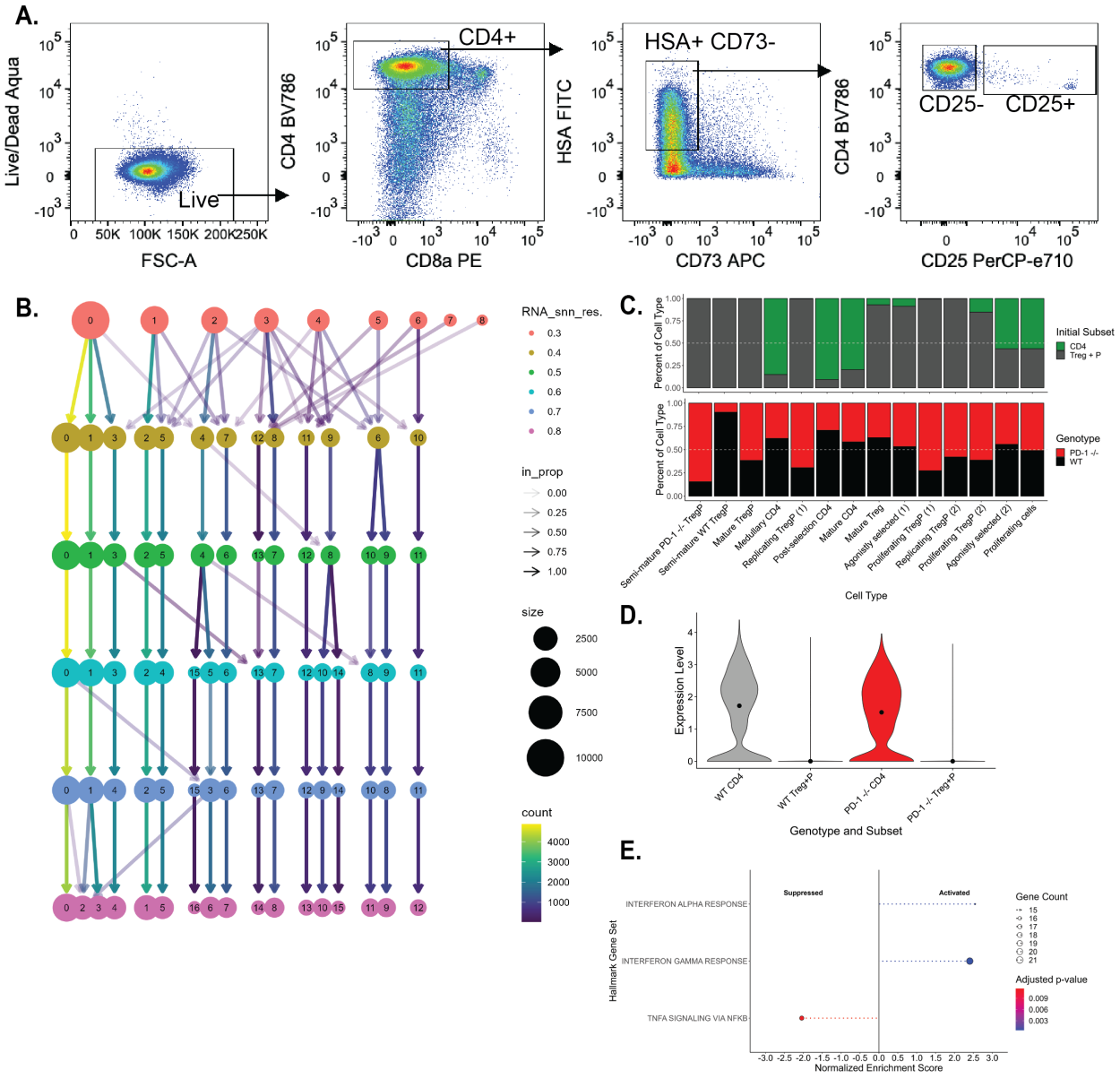

Figure 2.9 Single-cell RNAseq of CD4+ SP and CD25+CD4+ Treg+P

Figure 2.9: Single-cell RNAseq of CD4+ SP and CD25+CD4+ Treg+P. Thymi from 3-week-old WT or PD-1$^{-/-}$ animals were brought to single-cell suspension and CD8a+ cells were depleted prior to submission of samples for FACS. **A)** Gating scheme for FACS for samples submitted for scRNAseq. **B)** Clustree to demonstrate finalized clustering scheme. **C)** Stacked bar graphs showing the sample origin and genotype origin of each cluster. **D)** Cd5 gene expression from scRNAseq of WT and PD-1$^{-/-}$ CD4+CD25- (CD4) and CD4+CD25+ (TregP) (P = 8.691x10^{-96}). **E)** GSEA of cluster 0 (Semi-mature PD-1$^{-/-}$ TregP) versus cluster 1 (Semi-mature WT TregP).

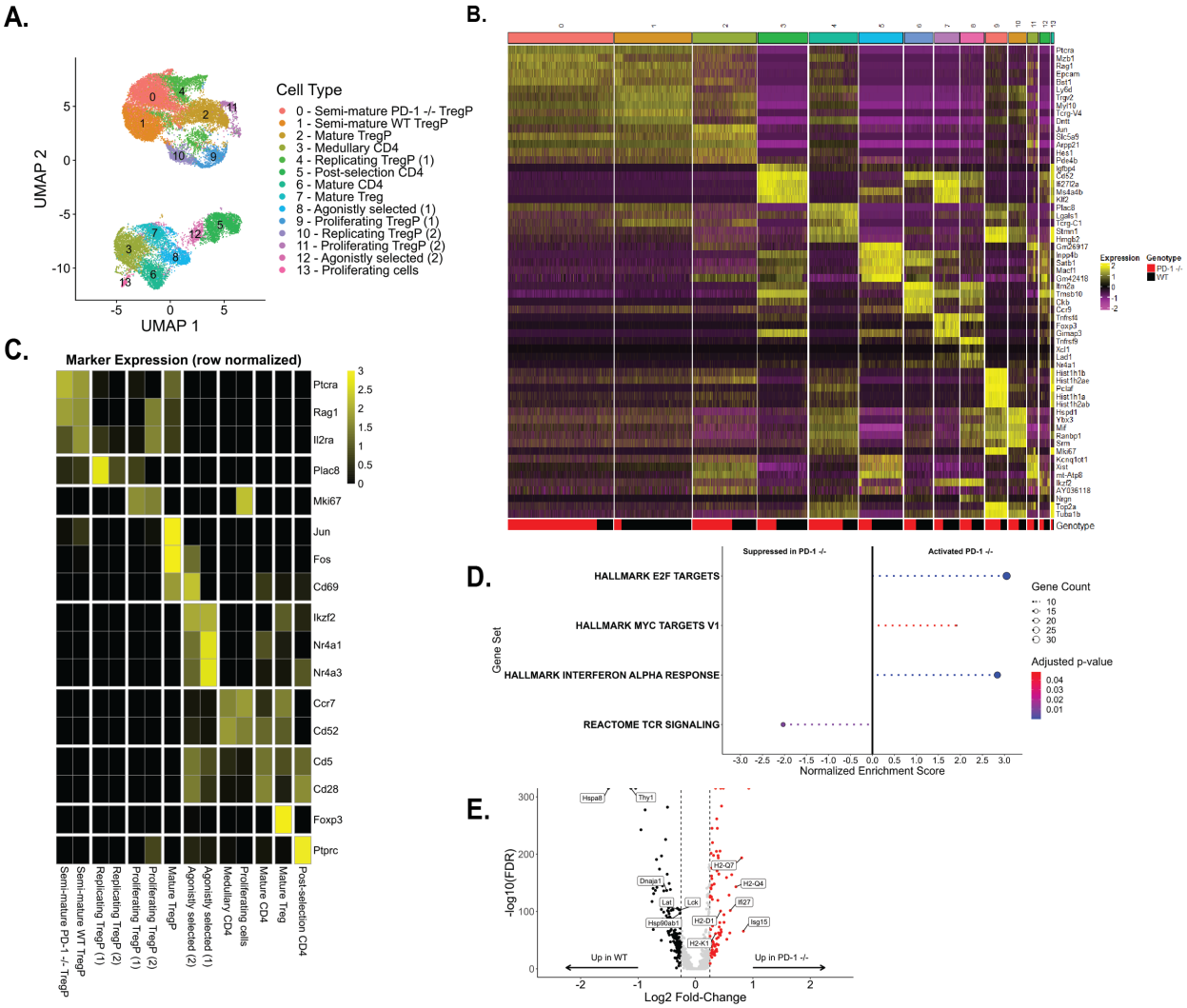

Figure 2.10 PD-1 deficient cells are more mature despite lower TCR signaling

Figure 2.10: PD-1 deficient cells are more mature despite lower TCR signaling. Thymi were harvested from 3-week-old WT or PD-1$^{-/-}$ mice (n = 3) and pooled for FACS. CD4+CD25- (CD4) and CD4+CD25+ (Treg+P) populations were sorted and scRNAseq was performed using the 10X Genomics platform. **A)** Uniform manifold approximation and projection (UMAP) of all cells that passed quality control (24,655 cells). **B)** Heatmap showing scaled expression of top markers ranked by fold change in each cluster. **C)** Heatmap showing top markers used to call clusters. **D)** Activated and suppressed hallmark pathways in PD-1$^{-/-}$ compared to WT in pseudo-bulk analysis. **E)** Volcano plot of differentially expressed genes in cluster 0 (Semi-mature PD-1$^{-/-}$ TregP) versus cluster 1 (Semi-mature WT TregP).

downregulated in PD-1$^{-/-}$ cells compared to WT cells, while MYC targets and E2F targets were significantly upregulated in PD-1$^{-/-}$ cells compared to WT cells (Fig. 2.10D). Indeed, we found that *Cd5* gene expression was down in PD-1$^{-/-}$ cells from our scRNAseq analysis (Fig. 2.9D, P = 8.691x10^{-96}), corroborating the findings of our protein analysis.

Upon further analysis of our scRNAseq data, we noted that cluster 0 and cluster 1 were the only clusters to separate primarily based upon the mouse genotype, representing TregP cells from PD-1$^{-/-}$ and WT respectively (Fig. 2.10B, Fig. 9C). By performing differential gene expression analysis, we identified Thy1 as one of the most differentially expressed genes (DEGs) between PD-1$^{-/-}$ and WT TregP cells (Fig. 2.10E). PD-1$^{-/-}$ cells were congenically marked with Thy1.1 and lack the WT Thy1.2 expression, reiterating the strong separation of PD-1$^{-/-}$ and WT cells between clusters 0 and 1 respectively. In addition, genes downstream of TCR/CD28 signaling (Lat and Lck) as well as heat shock genes (Hspa8, Hsp90aa1, Dnaja1) were significantly downregulated in PD-1$^{-/-}$ thymocytes. Interestingly, a recent study has demonstrated that signaling through LAT specifically regulates T cell development through its regulation of negative selection and promotion of Treg development (236). Genes with greater expression in PD-1$^{-/-}$ thymocytes included those associated with interferon signaling (Ifi27, Isg15) and histocompatibility genes (H2-Q7, H2-Q4, H2-K1, H2-D1). Importantly, H2-Q7 encodes the protein Qa2, which we identified was upregulated in PD-1 deficient thymocytes as these thymocytes were more mature (Fig. 2.5A, B). We also performed GSEA on the DEGs identified and found that interferon responses were activated while TNF signaling was suppressed in PD-1$^{-/-}$ cells (Fig. 2.9D).

PD-1$^{-/-}$ thymocytes have subtle increases in CD28 expression

PD-1 is also known to directly inhibit CD28 signaling, which is known to be critical for Treg development (127, 237). Therefore, we determined if there were any changes in CD28 expression in PD-1 deficient cells and found that there was a subtle increase in CD28 expression in FoxP3+ TregP and Treg in PD-1$^{-/-}$ animals (Fig. 2.11A, B). We also examined CD28 expression in PD-L1$^{-/-}$ animals and saw a subtle increase in CD4 SP, CD25+ TregP, FoxP3+ TregP, and Tregs (Fig. 2.12A, B).

PD-1 deficient T cells produce more IL-2

It has been reported that signaling through CD28 induces IL-2 production (238, 239). In light of the observation that WT thymocytes in the presence of PD-1 deficient thymocytes have an increased frequency of Tregs (Fig. 2.3G) and that IL-2 is a Treg niche limiting cytokine in the thymus (240-243), we speculated that perhaps PD-1 was regulating thymic IL-2 levels. We therefore examined IL-2 production in bulk thymocytes stimulated with PMA and ionomycin for 8 hours and observed about a two-fold increase in IL-2+ PD-1$^{-/-}$ thymocytes (Fig. 2.11C, D). Similar results were obtained when bulk thymocytes were stimulated with anti-CD3/CD28 (Fig. 2.12C). It has previously been reported that other CD4 SP thymocytes produce the majority of IL-2 in the thymus (244). To determine the dominant source of IL-2 in PD-1$^{-/-}$ thymocytes, we examined all IL-2+ cells for CD4 or CD8 expression. Surprisingly, we found that the increase in IL-2+ production was driven largely by CD8+ cells in PD-1$^{-/-}$ animals (Fig. 2.11E). This observation is intriguing given that CD8 SP thymocytes express about twice as much PD-1 compared to CD4 SP (Fig. 2.2D). To confirm that the PD-1 deficient cells were producing more IL-2, we harvested

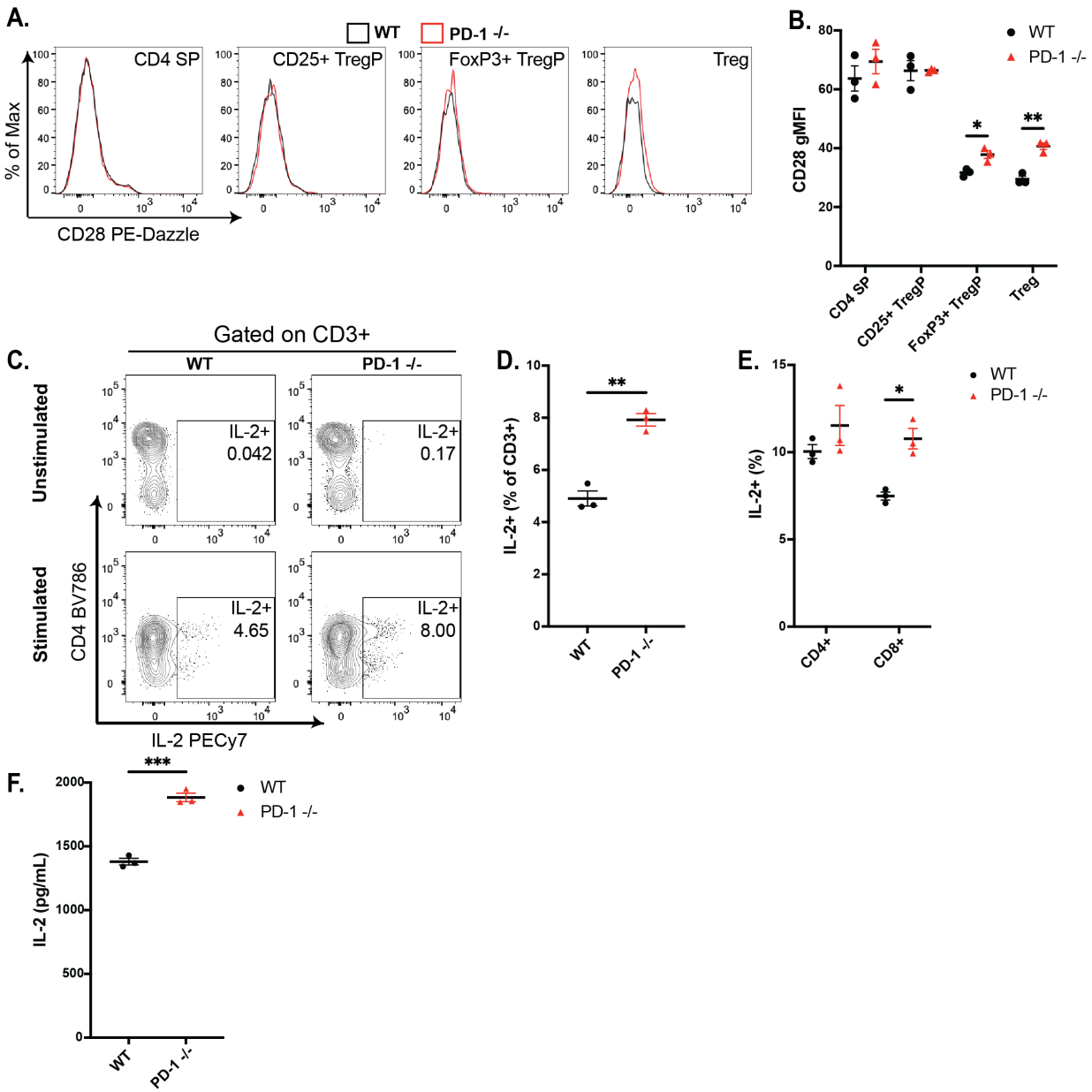

Figure 2.11 PD-1 deficient cells produce more IL-2 despite no change in response to CD28 signaling

Figure 2.11. PD-1 deficient cells produce more IL-2 despite no change in response to CD28 signaling. A) Thymi were harvested from 3-week-old WT or PD-1$^{-/-}$ mice (n = 3). Histograms depicting CD28 expression and **B)** quantification of CD28 gMFI. Data are representative of 2 independent experiments. **C)** Bulk thymocytes were harvested from 3-week-old WT or PD-1$^{-/-}$ mice (n = 3) and stimulated with PMA/I for 8 hours. Flow plots depicting gating of IL-2+ cells in unstimulated and stimulated cells from WT or PD-1$^{-/-}$ animals. **D)** Frequency of IL-2+ cells (% of CD3+). **E)** Frequency of CD4+ or CD8+ cells that are IL-2+. **F)** Quantification of the amount of IL-2 in the cell supernatants of the cultures by ELISA. Data are representative of 3 independent experiments. Unpaired Student T test or multiple unpaired T tests using the Holm-Šídák correction for multiple comparisons. * P<0.05, ** P<0.01, *** P<0.001

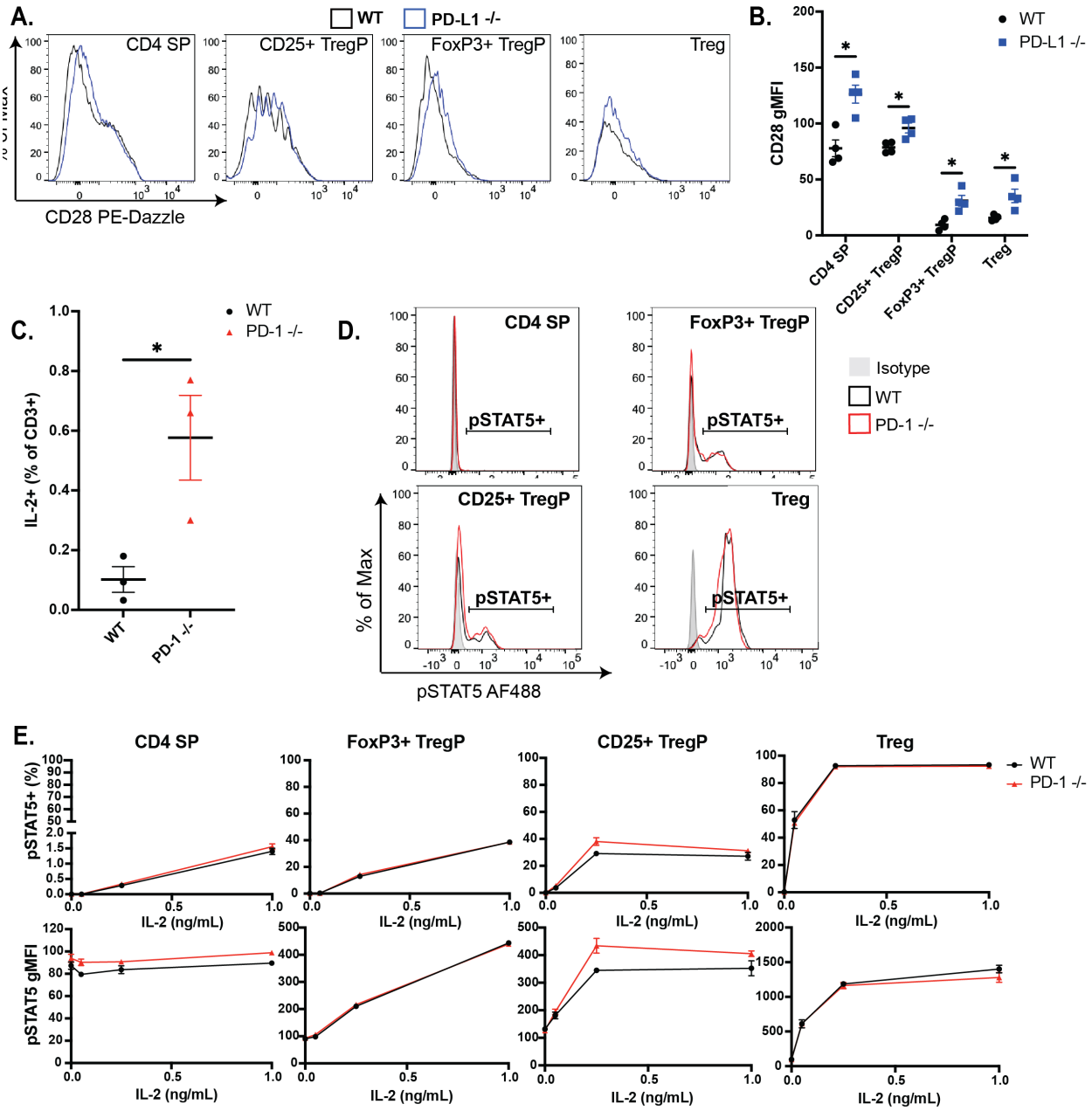

Figure 2.12 Markers downstream of CD28 and IL-2 signaling are unchanged in PD-1 deficient thymocytes

Figure 2.12: Markers downstream of CD28 and IL-2 signaling are unchanged in PD-1 deficient thymocytes. A) Thymi were harvested from 10-week-old WT or PD-L1$^{-/-}$ mice (n = 4). Histograms depicting CD28 expression and **B)** quantification of CD28 gMFI. Data are representative of 3 independent experiments. **C)** Bulk thymocytes were harvested from 3-week-old WT or PD-1$^{-/-}$ mice (n = 3) and stimulated with anti-CD3/CD28 for 8 hours. Frequency of IL-2+ cells (% of CD3+). Data are representative of 3 independent experiments. **D)** Bulk thymocytes from 3-week-old WT or PD-1$^{-/-}$ mice (n = 3) were stimulated with increasing concentrations of IL-2 (0 ng/mL, 0.05 ng/mL, 0.25 ng/mL, and 1 ng/mL). Histograms depicting pSTAT5 and the **E)** frequency of pSTAT5+ cells and pSTAT5 gMFI. Data representative of 3 independent experiments. Unpaired Student T test or multiple unpaired T tests using the Holm-Šídák correction for multiple comparisons. * P<0.05.

the cell supernatants and measured IL-2 concentration by ELISA and found that there was significantly more IL-2 present in the supernatants of PD-1$^{-/-}$ cell cultures (Fig. 2.11F).

The increase in IL-2 could drive an increase in Treg selection. Thus, we sought to determine if PD-1$^{-/-}$ thymocytes were more sensitive to IL-2 signaling. We measured STAT5 phosphorylation in WT and PD-1 deficient thymocytes stimulated with increasing concentrations of IL-2. We found no difference in the frequency of pSTAT5+ cells or pSTAT5 gMFI in WT compared to PD-1$^{-/-}$ thymocytes (Fig. 2.12D, E), suggesting that there is no difference in sensitivity to IL-2 in PD-1 deficient animals, rather, there is a global increase in the availability of this cytokine in the thymus.

Discussion

The emergence of PD-1 targeted therapies as standard of care treatment for many cancer patients has changed the clinical landscape of oncology. Over the past ten years, thousands of studies have sought to understand the mechanisms of anti-PD-1 response and resistance. However, very few studies have explored the importance of PD-1 during T cell development and differentiation. We propose that by studying a developmental role of PD-1 in the thymus, we might understand the biology of PD-1 blockade in the periphery. Herein, we demonstrate that PD-1 is expressed early on the cell surface of developing thymocytes at the double negative (DN) stage. These data agree with previous reports that describe PD-1 expression in DN thymocytes (193, 225). We further elucidate the expression of PD-L1 and PD-L2, finding that PD-L1 is expressed in cTEC and mTEC while PD-L2 expression is restricted to mTEC. Using immunohistochemistry, others have

reported PD-L1 expression distributed throughout the thymus while PD-L2 expression is restricted to the medulla in agreement with our findings (202, 203). In our study, we did not examine other thymic antigen presenting cells, such as dendritic cells, which have been reported to express PD-L1 and PD-L2 (203, 204). Interestingly, PD-L1 is known to be expressed by DN and CD8 SP thymocytes, while PD-L2 is not expressed on thymocytes at all (201). Our data expand these findings to demonstrate that some of the highest expressers of PD-L1 in the thymus are agonist selected thymocyte populations, including CD25+ TregP, FoxP3+ TregP, Treg, and NKT cells. This led us to ask how PD-1 signaling might impact thymocyte development, especially of agonist selected cell populations.

Several reports have shown that PD-1 regulates peripheral induction of Tregs through its interaction with PD-L1 via the Akt-mTOR pathway (213, 245, 246); however, the role of PD-1 in regulating Treg development in the thymus has not been well-studied. There is some evidence that PD-1 limits Treg development: a previous report by Ellestad and colleagues found PD-1$^{-/-}$ hematopoietic stem cells transplanted into Rag2 knockout mice were more likely to become Tregs compared to wildtype cells (211). Likewise, Chen and colleagues found an increase in the thymic development of Tregs using both PD-1$^{-/-}$ animals as well as mixed bone marrow chimeras (213). Interestingly, the authors of both studies determined that PD-1 is not necessary for promoting Treg selection in the thymus but did not follow up on the mechanism by which PD-1 restrains thymic Treg development. Interestingly, our scRNAseq data demonstrate that PD-1$^{-/-}$ and WT Tregs clustered together, indicating that these populations are similar. Additionally, we observed that WT

cells in the presence of PD-1 deficient thymocytes were more likely to become Tregs. These data highlight that the impact we observe in PD-1 deficient cells is not cell-intrinsic, but rather a change in the microenvironment. Here, we demonstrate that PD-1 limits the production of IL-2 within the thymus, thereby restraining the development of Tregs. Despite many indications that IL-2 is critical for Treg development (240-243), at least one recent study indicates that IL-2 is not the obligate cytokine leading to FoxP3 upregulation (247). This study concludes that FoxP3 upregulation requires disruption of agonist signaling by TGF-β under normal physiological conditions but in conditions with excessive *in vivo* IL-2, FoxP3 is upregulated in response to IL-2 signaling. In our model of PD-1 deficient animals, excessive IL-2 may be the driving factor for increased Treg development. One limitation of this study is that PD-1 is absent at all stages of T cell development. More nuanced studies limiting PD-1 deficiency to Treg progenitor populations could further elucidate the mechanism by which PD-1 is restraining Treg development.

Interestingly, we observed that the dominant increase in IL-2 production was from CD8+ thymocytes rather than CD4+ thymocytes. This contrasts with reports that have suggested that CD4+ thymocytes, specifically CD25+ TregP, are the main source of IL-2 in the thymus (243, 244, 248). Other studies have reported that PD-1 limits IL-2 in peripheral CD8+ T cells (249, 250); however, the exact mechanism remains unclear. One attractive hypothesis is that PD-1's inhibition of CD28 signaling leads to a decrease in IL-2 since CD28 signaling is known to facilitate IL-2 production (238, 239). In our study, we see a subtle increase in CD28 present on the cell surface of PD-1$^{-/-}$ Tregs and their

FoxP3+ progenitors, as well as in PD-L1$^{-/-}$ Tregs and both progenitor populations. This increase in CD28 expression could translate into the increase in IL-2 production observed. Future studies should seek to elucidate the mechanism by which PD-1 limits IL-2 production in T cells.

We observed a decrease in markers of TCR signal strength, specifically CD5 (233-235) and Nur77 (227), when PD-1 signaling was disrupted in thymocytes, which was corroborated by our sequencing data. These data suggest that the repertoire of Tregs may be different in PD-1$^{-/-}$ animals. One hypothesis that aligns with our findings is that less self-reactive clones are being selected into the Treg repertoire in the absence of PD-1. It would be interesting to explore this hypothesis using PD-1$^{-/-}$ animals on a fixed TCRβ chain background that allow for deep sequencing of the TCRα chains to examine the repertoire.

Most studies of the biology of PD-1 have focused on the role of this pathway in restraining peripheral effector T cell function. Blockade of this inhibitory protein is hypothesized to drive an increase in effector T cells (Teff), shifting the Teff:Treg ratio to enhance the immune response in tumor and infection models. However, the current findings suggest that blockade of this pathway might yield an increase in thymic Treg populations, which would be undesirable in the context of tumors or infections. By using therapies that systemically target PD-1, we may unknowingly alter the frequency, repertoire, and function of Tregs selected for in the thymus. These alterations could impact response to therapy or the development of adverse events in patients.

Chapter 3 : Summary and Future Directions

Summary

Chapter 2 describes a role for PD-1 in restraining Treg development in the thymus. We demonstrate that PD-1 and its ligands, PD-L1 and PD-L2, are expressed during thymocyte development. Using PD-1 deficient animals, we find that PD-1 signaling specifically limits the development of Treg, but not other agonist selected cells. Although we initially hypothesized that PD-1's inhibition of TCR signaling reduced Treg development, we found that there was a decrease in metrics associated with TCR signal strength in PD-1$^{-/-}$ thymocytes. Instead, we find that PD-1$^{-/-}$ thymocytes produce more IL-2, which could drive the increase in Treg selection observed in PD-1 deficient animals. Together, these data suggest that blockade of PD-1 might increase the production of thymically-derived Tregs which could contribute to an immunosuppressive TME.

Open questions and future directions

Our data are consistent with the selection of less self-reactive clones into the Treg repertoire; however, we were unable to examine the actual TCR repertoire in our studies due to low cell counts that resulted in under sampling of the TCR repertoire. I hypothesize that there should be increased overlap in the TCR repertoire of conventional CD4+ T cells and Tregs in PD-1 deficient animals. To explore this hypothesis, I could breed PD-1$^{-/-}$ mice with mice that have a fixed TCRβ chain. Fixing the TCRβ chain would allow me to sequence the TCRα chains to determine TCR diversity and clonality. I would take thymi from three-week-old WT TCRβ-fixed mice and PD-1$^{-/-}$ TCRβ-fixed mice and sort

conventional CD4+ T cells and Tregs, which would be submitted for TCRα chain sequencing. Since we have evidence that suggests that less self-reactive clones may be selected into the Treg lineage in the absence of PD-1, I would expect that there would be more shared clones between the conventional CD4+ T cells and Tregs in PD-1$^{-/-}$mice, which could also increase the TCR diversity of the Tregs in PD-1$^{-/-}$ mice. A greater breadth of Treg specificity could include tumor-associated antigens, which might result in preferential recruitment of Tregs into the tumor where they may exert their immunosuppressive functions.

We have observed an increase in IL-2 production by thymocytes of PD-1 deficient mice; however, whether this increase in IL-2 is causing the increase in thymically-derived Tregs remains unclear. I hypothesize that the increase in IL-2 would result in the corresponding increase in Tregs observed in PD-1$^{-/-}$ animals. To explore this hypothesis, I could inject IL-2 blocking antibody or a control antibody intrathymically in WT or PD-1$^{-/-}$ animals. Then, thymi are harvested and stained for flow cytometry to assess Treg induction. I would expect that blocking IL-2 would reduce Treg development in both WT and PD-1$^{-/-}$ mice since IL-2 is known to be critical for Treg development (240-243); however, blockade in PD-1$^{-/-}$ mice would return Treg development back to WT baseline. These data would establish a causal link between IL-2 production and Treg development in my model system.

A recent publication has suggested that TGFβ is necessary for FoxP3 induction and that IL-2 only drives FoxP3 expression when it is present at supraphysiologic levels(247). In

my model system, I hypothesize that IL-2 is present at supraphysiologic levels and, therefore, drives Treg induction independent of TGFβ. To assess this hypothesis, I could cross PD-1$^{-/-}$ mice to TGFβR1^{cKO} mice where TGFβR1 knockout is driven by the E8$_{III}$-Cre to knockout TGFβR1 in all pre-selection DP thymocytes. Then I could examine thymi of three-week-old mice by flow cytometry to determine the impact on all stages of Treg development. If Treg development is still higher in PD-1$^{-/-}$; TGFβR1^{cKO} mice compared to PD-1^{WT}; TGFβR1^{cKO} mice, then this is evidence that Treg development in this model is not primarily driven by TGFβ and may be driven by IL-2 instead. To further assess whether there are supraphysiologic levels of IL-2 in PD-1 deficient thymi, I could perform qPCR for IL-2 mRNA in thymocytes and I could perform an ELISA for IL-2 presence per gram of whole thymus. If there are elevated levels of IL-2 in PD-1$^{-/-}$ thymi, as I expect, this is additional evidence that IL-2 may be driving Treg generation in this model.

Whether PD-1 signaling alters the function of Tregs is still debated. Some studies have found that PD-1$^{-/-}$ Tregs are less proficient suppressors (245, 251), while other studies have found that PD-1 is not involved in regulating the suppressive capacity of Tregs in both *in vitro* and *in vivo* model systems (211, 213); however, at least one study did demonstrate that PD-1 deficient Tregs were more suppressive via PD-1's modification of the PI3K-Akt pathway (252). These studies were all performed on peripheral Tregs or induced Tregs, not thymically derived Tregs, so whether the Tregs produced in the thymus of PD-1 deficient animals are functionally distinct remains unclear. I hypothesize that thymic Tregs from PD-1$^{-/-}$ mice would be better suppressors compared to their WT counterparts. To determine if this is the case, I could sort thymic Tregs from either WT or

PD-1$^{-/-}$ mice and perform an *in vitro* suppression assay. To assess *in vivo* capacity for suppression, I could perform transfer studies of thymic Tregs sorted from congenically marked PD-1$^{-/-}$ or WT mice which are injected into host mice one day before experimental autoimmune encephalomyelitis (EAE) disease induction. Then mice are monitored daily starting at 8 days post induction for clinical disease score. At endpoint, the brain and spinal cord could be also assessed for infiltration of lymphocytes. Because EAE is mediated by CD4+ Th1 and Th17 cells, this model assesses the capacity of Tregs to control these populations specifically. I would expect that PD-1 deficient Tregs would be more suppressive, which would be indicated by less CD4+ T cell proliferation in the *in vitro* suppression assay and by a lower disease score with less lymphocyte infiltration into the central nervous system in the *in vivo* suppression assay. This would indicate that PD-1 deficient Tregs are more suppressive and could therefore contribute to an immunosuppressive TME in the context of a tumor.

Our lab has demonstrated that RTE generated because of ADT-induced thymic regeneration travel to prostate tumors where they get activated and produce effector cytokines, thereby highlighting their ability to contribute to the tumor immune microenvironment (Appendix A). Whether PD-1 blockade in the context of ADT-induced thymic regeneration would lead to greater Treg generation in the thymus remains unanswered. I hypothesize that PD-1 blockade in the context of ADT-induced thymic regeneration would result in more Tregs in the thymus and in the RTE exported from the thymus. To assess this hypothesis, I could take TCRδ-CRE^{ERT2};Rosa26-tdTomato mice and perform either a sham surgery or bilateral orchiectomy to induce thymic regeneration.

Then I would treat the animals with anti-PD-1 or a control antibody for three treatments, each three days apart, starting on day 3 post-orchiectomy. One day after the last treatment, I would harvest the thymi and the spleen to stain for flow cytometric analysis of Treg generation. If PD-1 blockade does induce greater Treg generation during thymic regeneration, I would expect there to be higher numbers of Tregs in the thymus of animals that received anti-PD-1 in addition to orchiectomy and I would expect there to be more Tregs exported into the periphery as tdTomato+ cells. This would indicate that PD-1 blockade does induce greater Treg production in the thymus while the thymus is regenerating in response to ADT. The next step would be to test this in a tumor model to see if more tdTomato+ Tregs end up trafficking to the tumor and to sort tumor infiltrating Tregs for use in *in vitro* suppression assays to determine how they might contribute to an immunosuppressive TME. These data would help us understand whether prostate cancer patients undergoing ADT-induced thymic regeneration might produce more Tregs that contribute to an immunosuppressive TME as a mechanism of therapeutic resistance.

Broader Implications

In prostate cancer patients who are receiving ADT, thymic regeneration will lead to increased thymic output and some of these newly generated T cells will travel to the tumor where they may participate in the anti-tumor immune response (Appendix A). If anti-PD-1 is applied in this context, the findings of this dissertation work suggest that there will be an increased generation of thymically-derived Tregs that might travel to the tumor to create an immunosuppressive microenvironment. This may be one of the reasons that this therapy has largely failed for most prostate cancer patients. The field should focus on

determining if anti-PD-1 therapy in prostate cancer patients receiving ADT does increase Tregs in the tumor microenvironment. If so, the functionality of these Tregs must be assessed. If less self-reactive clones are being selected into the Treg lineage, these Tregs might not be receiving the necessary baseline level of stimulation needed for their optimal survival and function. If this is the case, Tregs resulting from combination ADT and PD-1 blockade may be numerous, but less effective suppressors. Although it may be attractive to apply Treg-targeted therapies, immune related adverse events may limit the tolerability of such combinations. The field should focus on determining the best dosing schedule for patients to restrain the effect of immunosuppressive Tregs while limiting unwanted side-effects: perhaps Treg-targeted therapies would be best used early when combined with anti-PD-1 to reduce the burst of newly generated Tregs but may not be necessary for the whole course of anti-PD-1 treatment. This could address the issue of increased thymically-derived Tregs resulting from anti-PD-1 therapy while hopefully reducing immune related adverse events in these patients.

The findings of this dissertation can be applied outside of the context of prostate cancer and beyond T cell development in the thymus. The data herein imply that PD-1 regulates IL-2 production especially in CD8+ T cells, which has been previously reported in the periphery (249, 250); however, how PD-1 limits CD8+ IL-2 production remains unknown. It is attractive to speculate that blockade of PD-1 signaling leads to an increase in CD28 costimulation, which drives more IL-2 production, but this mechanism needs to be elucidated. Indeed, one potential mechanism for anti-PD-1's efficacy in many patients may be through an increase in IL-2 in the tumor microenvironment. Notably, IL-2 was one

of the first immunotherapies developed that demonstrated anti-tumor effects, some these responses being very durable (253-256). Based upon the durability of responses in single and multi-institutional studies in metastatic renal cancer, the U.S. Food and Drug Administration approved high-dose IL-2 in 1992, becoming the first immunotherapy approved for the treatment of cancer patients. However, IL-2 is also important for Treg cell development and homeostasis in the periphery (241). As stated, this dissertation has highlighted the importance of PD-1 in limiting the development of thymically-derived Tregs. Therefore, anti-PD-1 could enhance Treg production, leading to an increase in Tregs available to traffic to the tumor. If PD-1 were then to promote further IL-2 release in Treg-enriched tumors, the immunosuppressive tumor microenvironment could become dominant and may be a mechanism of resistance in some patients. If this is the case, it is rationale for combination therapies that reduce Treg function in the TME, such as blockade of CTLA-4, in combination with therapies that increase T cell function in the TME, such as anti-PD-1 therapy. This combination is already being applied in many clinical trials, but the work herein provides a potential mechanism of action to explore through IL-2 signaling. There may be ways to further exploit this mechanism for maximum anti-tumor efficacy while minimizing undesirable effects of the immunosuppressive cells such as Tregs in the tumor microenvironment and reducing immune related adverse events with combination therapies.

Chapter 4 Appendix A: Restored thymic output after androgen blockade participates in antitumor immunity

Adapted from published manuscript:

Polesso, F., *Caruso B., Hammond S. A., & Moran A.E.* Restored thymic output after androgen blockade participates in antitumor immunity. *Journal of Immunology* (2022). https://doi.org/10.4049/jimmunol.2200696

Contributions

A.E.M. conceived the study and designed all the experiments with F.P. F.P. performed all the experiments and analyzed the data. F.P., B.C., S.A.H., and A.E.M. interpreted the data and wrote the manuscript.

Abstract

The thymus is a hormone sensitive organ, which involutes with age in response to production of sex steroids. Thymic involution leads to a decrease in the generation of recent thymic emigrants (RTE), resulting in a reduced response to immune challenges such as cancer. Interestingly, the standard of care for prostate cancer patients is androgen deprivation therapy, which leads to thymic regeneration and an increase in thymic output. It remains unknown whether these newly produced T cells can contribute to the antitumor immune response. The present study defines the kinetics of thymic regeneration in response to ADT in mice, determining that thymic epithelial cell (TEC) proliferation is critical for the increase in RTE output. Using a novel mouse model to track RTE *in vivo*, we demonstrate that these newly generated RTE can traffic to tumors where

they become activated and produce effector cytokines at levels like more mature T cells. Collectively, these data suggest that RTE produced from ADT-induced thymic regeneration could be harnessed for the antitumor immune response.

Introduction

The thymus is critical in establishing and maintaining a diverse peripheral T cell pool, which is essential for mounting an effective immune response against a variety of pathogens. However, the thymus degenerates with age in part due to an increase in circulating sex steroids that begins in puberty (31). Thymic decline results in a decrease in thymic cellularity and a reduction in the export of naïve CD4+ and CD8+ T cells, called recent thymic emigrants (RTE) (26). Decreased production of RTE causes expansion of existing memory T cells (38, 39, 257), and decreases the diversity of the TCR repertoire (37, 39), which is thought to contribute to age-related reductions in the response to vaccines, infections, and cancer (258). Further, this decline in TCR repertoire diversity may make it more difficult for patients to respond to immunotherapies, such as checkpoint blockade (259).

The effect of sex steroids on the thymus can be reversed through androgen deprivation therapy (ADT), which can be achieved with bilateral orchiectomy or gonadotropin-releasing hormone (GnRH) agonists or antagonists (31, 60, 61). Interestingly, the standard of care for prostate cancer patients is ADT, which results in thymic regeneration and an increase in thymic output (60, 61). Though increased thymic output following ADT might be predicted to improve immunity, some evidence suggests that RTE require further

maturation in the peripheral secondary lymphoid organs to be fully functional (69, 72). Therefore, it is unclear whether RTE produced in the context of ADT can contribute to antitumor responses.

In the present study, we define the kinetics of thymic regeneration after ADT in male mice and highlight the importance of thymic epithelial cell proliferation as a driver of thymic regeneration. Using a novel mouse model to track RTE *in vivo*, we demonstrate that RTE produced because of ADT can traffic to tumors. Importantly, the RTE found in tumors were functional, expressed activation markers and produced similar levels of cytokines as non-RTE, suggesting that further maturation is not required in this context. Together, this evidence suggests that RTE produced because of ADT can be harnessed for antitumor responses.

Materials and Methods

Animals and tumor models

C57BL/6 (stock #000664), RIP-mOVA (stock #005431), OTI (stock #003831), C57Bl/6;CD90.1 (also known as Thy1.1, stock #000406), C57BL/6;CD45.1 (also known as B6.SJL, stock #002014), Rag2-GFP (stock #005688), TCRδ-CRE^{ERT2} (stock #031679) and Rosa26-tdTomato (stock #007914) were purchased from the Jackson Laboratory. All animals were maintained under specific pathogen-free conditions in the Oregon Health & Science University (Portland, OR) animal facility. Sexually mature 12-week-old males were used in all the experiments described. Murine *Pten*$^{-/-}$*;p53*$^{-/-}$*;Smad4*$^{-/-}$ (PPSM) castration resistant prostate tumor model (gift of Ronald DePinho) and TrampC1-OVA (gift of Michael Gough) were propagated *in vitro* using complete

media, RPMI 1640 (Lonza) containing 0.292 ng/ml glutamine, 100 U/ml streptomycin/penicillin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10mM HEPES (Sigma-Aldrich). All cell lines were tested and confirmed to be Mycoplasma and endotoxin-free using the MycoAlert Detection kit (Lonza) and the Endosafe-PTS system (Charles River Laboratories). All culture media reagents were purchased from Hyclone Laboratories unless noted otherwise. All animal experiments were approved by the Institutional Animal Care and Use Committee of OHSU.

Orchiectomy surgeries and androgen deprivation therapy treatments

Orchiectomy surgeries were performed as previously described (260). Briefly, male mice were anesthetized with isoflurane, and a small midline ventral skin and muscle incision made. Testes were pulled out through the incision, and the spermatic cords were sectioned and cauterized, and testes removed along with surrounding fatty tissue. The muscle layer and skin were closed with absorbable suture. For antigen deprivation therapy, mice were treated with 0.5 μg of degarelix (Medchem Express) by S.C. injection once every 14 days. Enzalutamide (Medchem Express) was administered daily for 2 weeks in the feed at 50 mg/kg in Purina chow 5053, Research Diet Inc. (~ 0.25 mg/mouse/day). Abiraterone (Medchem Express) was administered by oral gavage at 0.5 mmol/kg/d in vehicle (5% benzyl alcohol, 95% safflower oil), daily for 2 weeks.

Tumor challenge and adoptive transfers

$1x10^6$ PPSM tumor cells were injected on the hind flanks of C57BL/6, B6;SJL, and $1x10^6$ TrampC1-OVA were injected on the hind flanks of RipmOVA male mice (3-5 per group). On day 10 for PPSM or day 17 for TrampC1-OVA tumor bearing animals, $1x10^5$ flow

cytometry sorted Rag2-GFP+ (RTE), Rag2-GFP– (non-RTE), OTI;Thy1.1;Rag2-GFP+ (OTI RTE) or OTI;Thy1.1;Rag2-GFP– (OTI non-RTE), were adoptively transferred by i.v. injection in the tumor bearing animals. Tumor draining lymph nodes (dLN) and tumors were harvested 5 days post adoptive transfer. For RTE time-stamping experiments, $1x10^6$ PPSM tumor cells were injected in the hind flanks of TCRg-CRE^{ERT2}-R26-tdTomato animals. 7 days post tumor implantation, half of the animals were orchiectomized. 2 days post orchiectomy, all animals were treated with 2mg Tamoxifen by oral gavage daily for 14 days. Tumor dLN (inguinal) and tumors were harvested 21 days post orchiectomy.

TEC, thymocytes, and lymphocyte isolation

TEC were isolated and enriched as previously described (228) . For thymocyte isolation, thymi were harvested and processed to obtain single-cell suspensions using frosted ends of microscope slides. Cells were rinsed with PBS containing 1% FBS and 4 mM EDTA. Tumor infiltrating lymphocytes (TIL) were harvested by dissection of tumor tissue into small fragments in a 50-cc conical tube followed by digestion at room temperature in a bacterial shaker at 180 rpm for 30 min in 1 mg/ml collagenase type IV (Worthington Biochemicals), 100 μg/ml hyaluronidase (Sigma-Aldrich) and 20 mg/ml DNAse (Roche) in PBS. Cells were then further disrupted with a 1-cc syringe plunger through a 70-μm nylon cell strainer (BD Biosciences) and filtered to obtain a single cell suspension. Tumor dLN (inguinal) were harvested and processed to obtain single-cell suspensions using frosted ends of microscope slides. Cells were rinsed with PBS containing 1% FBS and 4 mM EDTA.

Flow cytometry and cell sorting

For flow cytometry analysis, cells were incubated for 20 min on ice with e506 fixable viability dye and the following antibodies: TCRβ (H57-597), CD4 (RM4-5), CD8 (53-6.7), TCRγδ (eBioGL3), NK1.1 (PK136), CD44 (IM7), CD69 (H1.2F3), Epcam (G8.8), CD45 (30-F11), PD1 (J43), CD45.2 (104), CD45.1 (A20) and Thy1.1 (HIS51). Intracellular proteins FoxP3 (FJK-16s), Ki67 (SolA15), IFNγ (XMG1.2) and TNFα (MP6-CT22) were detected using the FoxP3 Transcription Factor Concentrate and Diluent from eBioscience. All antibodies and viability dyes were purchased from eBioscience, Biolegend, or BD Biosciences. Data were collected with a Fortessa flow cytometer (BD Biosciences) and analyzed using Flowjo software (Tree Star). Unless noted otherwise in the figure legend, cells were gated through live/TCRβ+ gates for analysis. T cell sorts were performed on an Aria (BD Biosciences) with an 85-micron nozzle.

In vitro activation and intracellular cytokine staining

Splenocytes and bulk TIL were plated at $1x10^6$ cells/well in 96-well plates and stimulated for 5h with PMA (80 nM) and ionomycin (1.3 μM) or 1nM SIINFEKL peptide for OTI re-stimulation, in the presence of brefeldin A (BFA). Cells were then stained for surface markers, fixed and permeabilized using either the BD Cytofix/CytoPerm or eBioscience Foxp3 kit, and stained for intracellular cytokines.

Statistical analysis

Statistical analysis was performed using unpaired two-tailed Student *t* test (for comparison between two groups) or One-way ANOVA with Tukey multiple comparison

(for comparison between multiple groups) using GraphPad Prism 8 (GraphPad Software). Error bars represent SEM. Statistical tests and *p* values are specified for each panel in the respective figure legends, and *p* values < 0.05 were considered significant. Biological replicates (individual animals) for each experiment are indicated in the figure legends.

Study approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of Earle A. Chiles Research Institute at Portland Providence Cancer Center or the Institutional Animal Care and Use Committee of OHSU.

Results

ADT promotes thymic regeneration through proliferation of TEC but not thymocytes

It is well established that ADT leads to regeneration of the thymus in mice and humans (31, 60-62), but the kinetics of this regeneration process have not been explored in detail. Sexually mature (12-week-old) male mice were orchiectomized, and thymi harvested 2-, 5- and 12-weeks post orchiectomy. Thymic weight and cellularity increased over time after orchiectomy (Fig. 4.1A, B), peaking at 5 weeks. As a comparison, thymic weight, and cellularity in intact male mice at ages 2, 5 and 12-weeks remained unchanged (Fig. 4.2A, B). A known mechanism of age-related thymic atrophy results from a reduction of the thymic epithelial space and reduced numbers of TEC (261). We indeed observed a gradual reduction in Epcam+CD45- TEC numbers in the thymi of intact male mice at 5 and 12 weeks of age compared to 2-week-old neonates (Fig. 4.2C, D), reflecting

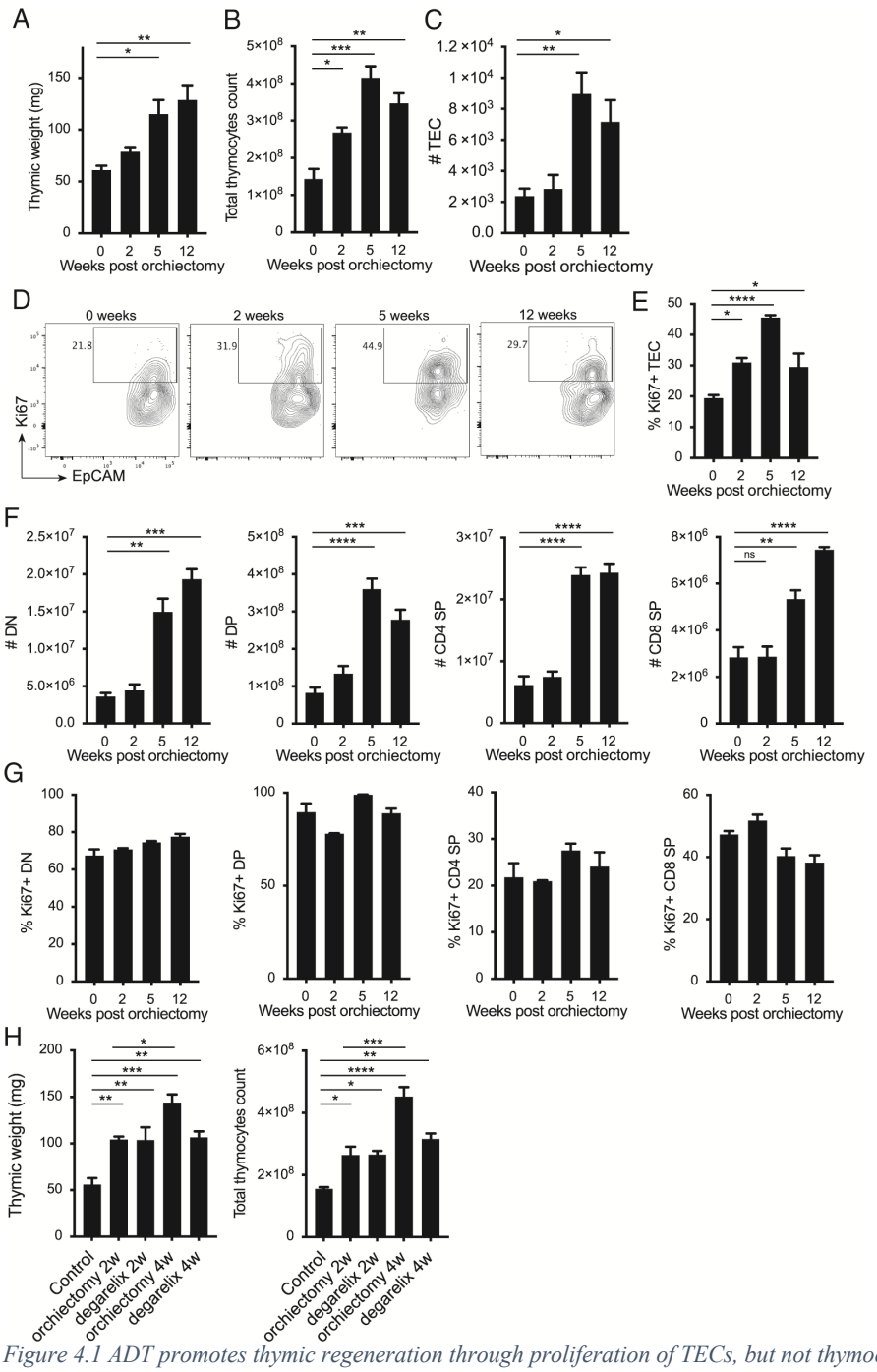

Figure 4.1 ADT promotes thymic regeneration through proliferation of TECs, but not thymocytes

Figure 4.1: ADT promotes thymic regeneration through proliferation of TECs, but not thymocytes. Twelve-week-old male mice were orchiectomized, and thymi were harvested 2, 5, and 12 wk later. (**A**–**C**) Thymic weight (A), total thymocyte count (B), and number of TECs (C) at time of harvest. (**D** and **E**) Representative flow plots gated on TECs ($CD45^{-}Epcam^{+}$) and showing EpCAM and Ki67 expression (D), and quantification of percent $Ki67^{+}$ TECs at 0, 2, 5, and 12 wk post orchiectomy (E). (**F** and **G**) Numbers (F) and percent $Ki67^{+}$ (G) of DN, DP, CD4 SP, and CD8 SP in the thymus at the indicated times. (**H**) Twelve-week-old male mice were orchiectomized or chemically castrated using degarelix, and thymi were harvested 2 or 4 wk later. Graphs show thymic weight and total thymocyte counts after the indicated treatments. Three animals per group. Data are representative of three experiments. One-way ANOVA with Tukey multiple comparison, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

thymic involution. Following orchiectomy, we observed a sharp increase in TEC numbers in the thymus at 5 weeks post-surgery (Fig. 4.1C), with increased numbers maintained up to 12 weeks. To establish whether the increase in TEC numbers was due to proliferation, we assessed Ki67 expression in TEC over time after orchiectomy. The frequency of proliferating TEC increased after orchiectomy, peaking at 5 weeks post-surgery (Fig. 4.1D, E) to reach proliferation levels like 2-week-old neonates (Fig. 4.2E).

Given the large increase in thymic cellularity post orchiectomy (Fig. 4.1B), we sought to identify which thymocyte subsets accounted for this increase during thymic regeneration and to elucidate the kinetics of this process. We observed a gradual increase in the number of double negative (DN) and double positive (DP) thymocytes over time after orchiectomy, as well as a gradual increase in CD4 and CD8 single positive (SP) thymocytes, with a 3 to 4-fold increase at 12 weeks post orchiectomy compared to pre-surgery (Fig. 4.1F). SP, DP, CD4 SP and CD8 SP thymocytes did not display increased expression of Ki67 after orchiectomy, contrary to observations made in TEC (Fig. 4.1G). This suggests that the increase in cell number was due to enhanced positive selection rather than proliferation of existing thymocytes in the thymus. We also observed a gradual increase in the number of $\gamma\delta$ T cells, NKT cells and regulatory T cells (Tregs) over time after orchiectomy (Fig. 4.2G-I), without an increase in proliferation as measured by Ki67 expression (Fig. 4.2J-L). These data establish that although thymic regeneration is measurable as early as two weeks post orchiectomy, this process continues over time

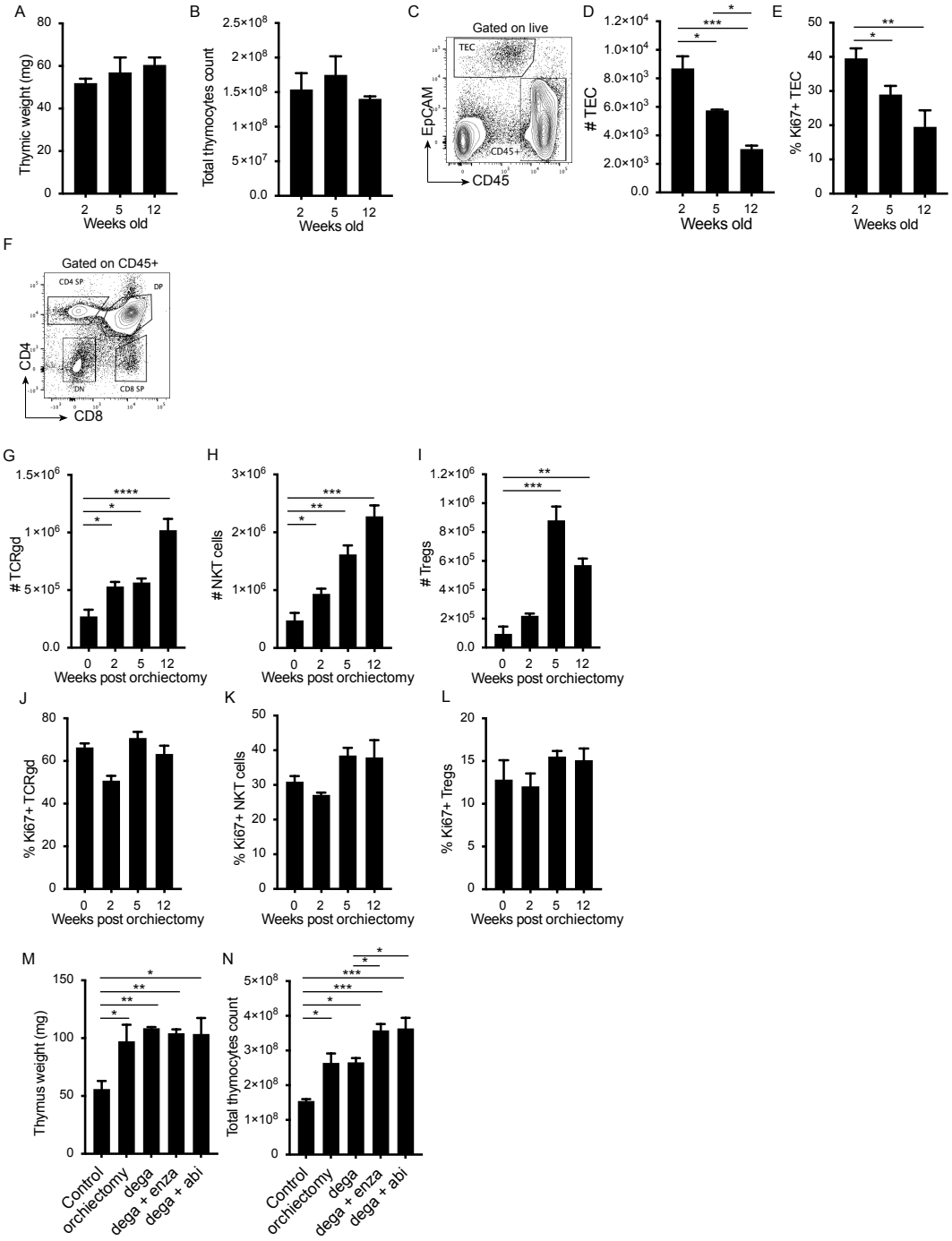

Figure 4.2 Thymic regeneration after orchiectomy or ADT

Figure 4.2: Thymic regeneration after orchiectomy or ADT. **A)** Thymic weight and **B)** total thymocyte count in intact male mice at 2, 5 and 12 weeks of age. **C)** Gating strategy to identify TEC. **D-E)** Number of thymic epithelial cells (TEC) (D) and percent Ki67+ TEC (E) in the thymi of male mice at 2, 5 and 12 weeks of age. **F)** Gating strategy to identify DP, SP, CD4 and CD8 SP in the thymus. Gated on live, CD45+. **G-I)** Numbers and **J-L)** percent Ki67+ of TCD$\gamma\delta$, NKT and Tregs in the thymus after orchiectomy. **M-N)** 12-week-old male mice were orchiectomized or chemically castrated using degarelix and treated with enzalutamide or abiraterone. Graphs show thymic weight (M) and total thymocyte counts (N) after the different treatments. 3 animals per group. Data representative of 2 to 3 repeat experiments. One-way ANOVA with Tukey multiple comparison, * P<0.05, ** P<0.01, *** P<0.001.

and increased thymic weight and cellularity is maintained up to 12 weeks post-surgery.

ADT is the mainstay of treatment for advanced prostate cancer patients, and thymic regeneration associated with such treatment has been reported (31, 60-62). Degarelix, a GnRH antagonist, is used clinically to reduce circulating testosterone levels. We compared the thymic regeneration achieved with orchiectomy to degarelix treatment in 12-week-old male mice. At 2 weeks post orchiectomy or degarelix treatment, the increase in thymic weight and total thymocyte number was similar between the two treatments compared to untreated control animals (Fig. 4.1H). Thymic weight and cellularity continued to increase in orchiectomized animals between 2 and 4 weeks whereas degarelix treated animals achieved maximal regeneration after 2 weeks (Fig. 4.1H). Second generation androgen receptor (AR) inhibitors such as enzalutamide or abiraterone are often added to GnRH inhibition to reduce AR signaling and adrenal androgens. The addition of either of these small molecules to degarelix did not significantly increase thymus weight over degarelix alone (Fig. 4.2M). We did observe a further increase in total thymocyte numbers 2 weeks post treatment (Fig. 4.2N).

RTE traffic to the tumor and display an activated phenotype

Given our observations and previously reported detection of RTEs in the blood of patients treated with ADT (26), we asked whether these new T cells could traffic into the tumor. To answer this question, we utilized Rag2-GFP mice, in which GFP is expressed under the control of the *Rag2* promoter, fluorescently tagging RTE in the periphery for ~5 days after exiting the thymus (72). We sorted TCRβ+Rag2GFP+ T cells from the spleens of

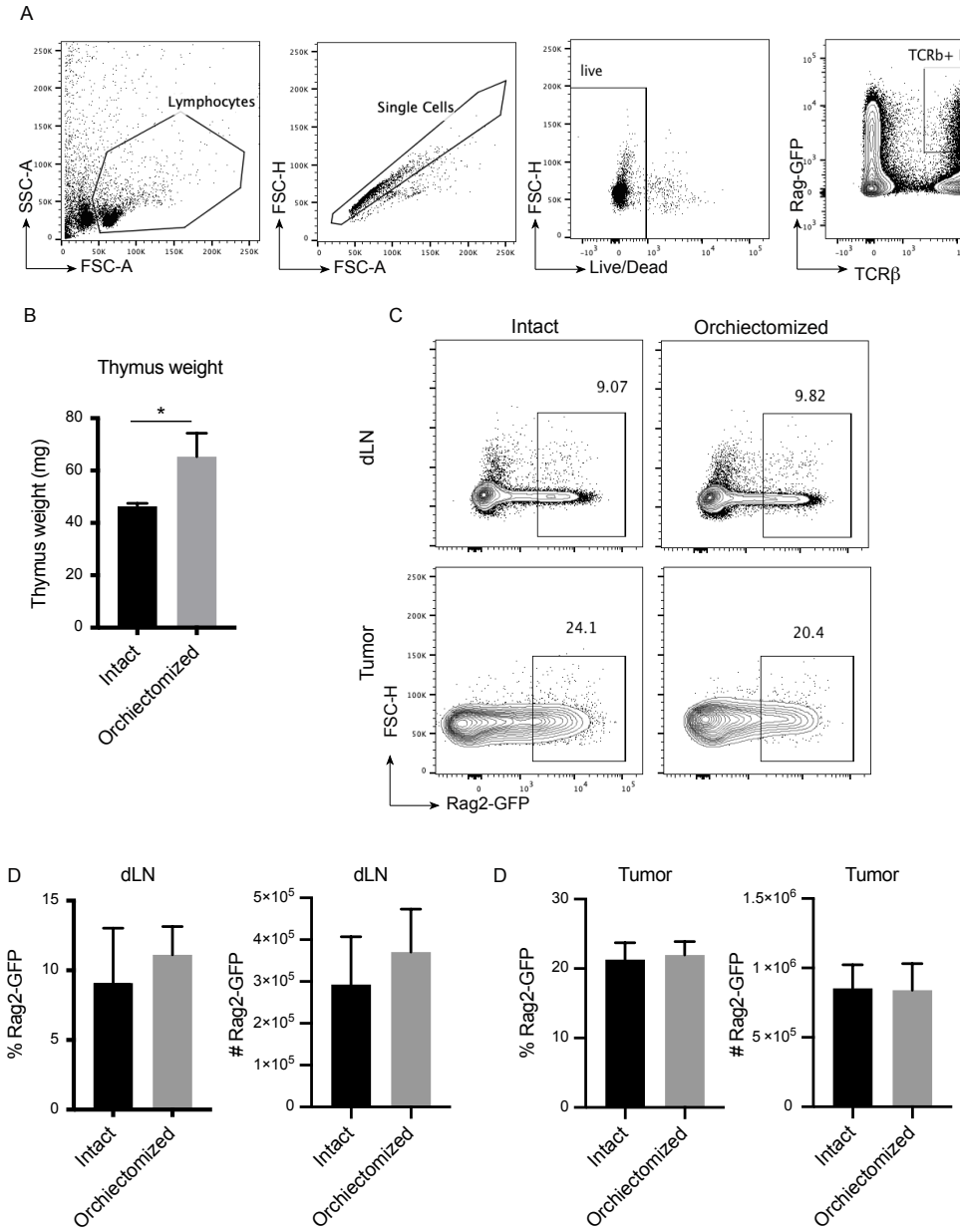

Figure 4.3 No difference in Rag2-GFP+ cells in orchiectomized mice

Figure 4.3: No difference in Rag2-GFP+ cells in orchiectomized mice. A) Gating strategy to sort Rag2-GFP+ RTE from the spleen of Rag2-GFP mice. **B-D)** Rag2-GFP male mice were implanted with PPSM tumors. 7 days post tumor implantation, animals were orchiectomized or left intact. B) Thymic weight 2 weeks post orchiectomy. C) Representative flow plots (gated on live, TCRβ+) showing Rag2-GFP expression in the dLN and tumor of intact and orchiectomized animals 2 weeks post-surgery. D) Frequency and number of Rag2-GFP TCRβ+ cells in the dLN and tumor (numbers are per gram of tumor). 3 animals per group. Unpaired two-tailed Student t test,* P<0.05.

Rag2-GFP male mice as a source of RTE (Fig. 4.3A), and adoptively transferred these RTE into congenic (CD45.1+) male recipient mice harboring a transplantable mouse prostate tumor (*Pten*$^{-/-}$;*p53*$^{-/-}$;*Smad4*$^{-/-}$, referred to as PPSM) (260, 262). Sorted Rag2GFP+ cells (RTE) were adoptively transferred 10 days post tumor implantation (Fig. 4.4A). dLN and tumors were harvested 5 days post adoptive transfer, and the presence of adoptively transferred RTE was assessed. CD45.2+ adoptively transferred RTE were recovered in both the dLN and tumors (Fig. 4.3C-E, Fig. 4.4B), highlighting that RTE can traffic to the tumor site within 5 days despite recent emigration from the thymus. Notably, the CD45.2+ T cells had lost Rag2-GFP expression in both the dLN (Fig. 4.4C) and the tumor (Fig. 4.4D) at time of harvest, reflecting time since undergoing positive selection. In the dLN, these cells expressed low levels of activation markers PD-1, CD44 and CD69, like host T cells (Fig. 4.4C). In the tumor, the RTE expressed PD-1, CD44 and CD69, markers consistent with activation. However, RTE expressed lower levels of PD-1 when compared to host T cells in the tumor (Fig. 4.4D, E). Together, these data demonstrate that RTE traffic to the tumor and exhibit an activated phenotype.

Tumor-specific RTE contribute to the antitumor immune response

Previous work suggests that RTE require further maturation in the peripheral secondary lymphoid organs (69) to be fully functional. Our findings that RTE can traffic to the tumor and appear activated is therefore novel and surprising. To further confirm that RTE can participate in the antitumor immune response, we tested whether tumor-specific CD8 RTE function as well as tumor-specific mature CD8 T cells sorted from the periphery. We generated OTI;Rag2-GFP;Thy1.1 animals, and sorted OTI RTE (Rag2- GFP+)

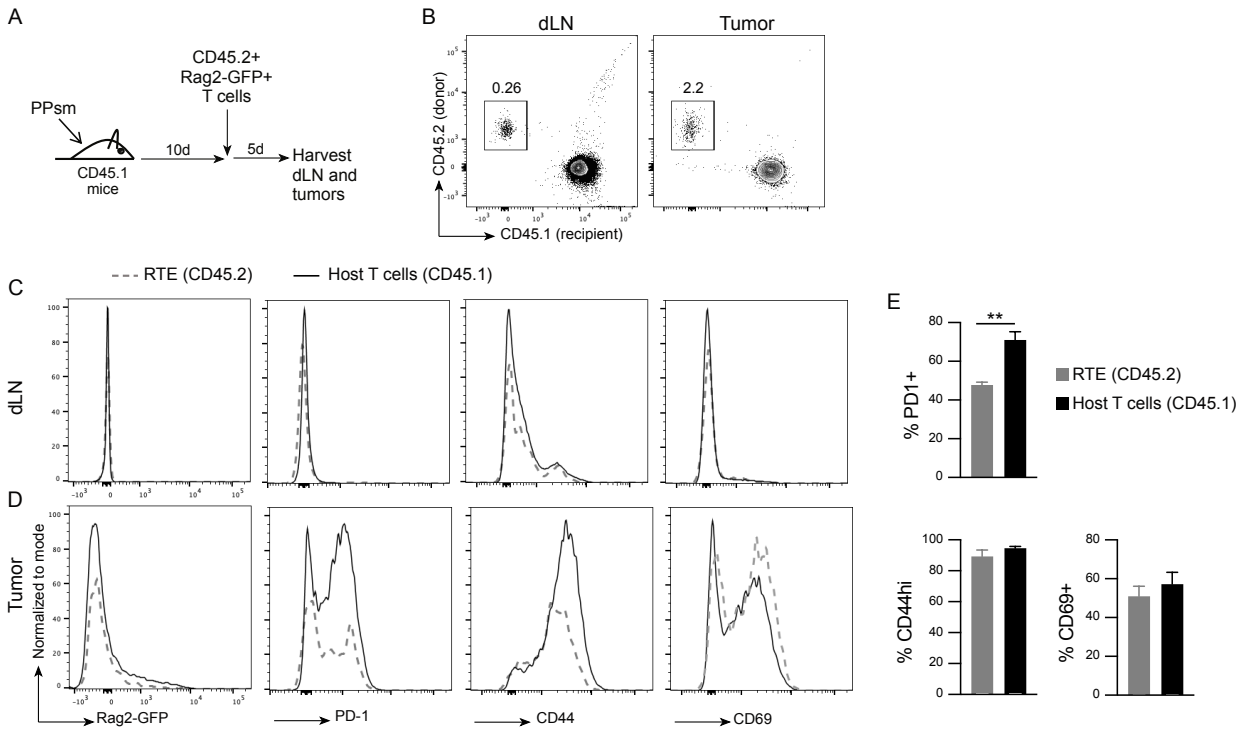

Figure 4.4 RTE traffic to the tumor and display an activated phenotype

Figure 4.4. RTE traffic to the tumor and display an activated phenotype. A) Experimental design. **B)** Representative cytogram (gated on live, TCRβ+) showing CD45.1+ (host) and CD45.2+ (adoptively transferred cells) staining in the dLN and tumor. **C-D)** Representative histograms, gated on live, TCRβ+, CD45.1+ (host T cells) or CD45.2+ (RTE), showing Rag2-GFP, PD1, CD44 and CD69 expression in host CD8 T cells and adoptively transferred RTE CD8 T cells in dLN (C) and tumor (D). **E)** Quantification of percent PD1+, CD44hi and CD69+ RTE and host T cells in the tumor. 3 animals per group. Data representative of 3 experiments. Unpaired two-tailed Student *t* test, * $P<0.05$, ** $P<0.01$.

and OTI non-RTE (Rag2-GFP-) from the spleens of these animals. Sorted OTI RTE or OTI non-RTE were adoptively transferred into OVA-expressing tumor-bearing male mice (TrampC1-Ova), allowing for direct comparison of tumor-antigen specific RTE and non-RTE T cell function in the tumor. Five days post adoptive transfer, dLN and tumors were harvested (Fig. 4.5A). Both RTE and non-RTE Thy1.1+ cells were recovered in dLN and tumors (Fig. 4.5B). The number of recovered RTE was slightly lower compared to the number of non-RTE in the dLN but was equivalent in the tumor (Fig. 4.5C), demonstrating that tumor-specific CD8 RTE have a similar ability to traffic to the tumor as non-RTE . As expected, the RTE had lost Rag2-GFP expression 5 days post transfer (Fig. 4.5D). Both the RTE and non-RTE OTI T cells displayed an activated phenotype compared to endogenous CD8 T cells in the dLN and tumor. RTE expressed similar levels of activation markers CD44 and PD-1 compared to non-RTE in both the dLN and tumor (Fig. 4.5D, E). The majority of both the RTE and non-RTE were proliferating in the dLN and tumors, with a small reduction in the frequency of Ki67+ RTE in the tumor compared to the non-RTE (Fig. 4.5F). These data further establish that tumor-specific CD8 RTE are similarly able to traffic to the tumor and exhibit an activated phenotype comparable to mature tumor-specific non-RTE CD8 T cells.

Activation markers on the RTE could be due to bystander activation in the tumor rather than direct activation by tumor antigen, and these cells might still be too immature to become fully activated and participate in the antitumor immune response. Therefore, we re-stimulated the RTE and non-RTE *ex vivo* with SIINFEKL peptide 5 days post adoptive transfer. The RTE produced IFNγ and TNFα cytokines at comparable levels to the non-

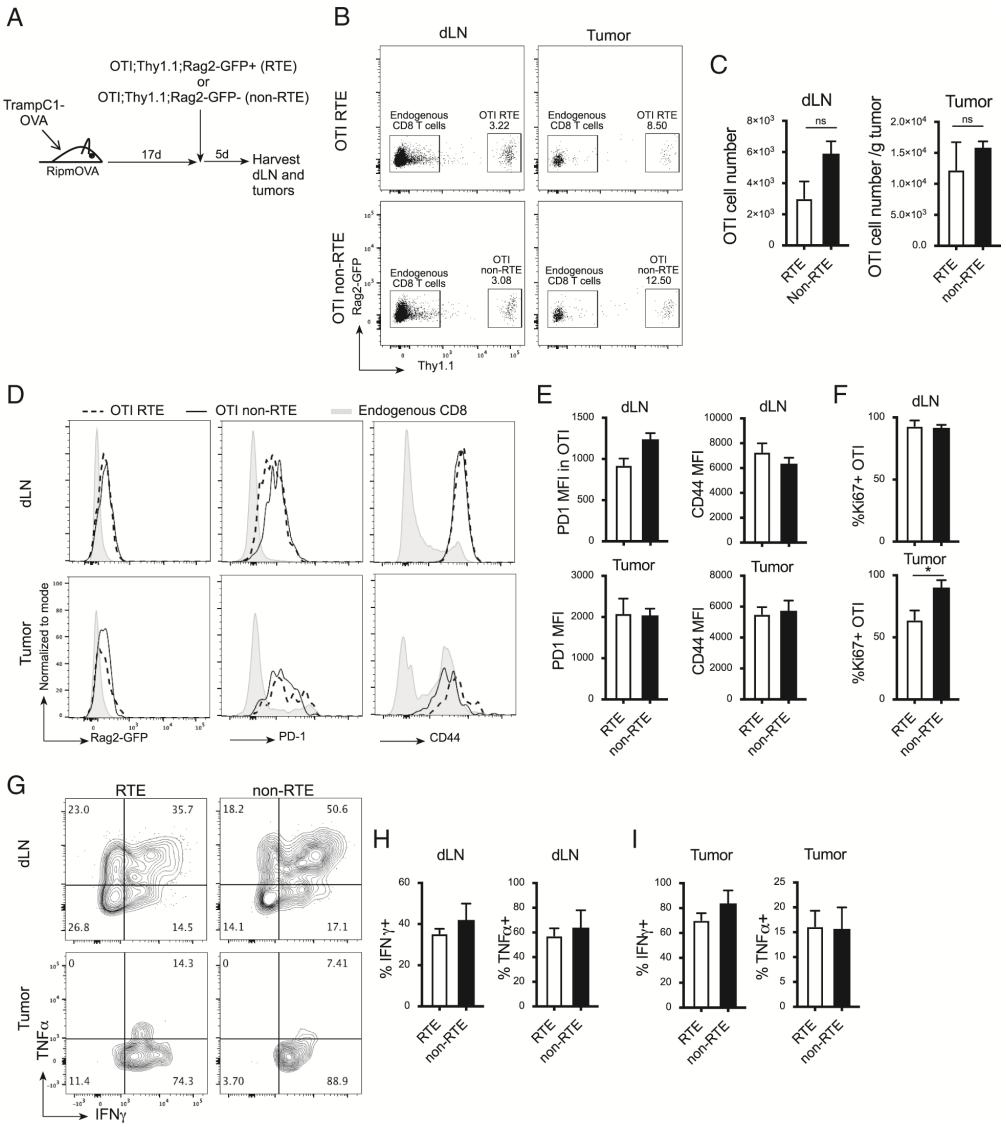

Figure 4.5 Tumor-specific RTE contribute to the antitumor immune response

Figure 4.5. Tumor-specific RTE contribute to the antitumor immune response. **A)** Experimental design. **B)** Representative flow plots (gated on live, TCRβ+) showing Rag2-GFP and Thy1.1 staining in the dLN and tumor of animals that received adoptive transfer of RTE OTI or non-RTE OTI. **C)** Cell number of recovered OTI cells in the dLN and tumor. **D)** Representative histograms (gated on live, TCRβ+, CD8+, Thy1.1+ or Thy1.1-) showing Rag2-GFP, PD1 and CD44 expression in the OTI cells and host endogenous CD8 T cells. **E)** Quantification of PD1 and CD44 MFI in OTI the dLN and tumor. **F)** Percent Ki67+ OTI cells. **G-I)** dLN cells and tumor infiltrating lymphocytes (TIL) were stimulated in vitro with SIINFEKL peptide and stained for intracellular cytokines. G) Representative flow plots (gated on live, TCRβ+, CD8, Thy1.1+) showing IFNγ and TNFα expression adoptively transferred RTE and non-RTE in the dLN and tumor. **H-I)** Quantification of percent IFNγ and TNFα among adoptively transferred RTE and non-RTE in the dLN (H) and tumor (I). 3 animals per group. Data are representative of 2 experiments. Unpaired two-tailed Student *t* test, * $P<0.05$.

RTE (Fig. 4.5G-I), suggesting that the RTE can actively participate in the antitumor immune response.

Orchiectomy leads to increased numbers of functional RTE in the tumor

We have shown that orchiectomy leads to thymic regeneration and increases thymocyte numbers, and that adoptively transferred RTE have the ability to traffic to the tumor and become activated. We therefore sought to directly test whether the RTE released following orchiectomy can traffic to tumors and participate in antitumor immune responses. To evaluate this, we initially implanted PPSM tumors in the flanks of Rag2-GFP male mice. Seven days after tumor implantation, animals were orchiectomized. Thymus, dLN, and tumors were harvested 2 weeks post-surgery. The thymic weights were increased 2 weeks post orchiectomy (Fig. 4.3B), consistent with thymic regeneration. To assess a potential increase in RTE release from the thymus post orchiectomy, we looked at the frequency and total number of Rag2-GFP+ T cells in the dLN and tumors. We did not observe an increase in GFP+ T cells in orchiectomized animals compared to intact animals (Fig. 4.3C-D). However, these data are confounded by the fact that RTE lose Rag2-GFP expression within 5 days of exiting the thymus, making it impossible to track them long-term in this system.

In order to follow RTE long-term, we developed a fate mapping system to allow for permanent marking of RTE following orchiectomy. TCRδ-CRE^{ERT2} mice have a tamoxifen-inducible Cre recombinase under the control of the TCRδ chain gene promotor. TCRδ expression starts in the thymus at the DN stage, and is lost in mature $\alpha\beta$ T cells due to

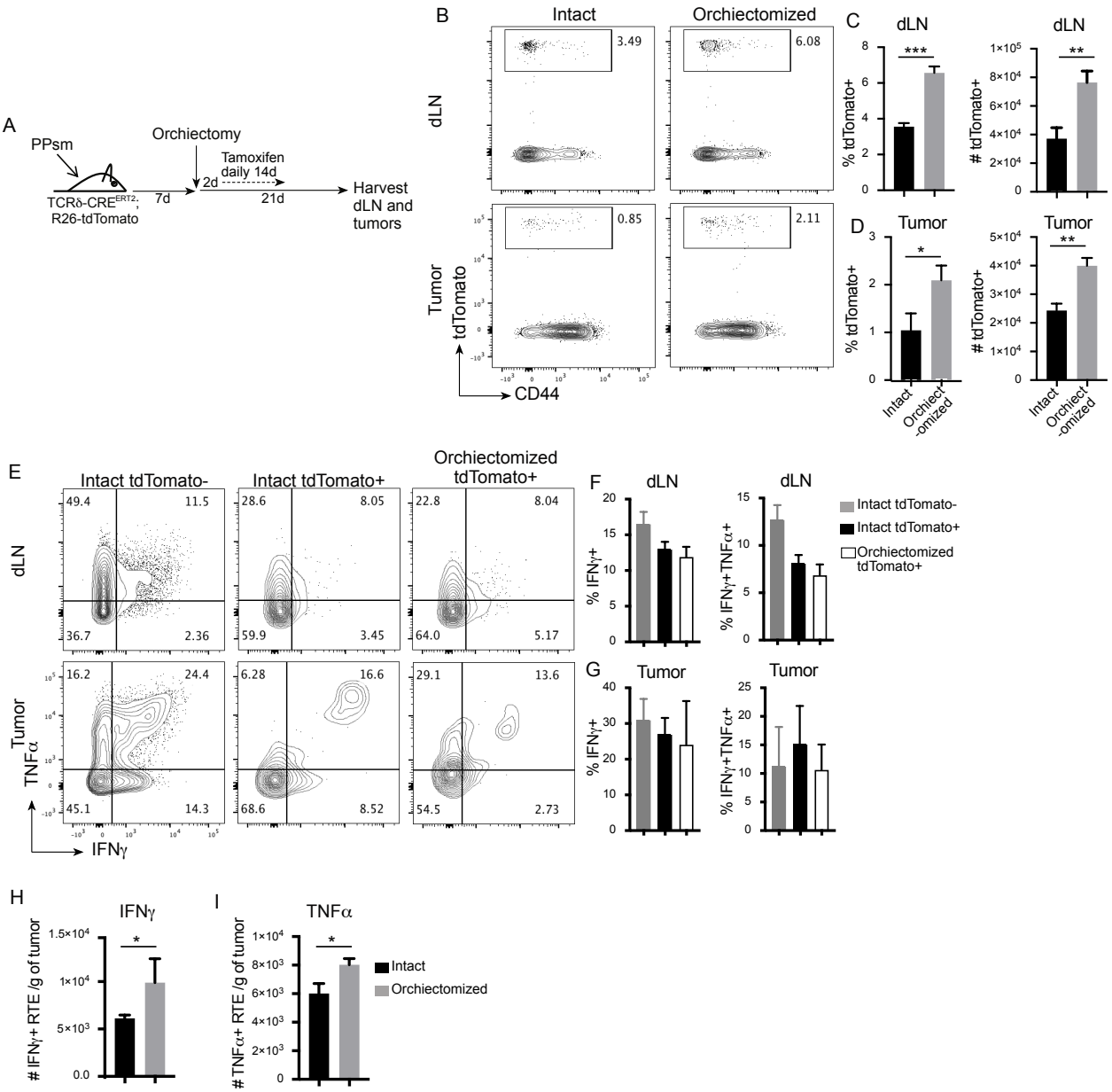

Figure 4.6 Orchiectomy leads to increased numbers of functional CD8+ RTE in the tumor

Figure 4.6: Orchiectomy leads to increased numbers of functional CD8+ RTE in the tumor. A) Experimental design. **B)** Representative flow plots (gated on live, TCR□+, CD8+) showing CD44 and tdTomato expression in the dLN and tumor of intact and orchiectomized animals. Quantification of percent and numbers of tdTomato+ CD8 T cells in the **C)** dLN and **D)** tumor (numbers are per gram of tumor). **E)** Representative flow plots gated on live, TCRβ+, CD8, tdTomato+ or - showing IFNγ and TNFα expression. Intact TdTomato+ cells are not shown but look identical to intact TdTomato- cells. **F, G)** Quantification of percent IFNγ+ and IFNγ+TNFα+ in the dLN **(F)** and tumor **(G)**. **H, I)** Number of CD8+ RTE expressing IFN□ **(H)** and TNFα **(I)** per gram of tumor. 3 animals per group. Data representative of 3 repeat experiments. Unpaired two-tailed Student *t* test, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

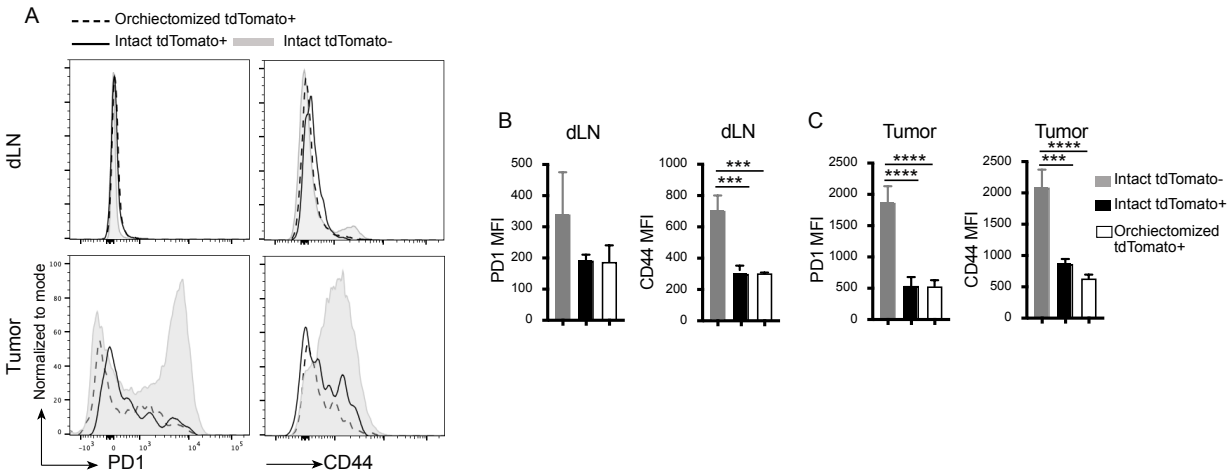

Figure 4.7 Activation markers in RTE from intact or orchiectomized animals

Figure 4.7: Activation markers in RTE from intact or orchiectomized animals. A) Representative flow plots (gated on live, TCRβ+, CD8+, tdTomato+/tdTomato-) of PD1 and CD44 expression. Quantification of PD1 and CD44 expression in the **B)** dLN and **C)** tumor. 3 animals per group. Data representative of 3 repeat experiments. Unpaired two-tailed Student t-test, *** P<0.001, **** P<0.0001.

somatic deletion at the DP stage (263). Therefore, TCRδ is only expressed in αβ T cells in the thymus. We generated TCRδ-CRE^{ERT2};Rosa26-tdTomato animals and implanted them with PPSM tumors. Ten days post tumor implantation, animals were orchiectomized to initiate thymic regeneration. Tamoxifen was administered daily for 14 days starting 2 days post orchiectomy, allowing for CRE expression in newly generated thymocytes and permanent expression of tdTomato in newly released RTE. dLN and tumors were harvested 21 days post orchiectomy (Fig. 4.6A). In both the dLN (Fig. 4.6B, C) and the tumor (Fig. 4.6B, D), a 2-fold increase in frequency and number of tdTomato+ CD8 T cells were recovered in orchiectomized animals compared to intact animals, reflecting an increase in the release of RTE following orchiectomy. In the dLN, the tdTomato+ CD8 T cells from both intact and orchiectomized animals showed very low levels of activation markers PD-1 and CD44, comparable to levels in tdTomato- T cells (Fig. 4.7A, B). In the tumor, tdTomato+ CD8 T cells from both intact and orchiectomized animals expressed PD-1 and CD44, although at lower levels than the tdTomato- CD8 T cells (Fig. 4.7A, C). Strikingly, the TdTomato+ CD8 T cells from both intact and orchiectomized animals were able to produce IFNγ and TNFα comparable to the more mature tdTomato- CD8 T cells in the dLN and tumor (Fig. 4.6E-G). Finally, orchiectomized animals had higher numbers of CD8 RTE expressing IFNγ and TNFα in the tumor than did intact mice (Fig. 4.6H, I). We observed a similar increase in the proportion and number of tdTomato+ CD4 T cells post orchiectomy in both the dLN and tumor (Fig. 4.8A-C). TdTomato+ CD4 T cells were able to produce IFNγ and TNFα cytokines in the dLN and tumor (Fig. 4.8D-F), and the number of CD4 RTE expressing IFNγ and TNFα in the tumor was higher in orchiectomized animals than in intact animals (Fig. 4.8G, H). These data establish

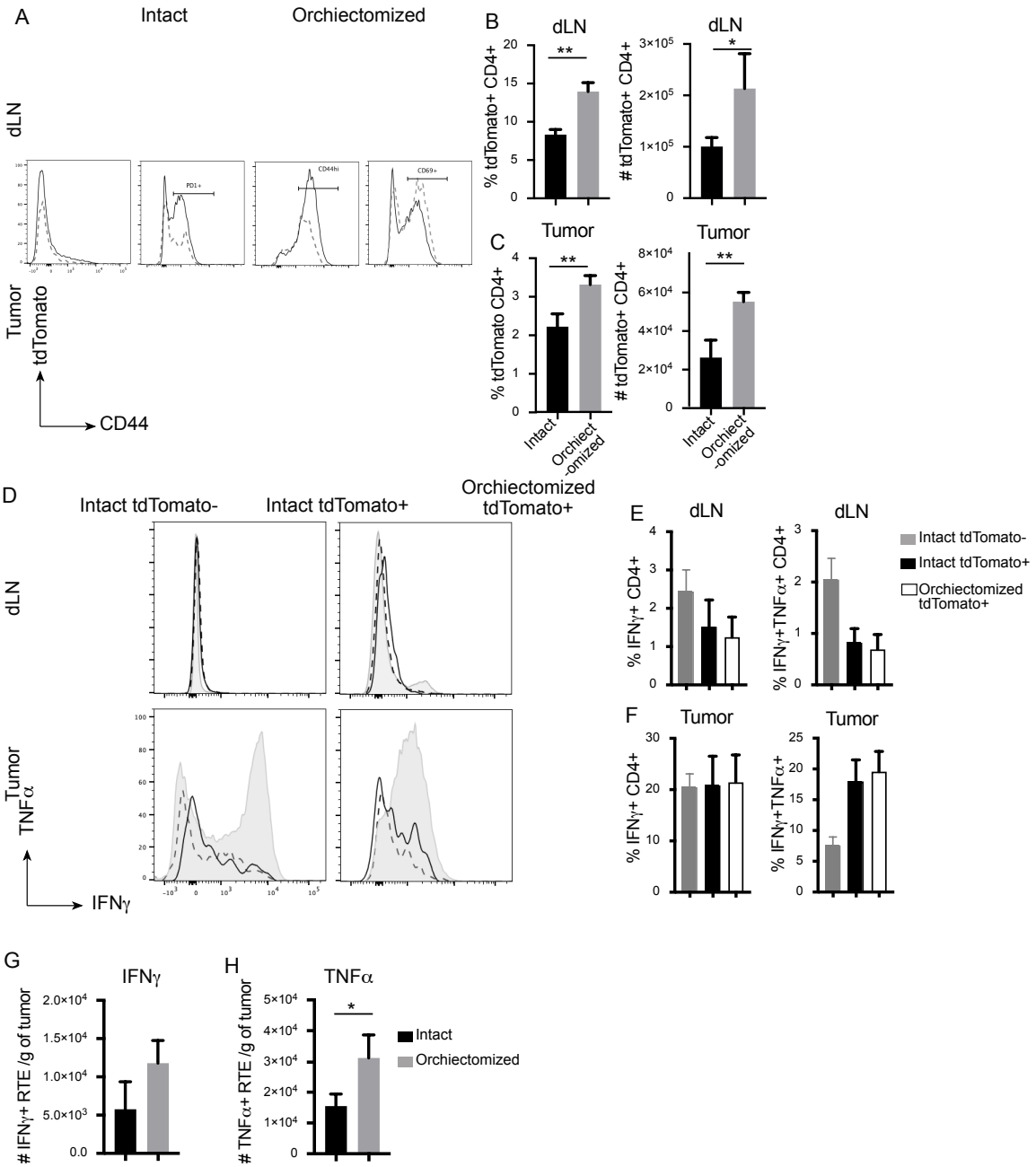

Figure 4.8 Orchiectomy increases the number of functional CD4+ RTE in the tumor

Figure 4.8: Orchiectomy increases the number of functional CD4+ RTE in the tumor. **A)** Representative flow plots (gated on live, TCRβ+, CD4+) showing CD44 and tdTomato expression in the dLN and tumor of intact and orchiectomized animals. Quantification of percent and numbers of tdTomato+ CD4 T cells in the **B)** dLN and **C)** tumor (numbers are per gram of tumor). **D)** Representative flow plots gated on live, TCRβ+, CD4, tdTomato+ or - illustrating IFNγ and TNFα expression. **E, F)** Quantification of percent IFNγ+ and IFNγ+TNFα+ in the dLN **(E)** and tumor **(F). G, H)** Number of CD4+ RTE expressing IFNγ (G) and TNFα (H) per gram of tumor. 3 animals per group. Data representative of 3 repeat experiments. Unpaired two-tailed Student t test, * P<0.05, ** P<0.01.

that RTE generated during thymic regeneration following orchiectomy are able to participate in the antitumor immune response.

Discussion

Thymic involution prevents aged hosts from mounting an effective response to vaccination and invading pathogens (264). We show in this report that both orchiectomy and ADT of male mice leads to proliferation of TEC, which correlates with an increase in thymic output of RTE. We demonstrate that RTE produced as a result of ADT can traffic to tumors where they are activated and produce levels of cytokines comparable to non-RTEs. Together, this evidence suggests that ADT induces an increase in RTEs that can participate in the antitumor response.

The impact of androgens on the function of the thymus has been well established. AR expression in the thymus has been demonstrated using ligand-binding assays in homogenate from whole thymus tissue in rats and humans (265, 266). More recent studies found AR expression in both thymocytes (267, 268) and thymic epithelial cells (TEC), with more abundant expression in TEC (30). We and others have demonstrated that blocking AR results in thymic regeneration (31, 60-62). Our findings indicate that this regeneration may be due to the effect of androgens on the TEC as orchiectomy led to the proliferation of TEC, but not thymocytes. This is in agreement with previous reports where the authors determined that the effect of androgens on TEC resulted in thymic enlargement (31, 32). A recent report in a murine model of prostate cancer demonstrated that ADT increased thymic area as well as output (61). Reports in prostate cancer patients

similarly demonstrated that the increase in RTE after ADT was due to an increase in thymic output rather than an increase in proliferation (26, 60). However, at least one study demonstrated an increase in thymocyte proliferation in response to orchiectomy (62).

RTE are thought to undergo further maturation in the periphery (69, 72), which, until now, has led to uncertainty regarding whether the RTE generated from ADT can meaningfully contribute to an immune response. Our data clearly show that RTE produced from ADT can traffic to tumors and participate in the antitumor immune response through the production of cytokines. Moreover, our data provide a potential mechanism to explain the positive effects of castration on disease outcome in a number of infection and tumor models. Orchiectomy of old mice that were then infected with human HSV-1 led to increased T cell activation in the draining lymph node as well as an increase in the specific lysis of HSV-1-infected cells (60). In an influenza A virus infection model, orchiectomy led to better viral control, which was due to an increase in virus-specific CD8+ T cell number and function (269). Other studies have shown that orchiectomized animals were protected from both chemically induced and transplantable tumors models, an effect that was dependent upon the thymus (269, 270). It's intriguing to consider the possibility of combining ADT with cancer vaccines to harness RTEs and their lack of long-term exposure to inflammatory cytokines. In fact, the lower expression of PD1 on RTEs within the tumor compared to non-RTEs further suggests that mechanisms to exploit the presence of these 'young' cells could enhance antitumor immunity. Finally, the use of androgen inhibition to regenerate the thymus not only induces a new wave of T cells, but

also relieves androgen mediated immune suppression (168, 271, 272); a phenomena that we believe would impact RTE and non-RTE alike.

Although the increased output of functionally competent RTEs likely plays a role in improved T cell responses following orchiectomy, our data do not preclude other mechanisms by which removal of androgens could boost T cell immunity. There may be a direct impact of the removal of androgens on peripheral T cells as AR is expressed by these cells (260, 273). Further, we cannot discount the effect of ADT on hematopoietic stem cells as hematopoiesis also increases with ADT (60, 274). Additionally, there is evidence that removal of androgens increases antigen presentation, which could also contribute to the reported findings (275).

Simple blockade of sex steroid production can return the thymus to its prepubertal state, allowing the thymus to utilize its inherent regenerative capacity to produce T cells without the addition of exogenous cytokines. Therefore, there is no evidence of the development of pathological conditions, such as autoimmunity, resulting from this strategy. Further, we do not think that the importance of sex hormones in regulating thymic output is limited to males as ovariectomy in aged female mice also led to an increase in thymic cellularity and output (269, 276). Further studies should be conducted to determine the role of hormones in regulating thymic output in females.

Collectively, we demonstrate that both physical and chemical castration lead to comparable levels of thymic regeneration, which results in an increase in RTE. These

RTE traffic to tumors, where they become activated and produce effector cytokines comparable to non-RTEs. Together, these data have implications for enhancing responses to tumor-vaccines, chemotherapy, immunotherapies, and for increasing immune reconstitution following chemotherapy.

Open questions and future directions

In our studies, we examined the functionality of CD8+ RTE; however, the fate of CD8+ RTE in the TME remains unclear. Because RTE are skewed towards anergy due to their phenotype (TCR^{hi} $CD28^{lo}$) (69), I expect that they do not become exhausted in the TME. To examine whether RTE become terminally exhausted T cells, I would utilize the fate-mapping system where RTE are permanently marked with tdTomato. TCRδ-CRE^{ERT2};Rosa26-tdTomato animals would have PPSM tumors implanted on the flanks. Ten days after tumor implantation, animals will be orchiectomized to induce thymic regeneration. Tamoxifen would be administered daily for 14 days starting 2 days post-orchiectomy to allow for CRE expression in the newly generated thymocytes that will mark them permanently with tdTomato. Tumors would be harvested at an exhaustion timepoint, 28 days following orchiectomy, and CD8+ T cells would be isolated, stained for flow cytometry, and examined for markers consistent with T cell dysfunction, including markers for progenitor exhausted T cells. I expect that a greater percent of tdTomato+ cells would form progenitor exhausted T cells, with fewer of these cells becoming terminally exhausted. To determine functionality of these cells at an exhaustion timepoint, tdTomato+ CD8+ TIL and tdTomato- CD8+ TIL could be restimulated for cytokine production.

It has been reported that progenitor exhausted T cells are responsible for driving the response to immune checkpoint blockade, especially anti-PD-1 therapy (101). Because I expect that fewer of the RTE would become terminally exhausted with a higher percentage of progenitor exhausted T cells, I hypothesize that RTE should respond more effectively to immunotherapy than non-RTE. To test this hypothesis, I would again utilize the tdTomato fate-mapping system for RTE. TCRδ-CRE^{ERT2};Rosa26-tdTomato animals would have PPSM tumors implanted on the flanks. Seven days after tumor implantation, animals will be orchiectomized to induce thymic regeneration and tamoxifen would be administered daily for 14 days starting 2 days post-orchiectomy to allow for CRE expression in the RTE to mark them with tdTomato. Fourteen days after tumor implantation, I would treat animals with anti-PD-1 or control antibody every three days for three treatments and would harvest tumors one day after the last treatment. CD8+ TIL would be isolated and stained for flow cytometry restimulated *ex vivo* with a proliferation dye to examine functionality and proliferation in response to restimulation of tdTomato+ RTE compared to tdTomato- non-RTE. I expect that the RTE would be more proliferative and more functional in the group treated with anti-PD-1, especially when compared to non-RTE, indicating a favorable response of RTE to ICB.

We did find an increase in functional CD4+ RTE in the tumors of orchiectomized mice, but we did not explore their helper phenotype. It has been demonstrated that CD4 RTE are skewed towards the Th2 lineage (71), which seem to contribute variably to the antitumor immune response, unlike Th1 cells. I hypothesize that CD4 RTE are Th2-skewed in the TME. I would examine this *in vitro* first by harvesting CD4+ GFP+ RTE or

CD4+ GFP- non-RTE from Rag2-GFP;OTII mouse spleens, which contain CD4 T cells that are specific for the OVA antigen. I would stimulate these cells *in vitro* under Th1- or Th2-polarizing conditions to determine if RTE more readily differentiate into Th1 or Th2 cells, then stain the cells for flow cytometry to examine markers of Th1 or Th2 differentiation and cytokine production. I expect the OTII GFP+ RTE to differentiate into Th2 cells at higher frequencies and produce greater Th2 cytokines compared to the OTII GFP- non-RTE. Additionally, to examine this *in vivo*, I would use a model like the one proposed above in the TCRδ-CRE^{ERT2};Rosa26-tdTomato animals harboring PPSM tumors. I would harvest tumors, isolate CD4+ T cells, and stain for Th lineage markers for analysis by flow cytometry. I expect similar results in that the CD4+ RTE will more readily be Th2-skewed compared to non-RTE in the TME.

It has also been reported that CD4+ RTE are more likely to become Tregs (78), but whether this occurs in the TME remains unknown. I hypothesize that the CD4+ RTE become Tregs at higher rates in the TME and that these cells would be superior suppressors compared to non-RTE Tregs. I would again utilize the TCRδ-CRE^{ERT2};Rosa26-tdTomato animals harboring PPSM tumors and isolate CD4+ TIL to examine Treg frequency by flow cytometry. Additionally, I would sort tdTomato+ Tregs derived from RTE or tdTomato- Treg derived from more mature CD4+ cells and examine their functionality in an *in vitro* suppression assay. I expect that a greater percent of CD4+ RTE will be Tregs and that these Tregs will be superior suppressors compared to CD4+ non-RTE Tregs.

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260. Guan, X., F. Polesso, C. Wang, A. Sehrawat, R. M. Hawkins, S. E. Murray, G. V. Thomas, B. Caruso, R. F. Thompson, M. A. Wood, C. Hipfinger, S. A. Hammond, J. N. Graff, Z. Xia, and A. E. Moran. 2022. Androgen receptor activity in T cells limits checkpoint blockade efficacy. *Nature*.
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272. Yang, C., J. Jin, Y. Yang, H. Sun, L. Wu, M. Shen, X. Hong, W. Li, L. Lu, D. Cao, X. Wang, J. Sun, Y. Ye, B. Su, and L. Deng. 2022. Androgen receptor-mediated CD8(+) T cell stemness programs drive sex differences in antitumor immunity. *Immunity* 55: 1268-1283.e1269.
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275. Koh, Y. T., A. Gray, S. A. Higgins, B. Hubby, and W. M. Kast. 2009. Androgen ablation augments prostate cancer vaccine immunogenicity only when applied after immunization. *Prostate* 69: 571-584.
276. Perisić, M., N. Arsenović-Ranin, I. Pilipović, D. Kosec, V. Pesić, K. Radojević, and G. Leposavić. 2010. Role of ovarian hormones in age-associated thymic involution revisited. *Immunobiology* 215: 275-293.

Curriculum Vitae

Breanna P. Caruso

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EDUCATION

Oregon Health & Science University **June 2023**
Portland, OR
PhD in Cancer Biology

Colgate University **May 2013**
Hamilton, NY
BA in Cellular Neuroscience
Cumulative GPA: 3.72, *magna cum laude*

GRANTS

Ruth L. Kirschstein Predoctoral Individual NRSA (F31) June 2021-June 2023
National Cancer Institute, #1 F31 CA261058-01 *$92,072*

Department of Cancer Biology, Stipend Award November 2020
Knight Cancer Institute *$40,000*

Graduate Research Fellowship Program Award April 2018-May 2021
National Science Foundation,#AVPRS0015 *$138,000*

MENTORSHIP

Summer Student Mentor, OHSU: Rachel Amadio June 2022-August 2022
Summer Student Mentor, OHSU: Ester Emlen June 2019-August 2019
Summer Student Mentor, OHSU: Yamini Naidu June 2017-August 2017
Summer Student Mentor, NIH: Benjamin Haner May 2015- July 2015
Summer Student Mentor, NIH: Benjamin Haner May 2014- July 2014

RESEARCH EXPERIENCE

Oregon Health and Science University
Portland, OR
PhD Candidate September 2017-present
Advisor: Amy Moran – Examined the role of the inhibitory molecule, PD-1, in regulating the development of regulatory T cells in the thymus with implications for men with prostate cancer undergoing anti-PD-1/anti-PD-L1 during ADT-induced thymic regeneration

Skills and Techniques:

- 12+ color flow cytometry – Symphony Analyzer, Fortessa Analyzer, FlowJo Software
- FACS
- Phosflow cytometry
- PCR – qPCR, RT-PCR
- Tumor models – tumor injections, cell culture, tumor measurements, tumor harvest
- *In vitro* assays – Treg suppression, Treg induction, T cell proliferation, T cell

stimulation
- *In vivo* mouse work – adoptive transfer, bone marrow chimeras, injections, bleeding, necropsy, tissue processing

Research Assistant II, September 2015-August 2017
Advisor: Pepper Schedin, PhD – Investigated the contribution of immunosuppressive dendritic cells to the tumor-promotional microenvironment of the involuting mammary gland. Analyzed the role of calponin in regulating myoepithelial cell function in the context of ductal carcinoma *in situ*.

Skills and Techniques:
- Animal husbandry – breeding, genotyping, colony maintenance
- Cell culture- 2D, 3D
- Immunohistochemical staining
- Immunofluorescence staining

National Institutes of Health
Bethesda, MD
Postbaccalaureate IRTA Fellow May 2013-July 2015
Advisor: Steven Jacobson, PhD – Investigated Human Herpesvirus 6A and 6B as potential environmental triggers for Multiple Sclerosis. Supported clinical trials examining the therapeutic potential of Raltegravir and Humaized MiK-Beta-1 on HTLV-1 proviral load. Identified a patient with PTLV-1, the first to demonstrate that this virus could cause neurological disease in humans.

Skills and Techniques:
- PCR – droplet digital, qPCR, RT-PCR
- Western blotting
- ELISA
- *In vivo* primate work – handling, necropsy, tissue processing

University of Rochester
Rochester, NY
Summer Scholar May 2012- August 2012
Advisor: John Olschowka, PhD – Determined the effects of high dose radiation on learning and memory.

Skills and Techniques:
- Mouse learning and memory tasks
- PCR – qPCR, RT-PCR
- Cryosectioning
- Immunohistochemical staining

Colgate University
Hamilton, NY
Honors Thesis August 2012-May 2013
Advisor: Jun Yoshino, PhD – Determined that melatonin inhibits astrogliosis in primary purified astrocyte cultures by modulating the COX-2 pathway.
Skills and Techniques:
- Cell culture – primary tissue culture
- ELISA

Research Assistant Dec 2010- August 2012

Advisor: Jun Yoshino, PhD – Examined the antioxidant effects of melatonin mediated via the nuclear translocation of NF-κB on the production of nitric oxide in primary glial cells.

Skills and Techniques:

- Cell culture – primary tissue culture
- Immunofluorescent staining
- Confocal microscopy

PRESENTATIONS

1. **Caruso B** and Moran AE. A lineage specific role for PD-1 in agonist selection in the thymus. IMMUNOLOGY 2022. May 2022. Oral Presentation.
2. **Caruso B** and Moran AE. The role of programmed cell death protein 1 (PD-1) in regulating T cell development in the thymus. OHSU Research Week. May 2021. Oral Presentation.
3. **Caruso B** and Moran AE. Determining the role of programmed cell death protein 1 (PD-1) in T cell development. STEM Village Immunology Seminar Series. November 2020. Invited Presentation.
4. **Caruso B** and Moran AE. The role of PD-1 signaling in T cell development. IVD Graduate Student Symposium. August 2019. Oral Presentation.
5. **Caruso B** and Moran AE. The role of PD-1 signaling in T cell development. OHSU Research Week. May 2019. Poster Presentation. **Poster Presentation Award.**
6. **Caruso B,** Brunetto G, Massoud R, Akahata Y, Ohayon J, and Jacobson S. Droplet Digital PCR for the Quantification of Human T-Lymphotropic Virus 1 DNA and Gene Expression in HAM/TSP Patients. 40 Years of Neuroimmunology Meeting. April 2015. Poster Presentation.
7. Massoud R, **Caruso B (presenting author)**, Akahata Y, Ohayon J, and Jacobson S. **Pilot study of Raltegravir, an Integrase inhibitor, in Human T-cell lymphotropic virus-1 (HTLV-1) associated myelopathy, tropical spastic paraparesis (HAM/TSP). 67th American Academy of Neurology Annual Meeting. April 2015. Poster Presentation.**
8. **Caruso B** and Jacobson S. Droplet Digital PCR for the Quantification of Human T-Lymphotropic Virus 1. 12th International Symposium on NeuroVirology. October 2013. Oral Presentation.
9. **Caruso B**, Brunetto G, Massoud R, Switzer B, and Jacobson S. Droplet Digital PCR for the Quantification of Human T-Lymphotropic Virus 1. 12th International Symposium on NeuroVirology. October 2013. Poster Presentation.

PUBLICATIONS

1. Caruso B, Weeder BR, Thompson RF, and Moran AE. (2023). PD-1 restrains thymic IL-2 production and regulatory T cell development. *Submitted to J Immunology.*
2. **Caruso B** and Moran AE. (2023). Thymic expression of immune checkpoint molecules and their implication for response to immunotherapies. *Trends in Cancer*. 10;S2405-8033(23)00063-8
2. Polesso F, **Caruso B**, and Moran AE. (2023). Restored thymic output after androgen blockade participates in antitumor immunity. *J Immunology*. 210(4): 496 – 503.
3. Guan X, Polesso F, Wang C, Sehrawat A, Hawkins RM, Murray SE, Thomas GV, **Caruso B**, Thompson RF, Wood MA, Hipfinger C, Hammond SA, Graff JN, Xia Z, and Moran AE. (2022). Androgen receptor activity in T cells limits checkpoint blockade efficacy. *Nature*. 606: 791-6.
   - Highlighted in Cancer Cell, Spotlight, May 9, 2022: https://doi.org/10.1016/j.ccell.2022.04.007
   - Highlighted in Cancer Discovery, Research Watch, April 1, 2022: https://doi.org/10.1158/2159-8290.CD-RW2022-055

- Highlighted in ACIR, In the Spotlight: https://acir.org/journal-articles/cancer-immunobiology/immune-cell-biology?entryId=32884

4. Betts C, Pennock N, **Caruso B**, Ruffell B, Borges VF, and Schedin P. (2018). Mucosal immunity in the female murine mammary gland. *J Immunology*. 201(2): 734-746
5. Leibovitch E, **Caruso B**, Ha S, Schindler M, Lee N, Luciano N, Billioux B, Guy J, Yen C, Sati P, Silva A, Reich D, Jacobson S. (2018) Herpesvirus trigger accelerates neuroinflammation in a nonhuman primate model of multiple sclerosis. *Proceedings of the National Academy of Sciences.* 115(44):11292-11297
6. Ginwala R, **Caruso B**, Zhan ZK, Pattekar A, Chew GM, Corley MJ, Zoonawat R, Jacobson S, Sreedhar S, Ndhlovu LC, Jain P. (2017). HTLV-1 Infection and Neuropathogenesis in the Context of Rag1-/-γc-/- (RAG1-Hu) and BLT Mice. *J Neuroimmune Pharmacol.* 12(3):504-20.
7. **Caruso B**, Massoud R, and Jacobson S. Human T-Cell Lymphotropic Virus Types 1 and 2. *Manual of Molecular and Clinical Laboratory Immunology*. Eighth ed. Washington, DC: ASM, 2016. 674-81. Print.
8. Enose-Akahata Y, **Caruso B**, Haner B, Charlip E, Nair G, Massoud R, Billioux, BJ, Ohayon J, Switzer WM, Jacobson S. (2016). Development of neurologic diseases in a patient with primate T lymphotropic virus type 1 (PTLV-1). *Retrovirology*. 13(1):56.
9. Ratner L, Rauch D, Abel H, **Caruso B**, Noy A, Barta SK, Parekh S, Ramos JC, Ambinder R, Phillips A, Harding J, Baydoun HH, Cheng X, and Jacobson S. (2016). Dose-adjusted EPOCH chemotherapy with bortezomib and raltegravir for human t-cell leukemia virus-associated adult T-cell leukemia lymphoma. *Blood Cancer J.* 6:e408.
10. de Castro-Amarante MF, Pise-Masison CA, McKinnon K, Washington Parks R, Galli V, Omsland M, Andresen V, Massoud R, Brunetto G, **Caruso B**, Venzon D, Jacobson S, Franchini G. (2015). Human T Cell Leukemia Virus Type 1 infection of the three monocyte subsets contributes to viral burden in humans. *J Virol*. 90(5):2195-207.
11. Leibovitch EC, Brunetto GS, **Caruso B**, Fenton K, Ohayon J, Reich DS, Jacobson S. (2014). Coinfection of human herpesviruses 6A (HHV-6A) and HHV-6B as demonstrated by novel digital droplet PCR assay. *PLos One.* 9(3):e92328.
12. Brunetto GS, Massoud R, Leibovitch EC, **Caruso B**, Johnson K, Ohayon J, Fenton K, Cortese I, Jacobson S. (2014). Digital droplet PCR (ddPCR) for the precise quantification of human T-lymphotropic virus 1 proviral loads in peripheral blood and cerebrospinal fluid of HAM-TSP patients and identification of viral mutations. *J Neurovriol.* 20(4):341-51.

OTHER EXPERIENCE AND PROFESSIONAL MEMBERSHIPS

2013 – 2014	Member, Psi Chi International Honor Society
2017 – present	Member, American Association for the Advancement of Science
2018 – 2019	Representative, OHSU All-Hill Student Council
2018 – 2019	Treasurer, OHSU Graduate Student Organization
2019 – 2021	President, OHSU Graduate Student Organization
2019 – 2021	Student Representative, OHSU Graduate Council
2020 – 2022	Member, OHSU Research and Training Conditions Task Force
2022 – 2022	Member, Oversight Committee