Investigation of CD4+ T cell heterogeneity and function in a genetic mouse model of lung adenocarcinoma

by

Amy Li

B.A., Molecular and Cellular Biology
Harvard University, 2010

Submitted to the Department of Biology
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Investigation of CD4+ T cell heterogeneity and function in a genetic mouse model of lung adenocarcinoma

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ABSTRACT

The clinical success of immune checkpoint blockade in the treatment of various cancers provides proof-of-concept that disrupting tumor immune tolerance is an effective therapeutic strategy. Nevertheless, only a subset of patients respond to immune therapies, suggesting that an improved understanding of immunosuppressive mechanisms is needed. CD4+ T cells, which are phenotypically diverse, orchestrate many diverse components of immune responses, and can contribute to tumor growth through a variety of mechanisms. Regulatory T cells (Tregs) in particular are an immunosuppressive subpopulation of CD4+ T cells that may play a central role in immune tolerance to tumors. Targeted manipulation of tumor-promoting CD4+ T cells could augment the efficacy of existing immunotherapies, but our understanding of the complexity and evolution of tumoral CD4+ T cell responses is limited.

We sought to characterize the transcriptional features of tumor-associated CD4+ T cells in order to identify pathways that may be used to inhibit their tumor-promoting functions. Using population and single-cell RNA sequencing, we longitudinally profiled conventional CD4+ T (Tconv) and Tregs in an inducible genetic mouse model of lung adenocarcinoma. We show that lung tumor-associated Tregs have a distinct transcriptional signature that is associated with survival in multiple cancers. Furthermore, we find that tumor-associated Treg and Tconv cells are varied and undergo shifts in gene expression that may represent strengthening immunosuppression as tumors progress. For example, a heterogeneous population of type 1 (Th1) cells is present throughout tumor development but adopts more characteristics that may be associated with immunosuppressive activity at late timepoints. Similarly, early Tregs appear more flexible and express genes associated with Tconv cells, but a terminally-differentiated effector Treg phenotype becomes predominant at late timepoints.

We identified ST2 as a potential mediator of effector Treg differentiation or expansion in tumors. Through IL-33 administration and Treg-specific genetic ablation of ST2 we find that IL-33 signaling may be necessary and sufficient for increased numbers of terminally-differentiated effector Tregs during tumor progression. Taken together, our insight on the evolution of CD4+ T cell heterogeneity in tumors provides a window into the natural history of tumor immunosuppression and may reveal critical mediators of the tumor immune response.

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I appreciate your enthusiasm for developing new tools and approaches to help the lab and our projects and I am glad that you were willing to share that process with me.

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CHAPTER 1

INTRODUCTION
The success of cancer immunotherapies in the last decade may lead one to believe that the study of immune regulation of tumor growth is a recent phenomenon. However, the relationship between inflammation and cancer has been a subject of scientific inquiry for hundreds of years. The interpretation of early accounts of immune reactions to tumors were limited by a poor understanding of cancer biology, immunology, and infectious disease. Later experiments attempting to demonstrate the importance of the immune system in preventing cancer were promising, but stymied by poor models. The advent of modern genetics had major implications for both cancer biologists and immunologists: the identification of oncogenes and tumor suppressors put much emphasis on the cell-intrinsic effects of genetic alterations, while transgenic mice allowed precise interrogation of the role of different types of immune cells. In spite of these divergent paths, a small group of physicians and scientists continued to investigate whether the immune system could prevent or eradicate established cancers. Eventually, advances in our understanding of immune suppression in the prevention of autoimmunity resulted in new, effective tools to modulate tumor immunity in patients, resulting in renewed interest in cancer immunology. Today, it is well-established that immune cells can both impair and promote tumor development. The tumor immune response is a complex nexus of immune cells with diverse phenotypes that are heavily informed by signaling from tumor cells and the tissue environment. Furthermore, checkpoint blockade therapies have demonstrated that mobilization of immune responses directed against tumors can have remarkably durable effects in some patients.
Much remains unknown about the factors that establish or impede a productive anti-tumor immune response, which are likely to vary by tumor type and patient history. Due to their ability to influence the activity of many other cell types, CD4+ T cells are well-positioned to be major orchestrators of tumor inflammation, and consequently targets for therapeutic intervention. However, CD4+ T cells are incredibly heterogeneous, and play divergent and often opposing roles in tumor development. Efforts to study CD4+ T cells in cancer have been further hindered by their considerable plasticity, which makes cell subsets difficult to define, track, and characterize, and their wide array of functions, which make assaying their activity difficult.

Here I will provide historical context for the rise of cancer immunotherapy and our current understanding mechanisms of immunosuppression in cancer. I will then focus on what is known about the role of CD4+ T cells, in particular regulatory T cells (Tregs), in the regulation of tumor immune responses. I will emphasize the recent use of transcriptional profiling to comprehensively characterize these and other tumor-associated populations and the implications of these studies. Finally, I will provide background on the biology of the interleukin 33 (IL-33) signaling pathway, which we are investigating as a mediator of Treg cell function in cancer.

I. Immune evasion and tolerance are hallmarks of cancer

Historical overview of cancer immunology

A parallel between cancer and inflammation was drawn long before scientists understood the pathophysiology of either process. Rudolf Virchow noted in 1858 the presence of leukocytes in tumors, and hypothesized that tumors formed at sites of “pathological irritation”, or inflammation (Virchow, 1863). In contrast, reports from as
early as the 18th century suggested that inflammation had an anti-tumor effect, as they described tumor regressions that were coincident with local or systemic infections (Hoption Cann et al., 2002). Throughout the 1800s, surgeons experimented with installing wounds near tumors or leaving surgical sites open to invite suppuration, or the formation of pus, while others attempted to induce regression through direct inoculation with malaria, syphilis, or erysipelas. In 1891, when William Coley at Memorial Hospital treated his first sarcoma patient with erysipelas, he was unaware that a number of German physicians had performed the same procedure just a few years earlier (Coley, 1893). Direct inoculation with erysipelas proved to be highly variable; the infection could be difficult to transmit in some cases, while other patients died of their infections. Coley pioneered the use of heat-killed preparations of *Streptococcus pyogenes* and *Serratia marcescens* to simulate infection, which improved the safety of the method (Coley, 1910). By the end of his career, Coley had treated nearly 1000 patients with his toxin, which induced remarkable cures or partial regressions in a substantial proportion of patients. It is now thought that Coley’s toxin, as this formulation became known, was able to stimulate a local and systemic tumor immune response, and was thus the first example of a cancer vaccine. However, in spite of his success, his treatments were met with skepticism and disfavor. Few physicians could replicate Coley’s success because of his use of multiple preparations and administration routes, some of which were more active than others, and he conducted poor long-term follow-up of patients (McCarthy, 2006). Coley’s toxin also appeared to be more effective in soft tissue sarcomas than in epithelial carcinomas (Starnes, 1992). Radiation therapy and chemotherapy became
increasingly popular due to their broad applicability, and Coley’s toxin fell into obscurity after his death in 1936.

At the same time, however, tumor and tissue transplantation studies inspired a number of scientists to propose a role for the immune system in preventing cancer. Through extensive study of tumor grafts, Paul Ehrlich and his contemporaries concluded that, with the exception of a few highly transplantable lines, grafts between different animals of the same species would regress after an initial period of growth (Tyzzer, 1907). Furthermore, they observed that some animals that had experienced regressions of inoculated tumor tissue could achieve stronger and faster regression of successive engraftments from the same source, suggesting that one could build immunity against tumors (Silverstein, 2001a). These findings led Ehrlich to hypothesize in 1909 that the immune system could restrain the growth of tumors, which would otherwise occur at great frequency (Dunn et al., 2004). In retrospect, many of these findings could be attributed to allograft rejection since most research animals at this time were not inbred. The observation that engraftment of normal tissue could produce histologically similar reactions, as well as immunity to tumors from the same source, led many leaders in the field to question whether tumor immunity was an independent phenomenon, or a consequence of tissue incompatibility (Bashford and Russell, 1910; Loeb, 1917; Scott, 1991). In fact, several years before he would isolate a cell-free extract capable of transmitting chicken sarcoma, Peyton Rous published a study on the similarities between the engraftment of normal embryo and tumor tissue, and concluded that it is essential to, “to discriminate between characters unique with tumor and those which it possesses in common with normal tissue” (Rous, 1910). Ironically, these tumor
transplantation studies fostered a fervent view of cancer as a disease necessarily transmitted by cells, which would make Rous’s groundbreaking discovery of cell-free transmission of a tumor deeply unpopular (Javier and Butel, 2008).

Nevertheless, reports that tumors were more readily transplantable within stocks of mice inspired scientists to identify heritable traits that conferred tumor resistance. The increasing availability of inbred lines of mice, heavily championed by Clarence Little at Jackson Laboratories, made it possible to study the genetic basis of susceptibility to tumor engraftment. In 1916, Tyzzer and Little found that while all F1 hybrid offspring are susceptible to tumors originating from either parental line, only a small percentage of F2 hybrids could be engrafted (Little and Tyzzer, 1916). They thus concluded, based on the laws of Mendelian inheritance, that 12-14 genetic factors were involved in tumor transplantation. Meanwhile, inspired by Karl Landsteiner’s use of sera to characterize the heritability of blood group antigens, Peter Gorer identified a blood group antigen, antigen II, that was required for engraftment of F2 and backcrossed hybrid mice (Gorer, 1937). Later, Gorer would collaborate with George Snell at Jackson Labs and discover that antigen II was identical or closely linked to the genetic locus associated with tumor rejection that Snell had recently identified, which they subsequently named H2 (Gorer et al., 1948). Although these findings laid the groundwork for transplantation immunology, they also cast doubt on tumor immunity, which was now deemed an artifact of using genetically mixed lines.

Nevertheless, there was soon evidence of tumor-associated, rather than strain-specific, antigens that could provoke immune-mediated tumor regressions. A report of spontaneous regression of transplanted methylcholanthrene (MCA)-induced sarcomas
in highly inbred mice by Ludwig Gross in 1943 offered some support for immunostimulatory tumor antigens (Gross, 1943). These findings were corroborated by additional studies, resulting in a surge of interest in identifying tumor antigens (Foley, 1953a; Prehn and Main, 1957). Lewis Thomas hypothesized in 1959 that mechanisms similar to those observed in tissue rejection had evolved to defend against neoplasia (Dunn et al., 2004), echoing ideas laid down by Ehrlich fifty years earlier. Taking stock of the ability of viruses and carcinogens to induce genetic changes, Macfarlane Burnet would extend upon the idea of cancer immune surveillance by proposing that tumors could host “new antigenic potentialities” that would lead to immune rejection (Burnet, 1957). Tumor outgrowth was thus a consequence of a failed or incomplete immunological reaction, perhaps due to insufficient antigen release early in the response, or “transient immune paralysis”, a remarkably prescient view that mirrors our understanding of cancer immunology today (Old and Boyse, 1964).

However, even as scientists began to build an extensive catalog of tumor antigens that had been described in experimental models (Old and Boyse, 1964), they were aware that tumor immunogenicity could only be described in chemically or virally induced models of cancer. In the same study describing immunity to MCA-induced sarcomas, Foley demonstrated that syngeneic mammary cancer lines derived from spontaneous tumors could not induce immunity (Foley, 1953a, 1953b). Similarly, Prehn and Main’s oft-cited comprehensive study of MCA-induced sarcoma also showed that histologically similar, but spontaneously-arising, fibrosarcoma cell lines failed to protect against subsequent transplantation (Prehn and Main, 1957). This led the field to conclude that these environmentally-induced tumors were distinct from spontaneous
tumors, likely due to their greater number of somatic mutations (Foley, 1953a). The idea that spontaneous tumors should be immunologically inert was likely bolstered by advances in our understanding of immune tolerance that also occurred at this time. In a landmark study, Billingham, Brent, and Medawar showed that fetuses from one inbred mouse strain that were engrafted with cells from a transplant-incompatible strain could accept skin grafts in adulthood from the other strain, but still reject grafts from a second unrelated strain (Billingham et al., 1953). Since it was not yet known that even spontaneous tumors had discrete genetic alterations, immune tolerance provided a mechanistic basis for why tumors derived from “self” should not mount an immune response. The field nevertheless continued its studies using experimental models where tumor immunogenicity could be demonstrated, and focused less on the spontaneous tumor models that brought into question the broad applicability of the theory of cancer immune surveillance. By 1974, 27% of one leading cancer journal’s articles were devoted to reports on tumor immunity, compared to 4% in 1966 (Hewitt et al., 1976). The reckoning began in 1974, when Osias Stutman used MCA to induce sarcomas in athymic nude mice and found, contrary to the predictions of the cancer immune surveillance model, no reduction in latency or increased incidence of sarcomas (Stutman, 1974). This implied that the immune response to tumors was only relevant in virally-induced cancers, of which few had been identified in humans. Two years later, Hewitt et al. dealt another blow by publishing a comprehensive study of spontaneous tumor lines that had all been unable to generate tumor immunity (Hewitt et al., 1976). He argued that reported observations of tumor immunity in animal models were a complete artifact of using chemical and virus-induced models that were inappropriate for
modeling human cancer. It is now known that athymic nude mice still have natural killer (NK) cells and detectable populations of conventional (TCRαβ) T cells (Kim et al., 2007b). Furthermore, a study done 20 years later using a lower dose of MCA would demonstrate that nude mice do develop tumors faster and with greater frequency (Engel et al., 1996). However, Stutman’s findings, and the emphasis on using spontaneous tumor models championed by Hewitt, would drastically slow the momentum of the cancer immunology field.

Advances in molecular biology and cellular immunology in the 1970s and 80s would provide the tools necessary to revive the theory of cancer immune surveillance in the 90s. The discovery of T cells in 1967 (Miller et al., 1967) was quickly followed by the discoveries of dendritic cells (Steinman and Cohn, 1973), major histocompatibility complex (MHC) restriction (Zinkernagel and Doherty, 1974), and NK cells (Kiessling et al., 1975). These breakthroughs would define the armamentarium of the cellular immune response to tumors, and provide a set of pathways that could be manipulated in experimental systems in order to learn more about cancer immunology. The observation that suppressor T cells could mediate tolerance to the growth of immunogenic tumors (Berendt and North, 1980) also broke ground for the study of cancer immunosuppression by providing a mechanism for how tumors could grow in spite of immune detection. Meanwhile, the identification and cloning of oncogenes and tumor suppressors and the development of transgenic mice led to novel mouse models of cancer that could precisely interrogate the role of the immune system in the growth of spontaneous tumors.
An early promising study came in 1983, which showed that transplanting mutagenized spontaneously-arising leukemia lines resulted in rejection of not only the mutant line, but also the parental line (Van Pel et al., 1983). These data hinted that a vigorous tumor immune response was possible if adequate priming of the immune response could be achieved to overcome immunosuppressive mechanisms. The first human tumor T cell antigen, MAGE-1, was described in 1991 by analyzing melanoma clones that could be lysed by autologous cytotoxic T lymphocytes (CTL), suggesting that immune recognition and clearance of tumors could occur in patients (van der Bruggen et al., 1991). A few years later, several groups contested Stutman’s results by showing that perforin-deficient mice, which have impaired cell-mediated cytotoxicity, have greater incidence and more aggressive growth of MCA-induced sarcomas (van den Broek et al., 1996; Smyth et al., 1999). In accompanying work, Robert Schreiber’s group showed that interferon gamma receptor (IFNγR)-deficient mice were more susceptible to not only MCA-induced sarcomas, but also to multiple tumor types when crossed to mice lacking the tumor suppressor p53 (Kaplan et al., 1998). These data provided critical evidence that immune surveillance may be relevant to the development of spontaneous tumors in addition to chemically or virally induced cancers. In a following study, they went on to show that mice deficient for recombinase activating gene 2 (RAG2), which lack T and B cells, also develop MCA sarcomas with greater frequency and rate (Shankaran et al., 2001). Remarkably, they found that transplantation of tumors from RAG2-deficient mice to syngeneic, wild-type hosts resulted in rejection of 40% of tumors, compared to 0% when transplanted tumors were from wild-type mice. These observations led them to conclude that immune pressure
selected for tumor cells that could better evade immune recognition, later coined "cancer immunoediting" (Dunn et al., 2004).

Further refinements to the re-invigorated model of cancer immunosurveillance were quickly made; Willimsky and Blankenstein generated a genetic mouse model of cancer dependent on the expression of SV40 T antigen (TAg) and found that robust anti-tumor adaptive immune responses to TAg persisted in tumor-bearing mice in spite of maintenance of antigen expression (Willimsky and Blankenstein, 2005). They therefore proposed that tumor escape occurs as a result of immune suppression rather than immunoediting in models of spontaneously-arising cancer. These studies have been corroborated by other groups, including our lab, which further demonstrated that tissue context may determine whether immunoediting or immune tolerance are dominant (DuPage et al., 2011, 2012). Willimsky and Blankenstein suggest that tumor immune tolerance may be mediated by host cell-derived transforming growth factor beta (TGFβ), which, along with interleukin 10, had recently been shown to impair dendritic cell (DC) function (Geissmann et al., 1999; Steinbrink et al., 1999). DC dysfunction had been an increasingly recognized barrier to tumor immune responses (Almand et al., 2000). In subsequent years the landscape of immunosuppressive elements in the tumor microenvironment would expand to include many cell types and pathways (Motz and Coukos, 2013), the most notable of which are immune checkpoint molecules (Pico de Coaña et al., 2015). Mechanisms of tumor immune suppression will be reviewed more extensively in the next section.

It is important to note that the experimental evidence for cancer immunosurveillance, immunoediting, and immune suppression provided a mechanistic
underpinning for ongoing work on cancer immunotherapy that had been continued by a small, but active, community of scientists and physicians. Indeed, the development of allogeneic bone marrow transplantation for the treatment of hematological malignancies led to a 1956 report of a graft-versus-leukemia (GVL) effect in a mouse transplantation model (Barnes et al., 1956). A decade later, Mathe and colleagues, who first used the term “adoptive immunotherapy” to describe allogeneic bone marrow grafts, administered donor leukocyte transfusions to a patient post-bone marrow transplant to stimulate an anti-leukemic effect (Mathe et al., 1965). Throughout the 1980s and 1990s, some held out hope that immune pathways could be harnessed to attack cancer, even though it was unclear whether endogenous immune responses could recognize and clear nascent tumors. For many years, histopathologists had noted that the presence of lymphocytes could be correlated with improved survival in a number of tumor types, while signs of chronic inflammation were associated with poor outcome (Coussens and Werb, 2002; Dvorak, 1986; Pagès et al., 2010). These observations motivated many efforts to produce vaccines or identify other immunostimulatory agents that could elicit effective immune responses against cancer. Interleukin 2 (IL-2) was heavily studied in late 1980s, leading to FDA approval for the treatment of metastatic renal carcinoma in 1992 and metastatic melanoma in 1998 (Lee and Margolin, 2011). High dose IL-2 achieved objective response rates of 15-20% in advanced melanoma, and durable complete responses in 5-7% of patients, but is quite difficult to tolerate, and thus reserved for the healthiest patients (Atkins et al., 2000). The only other cytokine therapy that received FDA approval was adjuvant therapy with interferon-alpha (IFNα) for
advanced melanoma, but it fails to have a consistently positive effect on overall survival, and 80% of patients suffer constitutional adverse effects (Lee and Margolin, 2011).

Although Coley’s toxin fell into disuse, oncologists continued to develop new therapeutic cancer vaccines. Bacillus Calmette-Guerin (BCG) vaccine was given to bladder cancer patients with promising effects as early as 1976 (Morales et al., 1976). Autologous tumor vaccines, which attempt to immunize patients against their own tumors, were first attempted in 1978 (Guo et al., 2013). In subsequent iterations, researchers enhanced the immunogenicity of these preparations by infecting tumor cells with a virus, or transducing them to express granulocyte-macrophage colony stimulating factor (GM-CSF). Allogeneic vaccines were also developed to overcome difficulties in producing autologous vaccines, but the efficacy of these strategies remained limited. The identification of tumor antigens and the ability to differentiate DCs ex vivo raised the possibility of using dendritic cell vaccines. Sipuleucel T, which consists of autologous DCs loaded ex vivo with a fusion protein of the cancer antigen prostatic acid phosphatase (PAP) and GM-CSF, was approved in 2010 for the treatment of hormone therapy-refractory prostate cancer, and was able to achieve a modest improvement in median survival (Burch et al., 2000; Guo et al., 2013).

Improvements in our ability to isolate and expand immune cell populations gave rise to adoptive cell therapy (ACT) approaches. In 1985, Steven Rosenberg and colleagues demonstrated that transfer of autologous, IL-2-stimulated peripheral blood lymphocytes (PBL) and high-dose IL-2 could achieve tumor regression in nearly half of a small group of patients with advanced cancer (Rosenberg et al., 1985). His group later demonstrated that a third of patients with advanced melanoma have objective
responses to tumor-infiltrating lymphocytes (TIL) and high dose IL-2 (Rosenberg et al., 1994). These treatments, however, were tailored for each patient and difficult to produce, and their effectiveness depended heavily on the source and functional status of individual TIL. The development of chimeric antigen receptors (CARs) that had enough signal strength to promote the persistence and expansion of T cells in the presence of antigen represented a major advance in this field, since this allowed redirection of PBL against a common tumor antigen (Lim and June, 2017). CAR T cells offer the promise of ACT without depending on the quality of the patient’s own T cell response, which may be blunted due to poor tumor immunogenicity and immune suppression.

Many of the cancer immunotherapies pursued in the 1980s and 1990s were aimed at augmenting the ongoing tumor immune response by sending stimulatory signals, or providing “backup” in the form of new cells. But these strategies often produced modest effect at high cost, highlighting the consequences of too much immune stimulation. Furthermore, as our appreciation of the immunosuppressive mechanisms restraining tumor immune responses grew, it became clear that these processes may need to be overcome to see better outcomes. The most influential breakthrough in cancer immunotherapy thus far has been achieved through the inhibition of immune checkpoints. The two-signal model of T cell activation first described in the 1970s established that T cell activation requires both antigen presentation and a second signal from a “costimulator” cell, later identified as cell-surface molecules expressed by antigen presenting cells (APCs) in the presence of danger signals (Bretscher and Cohn, 1970; Lafferty et al., 1978; Matzinger, 1994). This
model was refined by the discovery and characterization of cytotoxic T-lymphocyte associated protein 4 (CTLA-4) in the late 1980s, which provided critical insight that T cell-intrinsic inhibitory signaling restrains T cell activity and prevents autoimmunity by dampening T cell costimulation (Krummel and Allison, 1995; Tivol et al., 1996). Targeting these so-called “checkpoints” on TCR signaling thus became an active area of interest in cancer immunology research (Leach et al., 1996), culminating in the FDA approval of the anti-CTLA-4 antibody ipilimumab in March 2011 for the treatment of advanced melanoma. Remarkably, in long-term studies, even though only a modest proportion of patients respond to ipilimumab, overall survival reaches a plateau of ~20% at 3 years that, in some cases, extends over a decade. Similarly, anti-programmed death 1 (PD-1) therapy also appears to demonstrate a long survival curve “tail” in a greater and more diverse set of cancer patients. Checkpoint therapies thus offer the possibility of cancer cures in some patients, which has led to widespread and fervent interest in increasing the proportion of patients that respond to cancer immunotherapies.

In the same 1957 article in which he proposed that tumors may have neoantigens, Burnet concluded, “There is little ground for optimism about cancer,” reflecting his skepticism that much could be done to treat cancer once it had formed (Burnet, 1957). Among Burnet's doubts was a conviction that it would be difficult to find “some means whereby the protective mechanism of the body has its reactivity against minor deviations from self-patterns made more sensitive,” a sentiment deeply informed by the work of Medawar and colleagues on immune tolerance in mice. Fifty years later, improvements in our understanding of cancer and the immune system have allowed us to “break tumor tolerance” in some patients with astounding efficacy. The success of
checkpoint blockade and ACT have thrown a spotlight on experimental models of cancer immunology, to which many researchers increasingly look in order to contextualize and build upon these striking clinical observations (Dranoff, 2011). As the studies by Schreiber, Blankenstein, and many others have shown, this demand has been met with increasingly sophisticated models and genetic tools that offer new, exciting ways to dissect tumor immunity. Although the history of using mouse models to understand immune responses to cancer is long and varied, the current era of investigation is marked by the convergence of strong interest in understanding immune mechanisms in cancer and the availability of ever-improving research tools. Much remains to be done but hopefully the synchronized efforts of scientists and physicians to bring more patients benefit from immunotherapy warrants guarded optimism for the treatment of cancer in the years ahead.

Disruption of a productive tumor immune response by immunosuppressive mechanisms

The longitudinal growth of a transformed cell to a metastatic neoplasm is a remarkable feat of adaptation (Hanahan and Weinberg, 2011). Cells must acquire the cellular changes necessary to grow uncontrollably while also avoiding cell death, and they must support their growth by recruiting a protective stromal environment. Similarly, the interplay between a growing tumor and the immune system is a critical part of the natural history of cancer. A early, vigorous anti-tumor immune response may completely eliminate transformed cells, while tumor-intrinsic adaptations may promote immune cell dysfunction, thus providing an escape from immune pressure. Elimination and escape are often described as binary outcomes in the stepwise model of cancer immunosurveillance, while tumors in “equilibrium” are in an intermediate state where
cancer cells and immune cells are locked in an arms race, resulting in tumor dormancy until either elimination or tumor escape prevails (Dunn et al., 2004; Swann and Smyth, 2007).

The process of immune-mediated tumor elimination can be described as a cycle of events ("Cancer-Immunity Cycle") that repeat and reinforce each other to drive strong anti-tumor immunity (Chen and Mellman, 2013; Fearon, 2017) (Figure 1). This cascade of events is important to review as it represents what we hope to elicit with cancer immunotherapies, as well as the target of mechanisms of immune tolerance. First, tumor cells can be recognized by the immune system through a number of ways. Tumor antigens can come from a wide range of sources; protein-altering mutations can give rise to neoantigenic peptides, but many identified tumor antigens are aberrantly expressed germline proteins due to widespread DNA demethylation (Coulie et al., 2014). These antigens must be taken up and processed by APCs, which can occur as the result of tumor cell death. Dying tumor cells are also a potential source of danger associated molecular patterns (DAMPs) that can stimulate APCs and innate immune cells, thus facilitating expression of costimulatory molecules, secretion of additional inflammatory mediators, and the recruitment of more immune cells (Hernandez et al., 2016). For example, cancer cell-derived DNA can activate stimulator of interferon genes (STING) on CD11c+ DCs (Woo et al., 2014), leading them to produce type I interferon, which induces CXCL9/10/11 expression and is critical for tumor rejection (Diamond et al., 2011; Fuertes et al., 2011). CXCL10 further recruits CXCR3+ DCs (de Mingo Pulido et al., 2018), amplifying antigen uptake and presentation. Activated DCs traffic to lymphoid tissue where they prime T cell responses, resulting in the activation and
expansion of tumor-specific T cells. The phenotype and differentiation status of these T cells is dependent on the accompanying cytokine milieu, which varies with the characteristics of the innate immune response stimulated in the tumor environment. Differentiation and expansion of CXCR3+ type 1 helper (Th1) CD4+ T cells and CD8+ T cells, in particular, promote anti-tumor T cell activity. Perhaps in response to IFN-induced CXCL9 and CXCL10 (Mikucki et al., 2015), effector T cells then migrate to and infiltrate the tumor site, where they can recognize and kill tumor cells through degranulation of perforin/granzyme, expression of Fas ligand, and/or secretion of IFNγ and/or TNFα. Some effector T cells transition to long-lived memory cells, thus ensuring long-lived anti-tumor immunity. Antigen abundance and a favorable cytokine milieu (high IL-2 and IL-12) at priming improve the efficiency of memory generation (McKinstry et al., 2010). Tumor cell death releases additional antigen and immunostimulatory molecules, thus starting the cycle anew. There is evidence that tumoral expression of stress ligands, including NKG2D ligands, can also stimulate the activity of lymphoid populations such as NK cells, TCRαβ CD8+ T cells, and TCRγδ T cells (Smyth et al., 2005). Tumors and immunosuppressive cell populations can simultaneously inhibit multiple legs of the Cancer-Immunity Cycle (Figure 1), resulting in profoundly dysfunctional T cell responses and immune escape.

Tumor immune evasion is the most extensively-studied phase of cancer immunosurveillance because it is the phase in which we find most clinically detectable tumors. While it was once thought that the defeated immune system had no further role to play in the biology of “escaped” tumors, clinical observation and experimental evidence show that the immune system and tumor continue to influence each other
throughout tumor progression. Furthermore, individual tumors may demonstrate multiple, diverse mechanisms of immune evasion, resulting in tumor microenvironments that are as complex as they are deeply immunosuppressive. In this section, I will review major players that promote tumor immunosuppression with particular emphasis on how they impact the Cancer-Immunity Cycle.
Figure 1. Mechanisms of immune suppression in the tumor microenvironment disrupt the Cancer-Immunity Cycle.
Tumor cells, and many other cell types in the tumor microenvironment, can impair multiple stages of the development of anti-tumor immunity. Illustrated here are some direct effects cells have on the Cancer-Immunity Cycle, but in many cases immunosuppressive signaling can support the recruitment and activation of other tumor-tolerizing populations. Upregulated factors that promote tumor evasion are shown in red, and downregulated factors are shown in blue.

Adapted from (Chen and Mellman, 2013; Fearon, 2017).
Intrinsic and adaptive tumoral immune evasion

An earlier, immune system-centric view of tumor immune evasion focused heavily on immunoediting, the shedding of tumor antigens as a means of escaping immune pressure. Indeed, many tumors have been found to bear mutations or have epigenetically silenced MHC class I antigen presentation and processing machinery, or NK cell activating ligands (Chang et al., 2005a). Furthermore, some cases of relapsed tumors in patients treated with vaccines targeting one antigen have demonstrated loss of the immunizing antigen (Peterson et al., 2003). Recent data have shown that some patients who acquire resistance to anti-CTLA-4 and PD-1 therapy develop mutations in MHC I and β2M, thus reducing antigen presentation, while a few patients have JAK1/2 and other IFNγR pathway mutations, which may confer resistance to T-cell mediated cytotoxicity (Roh et al., 2017; Zaretsky et al., 2016). Other mechanisms used by tumor cells to avoid immune-mediated destruction have been described, including expression of FLIP and decoy receptors for TRAIL, or mutation of caspase 8, which all evade death receptor-mediated killing (Zitvogel et al., 2006). Tumoral expression of CD47 prevents macrophage-mediated phagocytosis of tumor cells, which contributes to limited antigen availability in the tumor microenvironment (McCracken et al., 2015). Tumors can also express inhibitory receptor ligands like PD-L1 and B7-H3 to further impair T cell activity (Motz and Coukos, 2013). As a means to prevent excess inflammation, IFNγ signaling also promotes tumoral expression of indoleamine 2,3-dioxygenase-1 (IDO1), which degrades tryptophan. Tryptophan is required for CD8+ T cell proliferation and CD4+ T cell survival, while the tryptophan metabolite kynurenine actually promotes Treg expansion (Platten et al., 2012). Recent reports have suggested that some tumors may
epigenetically silence their expression of chemokines that attract effector T cells (Peng et al., 2015), while other tumors express high levels of chemoattractants for Treg cells (Curiel et al., 2004; Facciabene et al., 2011; Ishida et al., 2006).

As a tumor continues to grow and invade, it can also adopt changes in gene expression that not only sustain its growth, but also have far-reaching effects the associated immune response. Interleukins 4 and 10 (IL-4 and IL-10), for example, are autocrine growth factors secreted by thyroid carcinomas that also polarize T cell responses toward a Treg or type 2 (Th2) response (Zitvogel et al., 2006). To promote angiogenesis, tumors also often express vascular endothelial growth factor (VEGF). VEGF promotes the immunosuppressive activity of endothelial cells and myeloid suppressor cell populations, and actively contributes to APC dysfunction (Gabrilovich et al., 1996; Motz and Coukos, 2013). Many malignant lesions also express cyclooxygenase-2 (COX2), which causes local overproduction of pro-inflammatory prostaglandin E2 (PGE2). In addition to inducing further VEGF expression, PGE2 can strongly influence T cell responses by inducing the myeloid cells to express IDO1. PGE2 can also promote the expression of IL-10 while suppressing the expression of interleukin 12 (IL-12), which drives Th1 T cell responses, and various chemokines (Motz and Coukos, 2013; Nakanishi and Rosenberg, 2013). Cancer cells can also secrete matrix metalloproteinases, which aid in invasion, but also cleave CD25 and the leukocyte adhesion molecules ICAM-1/2, as well as activate transforming growth factor beta (TGFβ) (Stefanidakis and Koivunen, 2006). Clearly, by adopting pathways associated with wound healing, tumors simultaneously promote a less inflammatory environment that is more conducive to tissue repair and regeneration.
There is also increasing evidence that oncogenic signaling may endow certain tumor genotypes with intrinsic immune evasion mechanisms. The best described cancer signaling pathway involved in immune modulation is Wnt/β-catenin signaling, which has been shown to downregulate CCL4 expression, leading to less recruitment of Batf3+ DCs, and preventing T cell accumulation in tumors (Spranger et al., 2015). The ability to attract these DCs is critical, as vaccination and ACT were ineffective in stimulating a productive anti-tumor T cell response as long as β-catenin signaling remained active (Spranger et al., 2017). These findings have been corroborated by a number of studies in different cancer types (Spranger and Gajewski, 2018), which collectively suggest that Wnt pathway activation should be targeted in tandem with immunotherapies to elicit effective tumor immunity. Similarly, PTEN loss in melanoma is also associated with poor immune infiltration of tumors, and PI3K inhibition has been shown to improve responses to checkpoint blockade (Peng et al., 2016). Other oncogenic signaling pathways appear to promote an inflammatory environment conducive to wound repair, and thus tumor growth. For example, N-ras and Braf-mutant tumors have been shown to produce high levels of PGE2 (Zelenay et al., 2015). Meanwhile, Wong and colleagues recently found that Lkb1-deficient lung cancers recruit more neutrophils, likely due to increased expression of CSF1 and the chemokines CXCL3/5/7. Interestingly, these tumors expressed lower levels of PD-L1 than mice with wild-type Lkb1, and were not susceptible to checkpoint blockade, but did see survival benefit with anti-IL-6 treatment (Koyama et al., 2016). Collectively, tumor-intrinsic signaling can disrupt the Cancer-Immunity Cycle chiefly by preventing the recruitment and activation of APCs and impairing the function of T cells that do manage to arrive in the tumor microenvironment.
Endothelial cells

Entry of T cells into the tumor microenvironment requires adhesion to endothelial cells, followed by extravasation through the vascular wall. Endothelins and VEGF coordinate neovascularization and endothelial cell proliferation. VEGF can prevent adhesion molecule expression on endothelial cells (Bouzin et al., 2007), thus impairing leukocyte tissue infiltration. Similarly, stimulation of the endothelin receptor type B (ETRB) by various endothelins can result in reduced T cell adhesion through suppression of ICAM-1 (Buckanovich et al., 2008). Nitric oxide (NO) produced by myeloid cells can also directly act on endothelial cells to prevent expression of P-selectin, ICAM-1, and VCAM-1 (De Caterina et al., 1995). Interestingly, endothelial cells can express a variety of molecules that promote T cell apoptosis, including FasL and TRAIL, as well as inhibitory receptors like PD-L1/L2, B7-H3, and TIM3 (Motz and Coukos, 2013; Motz et al., 2014). Cancer-associated vascular changes thus impair T cell migration into the tumor bed.

Fibroblasts

A population of activated fibroblasts expressing the fibroblast activation protein (FAP) was first described by Garin-Chesa et al. in 1990, who found that FAP+ fibroblasts could be found in all human adenocarcinomas (Garin-Chesa et al., 1990). In subsequent studies, FAP+ fibroblasts were found to express α-smooth muscle actin (α-SMA) protein, consistent with a myofibroblast phenotype. FAP+ cancer-associated fibroblasts (CAFs) express CXCL12, which prevents intratumoral accumulation of T cells (Feig et al., 2013). Depletion of FAP+ cells by diphtheria toxin (DT), or T cells expressing a CAR recognizing FAP, enhanced T cell infiltration in a genetic mouse
model of pancreatic ductal adenocarcinoma (Feig et al., 2013; Wang et al., 2014). Inhibition of CXCR4, the receptor for CXCL12, recapitulated the effect of FAP+ fibroblast depletion (Feig et al., 2013), suggesting that the primary contribution of fibroblasts to immune suppression lies in their ability to prevent effector T cell homing to tumors.

Tumor-associated macrophages

Macrophages may demonstrate tumoricidal properties in some contexts, but reports suggest that in many tumors they play a pro-tumoral role starting at cancer initiation (Noy and Pollard, 2014). Tumor-associated macrophages (TAMs) arise from both yolk sac-derived tissue macrophage populations and circulating bone marrow (BM)-derived precursors. TAMs can express classical and non-classical HLA molecules, which not only impair NK cell activity, but also promote a suppressor phenotype for T cells. Hypoxic conditions also induce TAMs to express inhibitory ligands like PD-L1 (Noman et al., 2014). TAMs recruit thymus-derived Tregs through their expression of various chemokine ligands (Curiel et al., 2004; Liu et al., 2011), and induce Treg differentiation through their secretion of TGFβ and IL-10. Expression of both arginase I (ARG) and nitric oxide synthase (iNOS) allows TAMs to deplete extracellular L-arginine (Biswas and Mantovani, 2010), which is essential for replenishing CD3ζ chain expression after internalization following TCR signaling. As a result, arginine-starved T cells proliferate less and are less functional (Rodriguez et al., 2003). TAMs can thus inhibit effector T cell function and promote an immunosuppressive environment through direct interactions and secreted factors.
Myeloid-derived suppressor cells (MDSCs)

In addition to TAMs, another group of myeloid cells deeply implicated in promoting tumor immune evasion are the myeloid-derived suppressor cells (MDSCs). This population was first described in late 1980s (Young et al., 1990) as a non-T, non-macrophage suppressive population that was induced by tumorigenesis. Similar cells were later identified in patient tumors (Almand et al., 2001). MDSCs arise in bone marrow from myeloid progenitors, where they differentiate to varying degrees of maturity, and then home to tumors through the bloodstream. Two main phenotypes of MDSCs have been described: monocytic MDSC (M-MDSC; CD11b+Ly6C+Ly6Glow) and granulocytic MDSC (G-MDSC; CD11b+Ly6c-Ly6G+). They share many markers with their non-suppressive myeloid cell counterparts (e.g. monocytes and polymorphonuclear granulocytes), and are thus defined by their suppressor activity (Ostrand-Rosenberg, 2016). MDSC recruitment and activity can occur as a result of pro-inflammatory signaling through IL-1b, IL-6, and PGE2, as well as by the release of HMGB1, an alarmin, from dying cells. TAMs and MDSCs have many overlapping functions, which include the expression ligands for immune checkpoints and depletion of extracellular L-arginine to impair T cell function. Their expression of iNOS, ARG, and NADPH oxidase (NOX) result in the generation of reactive oxygen and nitrogen species (ROS and RNS), which can directly induce T cell apoptosis, disrupt interleukin 2 receptor (IL-2R) signaling, alter peptide-MHC and TCR interactions, and chemically modify chemokines to recruit more immunosuppressive immune populations (Ugel et al., 2015). Similar to TAMs, MDSCs mostly impair the Cancer-Immunity Cycle by inhibiting the function of effector T cells once they have arrived. Indeed, depletion of
MDSCs with either CCR2 or CSF1R antibody results in increased accumulation of CD8+ T cells (Lesokhin et al., 2012; Strachan et al., 2013).

Antigen presenting cells/ dendritic cells

Mature DCs are able to effectively prime strong T cell responses because they present antigen along with costimulatory molecules, thus providing signal 1 and 2. Intratumoral CD103+ DCs have been shown to be particularly important for tumor rejection due to their ability to take up tumor antigen, migrate to draining lymph nodes via expression of CCR7, and prime strong anti-tumor T cell responses (Broz et al., 2014; Roberts et al., 2016; Salmon et al., 2016). Interestingly, CD11b+ migratory DCs are phenotypically more similar to TAMs and less able to deliver antigen to lymph nodes (Broz et al., 2014). Administration of FLT3 ligand can expand and activate CD103+ DCs, while GM-CSF expands CD11b+ DCs, highlighting the role of the cytokine milieu in determining DC phenotypes in the tumor microenvironment.

Furthermore, incompletely differentiated, “immature” DCs provide insufficient costimulation and/or inhibitory receptor signaling, resulting in a tolerogenic phenotype that promotes T cell anergy and induces Tregs (Lutz and Schuler, 2002; Mahnke et al., 2002). Chronic stimulation of naive CD4+ T cells may result in peripheral Treg differentiation. Indeed, FLT3 ligand has been shown to expand an immature CD103+ DC population that is associated with increased Treg infiltration of tumors unless a stimulus for DC maturation, such as the TLR3 agonist poly:C, is also co-administered (Salmon et al., 2016). Different DC subsets can also stimulate the differentiation of Th2 CD4+ T cells, which skews the immune response from Th1 cells, and begins a positive feedback loop wherein Th2 CD4+ T cells produce IL-10, which drives further APC
dysfunction (Mahnke et al., 2002). Immature and/or particular subsets of DCs thus disrupt the Cancer-Immunity Cycle by impairing T cell priming and activation and polarizing effector T cell function towards a more anti-inflammatory phenotype.

**T cell-intrinsic dysfunction**

Repeated antigen stimulation, like that seen in chronic viral infection, pushes CD8+ T cells towards an exhausted phenotype. Exhausted CD8+ T cells progressively become less functional; they proliferate less, and make fewer inflammatory cytokines (Wherry, 2011). During this process they also upregulate a number of inhibitory receptors, including CTLA-4, PD-1, lymphocyte activation gene 3 (LAG3), CD160, 2B4, T cell immunoglobulin- and mucin domain-containing molecule 3 (TIM-3), and B- and T-lymphocyte attenuator (BTLA). Signaling through these receptors appears to synergistically strengthen and reinforce the exhaustion phenotype through nonredundant pathways (Blackburn et al., 2009), and ligand availability may “tune” the negative signals received by T cells. For example, PD-1, which inhibits TCR signaling and T cell motility (Keir et al., 2008; Zinselmeyer et al., 2013), is thought to regulate T cell function primarily in tissues, where PD-L1 and PD-L2 are expressed. T cell exhaustion driven by inhibitory receptor signaling prevents progression of the Cancer-Immunity Cycle by severely limiting the function of effector T cells in the tumor microenvironment.

**CD4+ T cells**

CD4+ T cells regulate adaptive immunity by coordinating the functions of many other immune cells, and thus play a critical role in the pathogenesis of many diseases
Due to their considerable heterogeneity and potential plasticity, CD4+ T cell subsets have been shown to have varying, often conflicting effects on tumor immunity (Dobrzanski, 2013). Populations of CD4+ T cells that are likely to contribute to immune evasion, at least in some contexts, include type 1 regulatory (Tr1) cells, Treg cells, and T helper type 17 (Th17) cells. Th17 CD4+ T cells have been shown to directly promote tumor growth by inducing the expression of angiogenic factors, like VEGF, which have strong inhibitory effects on anti-tumor T cell responses (Numasaki et al., 2003). Th17 cells exposed to TGFβ also express the ectonucleotidases CD39 and CD73, which convert extracellular ATP to adenosine (Chalmin et al., 2012). Adenosine signaling impairs T cell infiltration, proliferation, and cytotoxicity (Vijayan et al., 2017).

Meanwhile, Treg and Tr1 cells are both highly immunosuppressive CD4+ T cell populations that have tissue-protective functions that aid tumor development (Adeegbe and Nishikawa, 2013). Treg cells are characterized by their expression of the transcriptional regulator Foxp3, and can inhibit tumor immune responses through the secretion of interleukin 35 (IL-35), IL-10, and TGFβ, direct killing of effector T and NK cells (Fehniger et al., 2007; Gondek et al., 2005; Ren et al., 2007), and generation of adenosine (Mandapathil et al., 2010). In other contexts, Treg cells have also been shown to compete with other T cells for metabolic and antigenic substrates, such as IL-2, and impair APC maturation (Caridade et al., 2013; Pandiyan et al., 2007; Vignali et al., 2008). Although Tr1 cells are less well-studied than their Treg cell counterparts, they have been identified in a number of human cancers (Adeegbe and Nishikawa, 2013) and are thought to exert their immunosuppressive effects primarily through their production of IL-10 and TGFβ (Grazia Roncarolo et al., 2006). CD4+ T cells thus not
only impair the function of effector T cells in tissue, but also disrupt priming of T cell responses to tumors. CD4+ T cell heterogeneity and function will be discussed further in a later section.

**Modeling tumor immunosuppression in mice**

A large array of interacting cells contribute to immune suppression in the tumor microenvironment, and their activities are likely to vary with tissue context and tumor-intrinsic characteristics. Mouse models therefore provide a whole-organism setting in which to study the complex network of cellular interactions and signaling pathways that restrain anti-tumor T cell responses. Transplantable tumor models using syngeneic cancer cell lines and immunocompetent mice have been a workhorse for tumor immunology, providing many insights on tumor antigens and the mechanisms of tumor immune recognition and evasion (Dranoff, 2011). The speed and reproducibility of these models also allow for rapid testing of therapeutics, and traditional and immune cancer therapies alike have been tested in these models (Kersten et al., 2017). However, transplantable tumors may be limited in their ability to capture faithfully the strength and diversity of tumor immunosuppressive mechanisms. A number of cancer cell lines were generated through chemical carcinogen-induced mutagenesis, resulting in highly immunogenic lines that may provoke stronger immune responses than most human cancers. The acquisition of additional mutations or epigenetic changes during *in vitro* passaging may further contribute to their divergence from patient tumors, not only in their immunostimulatory potential, but also in their genetic and phenotypic characteristics. Implantation of cells is also associated with considerable cell death, and the local inflammatory response may provide a boost to early anti-tumor immune
responses while blunting the activation of immunosuppressive pathways. Transplantable tumors are also often implanted in ectopic sites (e.g. subcutaneously) where the resulting immune response may look strikingly different from an autochthonous tumor, which must grow within and adapt to a tissue-specific cytokine milieu and immune infiltrate (DuPage and Jacks, 2013). Since these cell lines are highly proliferative and transplanted in relatively large numbers, they rapidly develop large tumors, and may thus fail to capture the longitudinal progression of tumor evasion mechanisms, which likely occur over the course of weeks to months, to even years, in patients (Dranoff, 2011; DuPage and Jacks, 2013; Wen et al., 2012). Notably, recent studies have demonstrated that transplantable cell lines derived from autochthonous tumors generate stronger and more effective anti-tumor immune responses compared to the parental tumors (DuPage et al., 2011; Garbe et al., 2006). Carcinogen-induced models are an option, but the long latency of tumor development and high degree of genetic variability are major drawbacks (Matsushita et al., 2012).

On the other hand, genetically-engineered mouse (GEM) models may be more appropriate models of immune evasion. Conditional transgenic models of cancer can enforce the expression of oncogenes and/or the ablation of tumor suppressor genes in a tissue-specific and/or inducible manner. Tumors that arise from these models often recapitulate the morphology and developmental stages of human cancers (DuPage et al., 2009; Frese and Tuveson, 2007). These models thus enable study of tumor immune responses in a setting where tumor genotype and progression are controlled, making them powerful tools for understanding the longitudinal development of immunosuppressive mechanisms in particular cancer contexts. Studies using these
models have highlighted the complexity of tumor tolerance, as well as the importance of the tissue environment in influencing immune responses. For example, tumors arising in the lung were found to induce strong immune tolerance, while sarcomas driven by the same genetic drivers and expressing the same antigens provoked a highly effective anti-tumor T cell response that forced immunoediting (DuPage et al., 2012). Oncogenic drivers also have an impact on the tumor immune milieu (Spranger and Gajewski, 2018; Wellenstein and de Visser, 2018). This is exemplified in a recent report showing that mouse lung adenocarcinomas (LUAD) driven by mutant epidermal growth factor receptor (EGFR) recruited far fewer T cells than LUAD driven by oncogenic K-ras and loss of p53 (Busch et al., 2016). A major limitation of GEM models is that they do not have many additional somatic mutations, resulting in less immunogenicity. GEM models can be engineered such that tumors express strong T cell antigens, however, thus boosting the tumor immune response and allowing study of tumor-specific T cell responses (Cheung et al., 2008; DuPage et al., 2011, 2012; Schietinger et al., 2012; Spranger et al., 2015; Willimsky and Blankenstein, 2005; Zhang et al., 2012). Less immunogenic GEM models may also provide good models for human cancers with lower mutation burdens.

II. CD4+ T cells in cancer

Historical overview of CD4+ T cells

The history of cellular immunology and cancer biology are inextricably linked. The first hint that lymphocytes were mediators of immune responses came from work on tumor and transplantation biology by James Murphy, working in the laboratory of
Peyton Rous. He joined the group shortly after Rous had reported that sarcomas in chicken could be transmissible by a cell-free agent (later discovered to be an oncogenic virus), and developed a method of engrafting tumor and healthy tissue in chick embryos. He observed that transplantation of a piece of lymph node or spleen from an adult animal into the embryo would lead to destruction of the grafted tumor or tissue, and that the rejected graft was associated with a dense bed of lymphocytes (Murphy, 1914). Similarly, he found that he could grow xenografts in the brains of rats—foreshadowing the identification of the brain as an immune-privileged site—that would also be rejected upon implantation of lymph node or spleen tissue into adjacent brain (Murphy and Sturm, 1923). Murphy concluded from his work that the lymphocyte was a primary driver of resistance to tumor, tissue, and infectious growth. But his discoveries were largely ignored by the prevailing immunologists of his day, who were heavily focused on defining the chemical basis of humoral immunity, and were not following developments in tumor research (Silverstein, 2001b).

It was not until the 1964 that James Gowans would establish that small lymphocytes continuously re-circulate from the thoracic duct, into which all lymphatics drain, to blood, to secondary lymphoid tissue, and back again through the duct (Gowans and Knight, 1964). This discovery offered critical insight into their function, as Gowans subsequently determined that lymphocytes were responsible for graft rejection and immunological memory (Gowans and Uhr, 1966; Howard and J. L. Gowans, 1972). Through his study of thymectomized animals, Jacques Miller proposed that bone marrow-derived antibody-producing lymphocytes (B cells) were distinct from thymus-derived “helper” lymphocytes (T cells), so-called because of the observation that
thymus-derived cells appeared to boost the activity of antibody-producing cells (Miller, 1999; Miller and Mitchell, 1968; Mitchell and Miller, 1968). By the early 1970s, several studies had indicated that T cells were responsible for anti-allogeneic cytotoxicity, and that they expressed clonal receptors that conferred specificity to their activity (Masopust et al., 2007). In 1975, the T cell population would experience its first subdivision, as it was found that treatment with CD8α and CD8β antiserum abrogated the cytotoxicity of T lymphocytes (Cantor and Boyse, 1975; Kisielow et al., 1975; Shiku et al., 1975).

It soon became clear that CD4+ T helper cells were functionally heterogeneous. Different subsets of CD4+ T cells were observed to provide antigen-restricted or unrestricted help to B cells, sometimes in an interleukin 4 (IL-4)-dependent fashion (Janeway, 1975; Killar et al., 1987; Kim et al., 1985; Marrack and Kappler, 1975; Tada et al., 1978). Meanwhile a separate population of helper T cells appeared to be responsible for delayed type hypersensitivity (DTH), which was inversely correlated with the antibody responses to the same antigen, suggesting that the cells responsible for each process are mutually suppressive (Liew and Parish, 1974). In the 1980s, the ability to clone T cells and assay for cytokines greatly enhanced the resolution of CD4+ T cell studies (Liew, 2002). Tim Mossmann and Robert Coffman provided a mechanistic basis for earlier observations of CD4+ T cell heterogeneity in 1986 when they generated two panels of T helper cell clones that could be distinguished by their cytokine expression. Both subsets could provide B cell help, but IL-4-producing Th2 cells strongly induced the production of IgG1 and IgE by mouse spleen cells, while IFNγ-producing Th1 cells preferentially stimulated IgG2A production (Coffman and Carty, 1986; Mosmann et al., 1986; Stevens et al., 1988). Furthermore, Th1 cells were shown to be primarily
responsible for DTH reactions (Cher and Mosmann, 1987). The observation that IFN\(\gamma\) and IL-4 reciprocally regulated Ig isotype production led to the hypothesis that Th1 and Th2 cells suppressed the other’s growth (Snapper and Paul, 1987). This model was confirmed in subsequent studies showing that IFN\(\gamma\) and IL-4 act as autocrine growth factors for their associated helper cell subsets, but inhibit the proliferation of the other subset (Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988). It has since been shown that differentiation into Th1 vs. Th2 cells is dependent on cytokine milieu, antigen dose and affinity, MHC haplotype, and co-stimulation by APCs (Liew, 2002). Moreover, the discovery that the development of Th1 and Th2 cells is mediated by the transcriptional regulators T-bet (Szabo et al., 2000) and Gata3 (Zhang et al., 1997; Zheng and Flavell, 1997), respectively, suggested that T helper cell lineage commitment is driven by the adoption of specific transcriptional programs.

A brief review of our current understanding of CD4+ T cell subsets

Based on our current understanding, CD4+ T cells can be divided into seven distinct subsets. For a recently activated naive CD4+ T cell in the periphery, the local cytokine milieu at the time of priming appears to determine its lineage specification and effector functions (Figure 2). As a result of this functional diversity, CD4+ T cells are able to support many branches of adaptive and innate immunity, as well as tailor and tune the immune response to suit different threats. In this section, I will provide an overview of the differentiation and function of each subset, followed by the role they may play in cancer immune evasion. Treg cells and other immunosuppressive CD4+ T cell subsets will be discussed in a separate section.
Differentiation of Th1 cells is driven by interleukin-12 (IL-12), which activates STAT4 and T-bet transcriptional activity (Szabo et al., 2000, 2003). T-bet is the major transcriptional regulator of Th1 lineage commitment, as it upregulates IFNγ, the signature Th1 cytokine, and the IL-12 receptor b2 (IL-12b2R) subunit, which confers responsiveness to IL-12 signaling, thus sustaining T-bet expression. Production of IFNγ by Th1 cells may be reinforced or amplified by eomesodermin (Eomes), which is also upregulated during Th1 differentiation and has been shown to coordinate with T-bet to produce IFNγ and promote effector activity in CD8 and CD4+ T cells (Intlekofer et al., 2008). Other effector cytokines produced by Th1 cells include IL-2, TNFα, and IL-10.

Th1 responses promote the activity of macrophages and CD8+ T cells, and are thus particularly important for controlling infection by intracellular pathogens (Mosmann and Coffman, 1989). It is perhaps unsurprising, then, that Th1 cells are the CD4+ T cell subset most strongly associated with anti-tumor immune responses. Th1 effector cell infiltration is associated with better survival (Fridman et al., 2011). Th1 cell-derived IFNγ can activate dendritic cells, enhancing the priming and maturation of CD8+ T cells (Corthay et al., 2005; Quezada et al., 2010). CD8+ T cell memory formation is aided by Th1 cell-derived IL-2. IFNγ further promotes Th1 cell differentiation since it can shift macrophages to the M1 phenotype, which is characterized by greater microbicidal activity and IL-12 production. CXCL9, CXCL10, and CXCL11 are type I and II interferon-inducible cytokines that can attract other T cells to tumors, while Th1 cell expression of CCL3 and CCL4 can also attract CCR5+ Th1 cells and naive CD8+ T cells (Laidlaw et al., 2016; Loetscher et al., 1998). Upregulation of antigen processing and presentation
machinery by IFNγ also results in better recognition and killing of tumor cells by T cells. IFNγ can also have a direct inhibitory effect on cancer cell proliferation and angiogenesis, and/or induce tumor cell death directly through expression of caspases, Fas, and TRAIL (Dobrzanski, 2013). Recent studies have suggested that IFNγ can also inhibit the generation and activity of Treg cells (Caretto et al., 2010; Nishikawa et al., 2005; Overacre-Delgoffe et al., 2017a).

Th1 cells have also been associated with cytolytic CD4+ T cells (CD4+ CTL), which have been described to kill both virally-infected and tumor cells through secretion of IFNγ and TNFα, degranulation of cytotoxic granules, or expression of FasL (Pardoll and Topalian, 1998; Trapani and Smyth, 2002). CD4+ CTLs rely on the expression of Eomes, developmentally linking them to the Th1 lineage, and also express genes associated with the Th1 phenotype, including IFNγ. Indeed, transferred tumor-reactive CD4+ T cells that have cytotoxic properties demonstrate features of Th1 differentiation (Quezada et al., 2010; Xie et al., 2010). However, recent reports suggest that CD4+ CTL may arise from other T helper subsets, suggesting that CD4+ CTL development is independent from Th1 polarization (Takeuchi and Saito, 2017). Class I-restricted T cell-associated molecule (CRTAM) has recently been described as a marker of CD4+ CTL that may facilitate further characterization of this population (Takeuchi et al., 2016).

A number of reports, however, suggest that Th1-associated cytokines may also have immunosuppressive effects. IFNγ can drive T-bet expression in Tregs, resulting in a Treg population that is specialized in suppressing Th1 responses (Koch et al., 2009, 2012; Levine et al., 2017). Furthermore, a number of IFNγ-inducible genes promote tumor immune tolerance, including PD-L1 and IDO1 (Dobrzanski, 2013). While a few
studies have shown that IL-10 may inhibit tumor growth (Emmerich et al., 2012; Mocellin et al., 2005; Tanikawa et al., 2012), IL-10 is best described as a mediator of tumor immune evasion due to its widespread immunosuppressive effects. IL-10 impairs APC function by lowering their expression of MHC and costimulatory molecules. Effector T cell functions can also be disrupted by IL-10 due to its negative effect on IL-12 production and upregulation of checkpoint molecules (Moore et al., 2001). Dual expression of IFNγ and IL-10 may represent an auto-regulatory mechanism to finely tune the activity of Th1 cells, as cells may express variable levels of either cytokine in a context-dependent fashion (Cope et al., 2011; Jankovic et al., 2010). This dynamic balance of signals may explain the observed pleiotropic effects of IL-10, and consequently Th1-like cells, on tumor immunity.

**Th2**

IL-4 drives the differentiation of Th2 cells by activating STAT6 signaling, drives the expression of GATA-3, the major transcriptional regulator of the Th2 response (Zhang et al., 1997; Zheng and Flavell, 1997), although it is also involved in the development of CD4+ T cells in general (Ho et al., 2009). GATA-3 is critical for suppressing Th1 differentiation and for the expression of other Th2 effector cytokines, including IL-5 and IL-13 (Zhu et al., 2004, 2006). IL-4 regulates isotype switching to IgE in B cells, which mediates allergic immune responses. IL-5 and IL-13 recruit eosinophils and promote hypersensitivity reactions that help expel helminths, respectively (Zhu and Paul, 2008). Th2 cells can also express IL-10 and IL-24, an IL-10 family member (Ouyang et al., 2011). Collectively, Th2 cells protect against helminth infections, induce and maintain allergic inflammation and asthma, and promote tissue repair.
Th2 cells have a controversial role in tumor immunity. Their induction of the expression of eotaxin, an eosinophil chemoattractant, was found to be critical for the clearance of melanoma metastases in the lung (Mattes et al., 2003). IL-24 has also been shown to suppress tumor cell growth and induce tumor cell apoptosis (Dash et al., 2010; Emdad et al., 2009). Conversely, Th2 responses have been associated with tumor development in melanoma and pancreatic cancer (Ochi et al., 2012; Tatsumi et al., 2002), suggesting that the effect of Th2 responses on tumors may also be context-specific.

**Th17**

Th17 cells are characterized by their expression of IL-17, which is driven predominantly by the major Th17 transcriptional regulator, RORγT (Ivanov et al., 2006). TGFβ and IL-6 promote differentiation of Th17 cells in mice through STAT3-dependent induction of RORγT, IL-17A, and IL-21 (Murugaiyan and Saha, 2009). Since IL-21 can also activate STAT3 signaling, IL-21 amplifies and stabilizes Th17 differentiation. Th17 cells also upregulate the related transcription factor RORα, which appears to be responsible for residual IL-17 production in RORγT-deficient mice (Yang et al., 2008). RORγT also promotes the expression of IL-17F, IL-26, and CCR6, while downregulating IFNγ expression. IL-17A can induce the expression of many pro-inflammatory cytokines, and both IL-17A and IL-17F recruit neutrophils, which are critical to the defense against extracellular bacteria and fungi (Zhu and Paul, 2008).

Th17 cells have been implicated in promoting carcinogenesis due to their role in coordinating chronic inflammation (Wang et al., 2009; Wu et al., 2009). Studies using GEM models have also suggested that IL-17 may have a direct effect on tumor growth by inducing angiogenesis (Dobrzanski, 2013; Numasaki et al., 2003). IL-17 appears to
promote tumor vascularization by inducing tumoral and stromal expression of VEGF, as well as the pro-angiogenic chemokines CXCL1, CXCL5, CXCL6, and CXCL8 (Numasaki et al., 2004, 2005). IL-17-induced expression of IL-6 can also activate STAT3 signaling on tumor cells, which promotes survival and angiogenesis (Wang et al., 2009).

The data implicating IL-17 in tumor initiation and growth stand in contrast to many studies of patient samples that have shown a correlation between the presence of Th17 cells and better clinical outcomes (Dobrzanski, 2013). It is possible that other effector molecules produced by Th17 cells are responsible for their anti-tumor effects; IL-21 can enhance CD8+ T cell responses (Frederiksen et al., 2008; Moroz et al., 2004; Zeng et al., 2005), while CCL20 can recruit CCR6+ DC populations. IL-17 may also directly promote DC maturation (Antonysamy et al., 1999) and IL-12 production by macrophages (Jovanovic et al., 1998).

The identification of IL-17+IFNγ+ CD4+ T cells in human and mouse Th17 populations may provide a mechanistic basis for understanding the conflicting roles Th17 cells appear to play in cancer (Annunziato et al., 2007; Huber et al., 2011). These cells are thought to arise due to Th17 cell plasticity, which appears to be due to expression of IL-12Rb2 on Th17 cells, enabling IL-12-induced downregulation of IL-17 and induction of IFNγ (Annunziato et al., 2007; Hirota et al., 2011). Adoption of a Th1 phenotype by some Th17 cells is likely to occur as a result of a particular cytokine and immune cell milieu, and contribute to changes in the balance of IL-17 and IFNγ-mediated effects on tumor immunity. The close relationship between Th1 and Th17 cells may thus explain the variable effect of Th17 responses on tumor immunity in different cancer types.
**Th22**

Th22 cells are a distinct CD4+ T cell subset that produces IL-22 independently of IL-17. First identified in patients with inflammatory disorders of the skin and other barrier tissues (Eyerich et al., 2009; Sonnenberg et al., 2011), they arise in many of the same conditions where Th17 cells are observed, and their development may be regulated by the aryl hydrocarbon receptor (Ahr) and RORγT (Eyerich and Eyerich, 2015; Plank et al., 2017). Th22 cells have been associated with inflammation in a number of cancers, and may promote the recruitment of immune cells to tumor sites (Dobrzanski, 2013). Recent data also suggest that IL-22 can directly activate STAT3 signaling on colon cancer cells and promote cancer cell “stemness” and proliferation (Kryczek et al., 2014; Sun et al., 2016).

**Th9**

The production of IL-9 was originally attributed to Th2 cells, but was later found to be associated with Th9 cells, which differentiate from naive CD4+ T cells in response to TGFβ and IL-4 (Dardalhon et al., 2008; Veldhoen et al., 2008). IL-4 drives STAT6 signaling, while TGFβ activates PU.1, resulting in expression of IRF4 and IL-9, respectively. Interestingly, Th2 cells can adopt the Th9 phenotype when exposed to TGFβ, which downregulates GATA-3, IL-4, and IL-5, while Th1 and Th17-associated cytokines appear to suppress Th9 polarization (Végran et al., 2015). Similar to Th2 cells, Th9 cells have been associated with allergic inflammation and airway hypersensitivity, as well as resistance to helminth infections.

Recent studies have suggested that Th9 cells have potent anti-tumor activity. Blockade of IL-9 in RORγT-deficient mice enhanced melanoma growth, while adoptively
transferred, tumor-specific Th9 cells had better anti-tumor activity than Th1 and Th17 cells (Lu et al., 2012; Purwar et al., 2012). Interestingly, Th9 cells may be able to suppress tumor growth through both contact-dependent and independent mechanisms. Th9-mediated antagonism of melanoma growth was peptide-specific and dependent on granzyme B expression (Purwar et al., 2012), while IL-9 may induce tumor cells to express CCL20, thus recruiting CCR6+ DCs and CD8+ T cells (Lu et al., 2012). Notably, IL-9 can also be secreted by Th1, Th17, and Treg cells (Végran et al., 2015), and IL-9 signaling has been shown to directly induce further production of IL-9 in these cells (Elyaman et al., 2009), suggesting that IL-9 and Th9 cells may have independent effects on tumor development in different contexts.

Tfh

Follicular helper T cells (Tfh) reside in lymphoid tissues, provide B cell help, and support B cell proliferation (Ma et al., 2012; Reinhardt et al., 2009). In mice, their differentiation appears to be driven by the coordinated action of IL-6, ICOS, IL-2, and TCR signaling (Crotty, 2014). They are CXCR5+, which promotes their migration to the B cell follicle, and they express the transcriptional regulator Bcl6 and secrete IL-21. The role of Tfh cells in tumor immunity is unclear, but recent reports suggest that while lymph node-resident Tfh may suppress anti-tumor immunity through their production of IL-4 (Shirota et al., 2017), circulating tissue Tfh may promote local B cell responses in the tumor microenvironment, perhaps stimulating humoral immunity (Gu-Trantien et al., 2013).
Regulatory T helper cells

A number of immunosuppressive CD4 T helper populations have been identified. The best described are Foxp3+ Treg cells, which will be discussed in the next section. Other suppressive cell populations can be induced from naive CD4+ T cells and include IL-10-producing T regulatory 1 (Tr1) cells (Groux et al., 1997; O'Garra et al., 2004), TGFβ-producing T helper 3 (Th3) cells (Carrier et al., 2007; Chen et al., 1994), and IL-35-producing iTreg35 cells (Collison et al., 2010). Notably, IL-10, TGFβ, and IL-35 expression are all also characteristics of Foxp3+ Treg cells, and the lack of distinguishing markers for these regulatory CD4+ T cell populations has limited our understanding of their role in the pathogenesis of cancer and other diseases (Olson et al., 2013; White and Wraith, 2016). Tr1 cells have been identified in human tumors, however, and are thought to arise due to priming of naive CD4+ T cells by immature DCs (Adeegbe and Nishikawa, 2013; Chaudhary and Elkord, 2016). In one study of colorectal cancer, Tr1 cells expressed both IL-10 and TGFβ, and appeared to be more immunosuppressive than Treg cells (Scurr et al., 2014). Interleukin 27 (IL-27) has recently been shown to induce Tr1 cells by coordinating the activity of the transcription factors c-Maf and Ahr (Vasantakumar and Kallies, 2013). This may suggest a developmental link between Tr1 cells and Th17 cells, and indeed, c-Maf has been shown to promote IL-10 expression in Th17 cells. On the other hand, Tr1 cells have been shown to share some characteristics with CD4+ CTL, including the expression of IFNγ (White and Wraith, 2016), CRTAM (Burton et al., 2014), and Eomes (Zhang et al., 2017a). Furthermore, Tr1 cells have been shown to express granzyme B, which enables them to directly kill APCs (Grossman et al., 2004a; Magnani et al., 2011).
These observations, along with the recognition of IFNγ+IL-10+ Th1 cells discussed previously, suggest that Tr1, CD4+ CTL, and Th1 cells may represent a spectrum of closely-linked states (Cope et al., 2011). Subtle shifts in the phenotype of these cells may alter the balance of their pro-tumorigenic and anti-tumor effects, which may explain the context-dependence and variability of the effects of these cells in tumors.
Figure 2. Overlapping cytokine signals drive the diversity and plasticity of CD4+ T cells
Diverse CD4+ T cell phenotypes are dictated by the complexity of the cytokine milieu, which can promote either particular cell fates, such as Th9 induction by IL-4 and TGFβ, or more plastic phenotypes that resemble intermediate states (e.g. Th1/Th17 cells, which can simultaneously produce IFNγ and IL-17). Cytokine cues for CD4+ T cells are depicted as “circles of influence”. Lineage-defining transcription factors induced by cytokine signals are located within these circles. Cellular phenotypes have been drawn based on the cytokine and transcriptional regulatory signalings thought to drive their differentiation.

Adapted from (DuPage and Bluestone, 2016).
Increasing recognition of CD4+ T cell plasticity and heterogeneity

The discovery of additional phenotypic subsets of CD4+ T cells has added further complexity to our understanding of helper T cell responses. Nevertheless, the Th1/Th2 paradigm, which was itself heavily influenced by the “suppressor” vs. “helper” T cell framework of the 1970s (see next section), laid the groundwork for how the field has defined CD4+ T cell heterogeneity since the 1980s. In this model, CD4+ T cells differentiate along discrete developmental pathways that result in stable transcriptional states. Not surprisingly, much effort has also been devoted to attributing various disease states and immunological phenomena to specialized CD4+ T cell populations. For example, the roles of Th1 responses in controlling infections by intracellular pathogens and Th2 responses in protecting against helminth infections have been described since the early 1990s. However, further study of CD4+ T cell subsets, especially in vivo, have revealed a considerably muddier picture of helper cell heterogeneity and plasticity (Zhu et al., 2010) (Figure 2). Phenotypic diversity within “classical” helper cell lineages has been observed. For example, Th2 cells express variable levels of the lineage-defining cytokines IL-4, IL-5, IL-10, and IL-13, which may be due to stochastic epigenetic modifications, or variable expression of transcription factors. Notably, Th2 cells that do not produce IL-4 selectively express PU.1 and also produce more CCL22 (Chang et al., 2005b). CD4+ T cells also demonstrate plasticity and appear to be able to alter their phenotype in response to environmental stimuli. Th2 cells can produce IFNγ under the influence of IL-12, while isolated Th17 cells can lose expression of IL-17 and produce IFNγ instead (DuPage and Bluestone, 2016). There is increasing evidence that differentiated CD4+ T cell subsets can retain the ability to
adopt features of other subsets, or perhaps undergo “reprogramming” altogether. Acquisition of Th1-like features by Th17 cells, and Th9-like features by Th2 cells, as well as the phenotypic overlap between Th1, Tr1, and CD4+ CTL cells (discussed above) exemplify CD4+ T cell plasticity. These findings have been corroborated by in vivo characterizations of CD4+ T cells that develop in response to infection, cancer, or other physiologic stimuli. In these settings, CD4+ T cells are primarily found in “partial” states of differentiation in which they have simultaneously adopted multiple phenotypes to varying degrees (Openshaw et al., 1995; Zhou et al., 2009). The characteristics of CD4+ T cells differentiated in vitro may thus represent extreme versions of physiological phenotypes, or features that arise under unusual circumstances that are not recapitulated in vivo. Ongoing single cell RNA sequencing and other comprehensive profiling efforts may offer insight on the determinants of CD4+ T cell heterogeneity that are beyond the markers we have already identified. Collectively, these data suggest that CD4+ T cells may occupy a continuum of states rather than fixed categories.

III. Foxp3+ Regulatory T cells and cancer

Discovery of Tregs

The identification of a suppressive T cell population pre-dates the classification of CD4 and CD8+ T cells. In 1970, Gershon and Kondo showed that a population of T cells distinct from the helper cells that augmented antibody production could dampen allogeneic responses (Gershon and Kondo, 1970). This led to immense interest in the characterization of suppressor T cells, and soon suppressor T cell activity would be described in a wide array of experimental models (Germain, 2008; Sakaguchi et al., 2007). Both CD4+ and CD8+ suppressor T cell populations were described, suggesting
the presence of multiple suppressor T cell subsets. A region of the MHC, the previously-uncharacterized I-J region, was reported to be expressed by suppressor T cells, and antisera to this region appeared to also bind a soluble factor expressed by suppressor T cells that could inhibit immune responses alone. As the field grew, different laboratories using different models and reagents reported discordant findings; suppressor T cell factor acted in an antigen-specific manner in some reports, but was indiscriminate in others. The rise of monoclonal antibody technology and molecular cloning techniques in the 1980s gave the field more standardized and precise tools to study the suppressor T cell population. However, major tenets of suppressor T cell biology quickly crumbled under the scrutiny. First, a report claiming to have purified an antigen-specific suppressor T cell factor was quickly invalidated by a second paper showing that the purified protein was actually an apolipoprotein (Breslow et al., 1982; Krupen et al., 1982). Second, and perhaps most disappointingly, no transcripts from suppressor T cells mapped to the region of the MHC locus where the I-J region was purported to be (Kronenberg et al., 1983). Furthermore, sequencing of the genomic DNA of the mice in which I-J had first been identified revealed no evidence of recombination in the region (Kobori et al., 1986). Finally, TCRβ chain rearrangements could not be found in DNA from suppressor T cell hybridomas (Hedrick et al., 1985). These findings, along with the persistent difficulty in finding markers for and identifying antigen-specific suppressor T cell clones, prompted the field to lose confidence in suppressor T cells altogether. Even though the field continued to find evidence for T cell-mediated inhibition of immune responses, the word “suppression” would remain, as Green and Webb put it, “the nearest thing to a dirty word we have in cellular immunology” (Green and Webb, 1993).
Instead, immune tolerance was largely attributed to clonal deletion and anergy, characterized in the late 1980s, and to the effects of immunosuppressive cytokines like IL-10, which T cells could express on occasion. Interestingly, the characterization of IL-10-producing Tr1 and TGFβ-producing Th3 cells in the 1990s avoided stoking the field’s resistance to the notion of suppressor T cells. This is likely because they were described as T cells that had acquired immunosuppressive functions as a result of particular stimuli, rather than a specialized subset of T cells dedicated to immunosuppression (Sakaguchi et al., 2007).

In spite of the reticence of immunologists to recognize suppressor T cells in the wake of their disappointment in the 1980s, the evidence for an inhibitory T cell population continued to grow. In 1969, Nishizuka and Sakakura made the observation that mice thymectomized shortly after birth were susceptible to ovarian destruction, which was later found to be due to autoimmunity. Shortly after, Penhale and colleagues showed that thymectomized rats that received sublethal irradiation developed autoimmune thyroiditis and type 1 diabetes, which could be prevented by inoculation with normal T cells from syngeneic animals (Penhale et al., 1973, 1976, 1990). Notably, CD4+ T cell and CD4 single-positive (SP) thymocytes were particularly effective at inhibiting these autoimmune reactions (Sakaguchi et al., 1982a). Furthermore, CD4+ T cells from animals that had developed autoimmunity could propagate the disease upon transfer into T-cell deficient hosts (Sakaguchi et al., 1982b). Collectively, these data suggested that a thymus-derived CD4+ T cell population could suppress the activity of a second population of CD4+ T cells that could mediate autoimmune disease (Sakaguchi et al., 2007).
Using a cell markers, several groups further characterized the CD4+ suppressor cell population. Sakaguchi and colleagues showed that instigation of autoimmunity was associated with a CD5^low population of CD4+ T cells, while CD5^high cells could suppress their activity (Sakaguchi et al., 1985). These findings were followed by the work of Powrie and Mason, who reported that OX-22^high CD4+ T cells induced autoimmune disease upon transfer into nude mice that was preventable by an OX-22^low CD4+ T cell population (Powrie and Mason, 1990). Subsequent work determined that the protective CD4+ T cell population was CD45RB^low (Morrissey et al., 1993; Powrie et al., 1993). However, these markers were quite broadly expressed, which prompted efforts to find a more specific marker of suppressive CD4+ T cells (Sakaguchi et al., 2007; Shevach, 2011). Sakaguchi’s group investigated CD25, the IL-2 receptor a-chain, as a candidate because they were CD5^highCD45RB^low, and represented just 5-10% of peripheral CD4+ T cells, and <1% of CD8+ T cells. Remarkably, splenocytes depleted of CD25+CD4+ T cells produced a wider range and greater frequency of autoimmune effects in athymic nude hosts than did CD5^low or CD45RB^high cells prepared similarly (Sakaguchi et al., 1995). Furthermore, co-transfer of CD25+CD4+ T cells prevented these autoimmune reactions. The earlier observations made in neonatal thymectomized mice were also reconciled by the group’s findings that CD4+CD25+ cells appeared around day 3, and rapidly expand to adult levels in three weeks (Asano et al., 1996). CD4+ T cells from euthymic mice younger than day 3, or CD25-depleted splenocytes from adult mice were able to induce autoimmune disease in athymic nude hosts, while transfer of CD25+CD4+ T cells from a normal host into thymectomized mice prevented autoimmunity. Although much of the work done on this suppressor population had
focused on their role in preventing autoimmune disease, the study by Sakaguchi et al.
also established a role for CD25+CD4+ T cells in inhibiting immune reactions to nonself
antigens, including xenogeneic proteins and allografts (Sakaguchi et al., 1995). Perhaps
in avoidance of prior terminology, this thymus-derived suppressor CD4+ T cell
population was given the name "regulatory T cells", or Treg. Subsequent work showed
that CD25 was not a mere marker of Treg cells, but that IL-2 signaling was critical for
the development of this population (Sakaguchi et al., 2007).

A functional assay for Treg cell activity was soon developed. Two groups
demonstrated that CD25+CD4+ T cells could inhibit the proliferation of CD8+ and
CD25-CD4+ T cells in vitro in response to specific antigen or nonspecific T cell stimuli
(Takahashi et al., 1998; Thornton and Shevach, 1998). The in vitro suppression assay
would prove invaluable in confirming the existence and identity of human Treg cells in
2001 (Shevach, 2001). Another major milestone for the study of Treg cells was the
discovery, through genetic mapping and the generation of transgenic mice, that
mutation of the forkhead/ winged-helix family member Foxp3 was responsible for the
scurfy mouse, which develops spontaneous, severe autoimmunity (Brunkow et al.,
2001). Mutations of human FOXP3 were subsequently found to be associated with
immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome,
which is characterized by autoimmunity of the bowel and various endocrine organs, and
severe allergies (Bennett et al., 2001; Chatila et al., 2000; Wildin et al., 2001). Several
groups began investigating similarities between IPEX syndrome and the pathology
observed in Treg-depleted mice, resulting in identification of Foxp3 as a critical driver of
Treg cell development and function in 2003 (Fontenot et al., 2003; Hori et al., 2003;
Khattari et al., 2003). Foxp3 mRNA was exclusively expressed by CD4+CD25+ thymocytes and peripheral T cells, while Foxp3 overexpression in CD4+CD25- T cells converted them into Treg-like cells with in vivo and in vitro suppressive activity. In mixed bone marrow chimeras, Foxp3-deficient bone marrow could not give rise CD25+CD4+ T cells, while Treg cells derived from Foxp3-wild-type bone marrow cells were sufficient to prevent autoimmunity. The generation of Foxp3 reporter mice that expressed diphtheria toxin receptor and/or GFP under the control of the Foxp3 promoter (Fontenot et al., 2005a; Kim et al., 2007a; Lahl et al., 2007) demonstrated unequivocally the importance of Treg cells in actively preventing autoimmunity in adult animals. Interestingly, Treg cells bear strong similarities to previously-described suppressor T cells, including their poor growth in vitro and susceptibility to cyclophosphamide treatment (Germain, 2008). Earlier studies of suppressor T cells generated confusing, discordant results due to poor experimental systems and tools, prompting considerable skittishness in the community. However, the use of monoclonal reagents and transgenic mice enabled consistent and definitive characterization of suppressive T cells, allowing for their “re-discovery” on much more solid ground in the 1990s. In a review in 2007, Sakaguchi and colleagues highlighted key questions concerning Treg cell biology (Sakaguchi et al., 2007): 1. How do Treg cells develop in the thymus and the periphery? 2. What is the mechanistic basis of Treg cell-mediated immunosuppression? and 3. How does Foxp3 control the development of Treg cells? These questions have been areas of active investigation, and in the following sections I will summarize our current understanding of these topics, as well as discuss the role of Treg cells in cancer.
Developmental origins of Tregs

Although initial experiments seemed to indicate that CD4+CD25- T cells could not turn into Treg cells without ectopic expression of Foxp3, subsequent studies demonstrated that peripheral, naive CD4+ T cells could acquire Foxp3 expression in the setting of antigenic stimulation in the presence of TGFβ, or chronic suboptimal antigen stimulation (Apostolou and von Boehmer, 2004; Chen et al., 2003). This led to the recognition that peripheral Treg cells were composed of a population of thymus-derived, “natural” Treg cells (tTregs), and periphery-derived, “induced” Treg cells (pTregs), which are so-named to distinguish them from Treg cells induced in vitro using antigenic stimulation and TGFβ (iTregs) (Shevach and Thornton, 2014).

Immature thymocytes are thought to differentiate into tTregs in response to self-antigen/MHC presented by thymic epithelial cells. Elegant studies performed in mice expressing a fixed, transgene-encoded TCRβ chain, or an otherwise restricted repertoire, demonstrated that Treg TCRα chains were diverse and only partially overlapped with non-Treg TCRα chains, suggesting that the tTreg developmental pathway selects for a distinct, TCR repertoire directed toward self-antigen (Hsieh et al., 2004; Pacholczyk et al., 2006; Wong et al., 2007). Retroviral transduction of non-Treg CD4 effector T cells with TCRs derived from Tregs, but not CD25- CD4+ T cells, conferred reactivity to autologous APCs, and resulted in expansion and induction of autoimmune disease when transferred into lymphopenic hosts, confirming that Treg TCRs are self-reactive (Hsieh et al., 2004). Notably, the in vitro response of effector T cells expressing Treg TCRs to autologous APCs was weak compared to reactions seen with transgenic TCRs and foreign ligand, suggesting that the affinity of Treg TCRs for
self-antigen is within the range of affinities that enable conventional CD4+ T cells to pass negative and positive selection. Indeed, mice with impaired negative selection due to MHC class II expression by thymic medullary epithelial cells (mTECs) have a greater proportion of Tregs (Hinterberger et al., 2010), while increased negative selection results in fewer Tregs (Ouyang et al., 2010a). The interplay between negative selection and tTreg differentiation may be further regulated by Aire, which induces expression of tissue-restricted antigens by mTECs (Anderson et al., 2002). Aire is critical for negative selection of autoreactive T cells, but also redirects some autoreactive CD4+ T cells to differentiate into tTregs (Aschenbrenner et al., 2007; Malchow et al., 2016). Collectively, TCR signaling strength is thought to instruct tTreg development such that intermediate signal strength favors tTreg differentiation, while low signal strength favors conventional CD4+ T cell differentiation (Figure 3). Signaling through both the TCR and CD28 are critical for induction of Foxp3 and Treg differentiation (Josefowicz et al., 2012a). NF-kB signaling appears to be particularly important for Treg differentiation, and c-Rel has been shown to bind a Foxp3 intronic region, conserved noncoding regulatory sequence (Foxp3-CNS3), a “pioneer” element that greatly increases the probability of Foxp3 expression (Isomura et al., 2009; Josefowicz et al., 2012a; Long et al., 2009; Ruan et al., 2009). IL-2, IL-7, and IL-15 signaling also appear to be critical, with IL-2 playing a dominant role in promoting Treg differentiation, likely through its induction of STAT5 (Burchill et al., 2008; Josefowicz et al., 2012a). A two-step model has thus been proposed for tTreg differentiation wherein high TCR signal strength upregulates CD25, and subsequent IL-2 signaling induces Foxp3 expression (Lio and Hsieh, 2008). This study identified a larger pool of IL-2-responsive thymic precursors than expected,
suggesting that an *in vivo* bottleneck imposed by limited levels of IL-2 may select clones with stronger self-reactivity for tTreg differentiation. Interestingly, initial studies of transgenic mice expressing a single Treg-derived TCR demonstrated infrequent differentiation of Foxp3+ Treg cells. In contrast, subsequent studies where the transgenic Treg TCR-expressing precursors were limited to <1% of the thymocyte population revealed efficient Foxp3+ Treg differentiation (Bautista et al., 2009; Leung et al., 2009). These observations indicative of strong intraclonal competition suggest that ligand may also be limiting in the thymic environment for tTreg differentiation. The observation that CD3ζ− mutant mice with attenuated TCR signaling have increased Treg development (Hwang et al., 2012) has suggested that high-affinity, transient TCR signaling is most efficient at inducing tTreg differentiation. In this “hit-and-run” model, short-lived TCR activation upregulates CD25 and promotes the conditions necessary for tTreg differentiation, while avoiding the inhibitory effects of prolonged TCR activation, including PI3K/ Akt activation (Li and Rudensky, 2016; Ouyang and Li, 2011).

Differentiation of pTregs is thought to arise in response to non-self antigens, including those derived from allergens, commensal bacteria, dietary metabolites, or infectious pathogens (Arpaia and Rudensky, 2014; Josefowicz et al., 2012a) (Figure 3). Indeed, selective deficiency of pTreg development results in allergic inflammation in the gut and lung, both mucosal barrier tissues (Josefowicz et al., 2012b). Consistent with these distinct sources of ligand, the TCR repertoire of Foxp3+ Treg cells arising from CD4+Foxp3- T cells transferred into lymphopenic hosts is distinct and only partially overlaps with the TCR repertoire of unmanipulated CD4+Foxp3+ mice (Lathrop et al., 2008). Studies using TCR transgenic and CTLA-4-deficient mice have demonstrated
that pTreg differentiation is driven by high affinity TCR signaling in the context of TGFβ availability and suboptimal priming, e.g. due to signaling through CTLA-4 (Josefowicz et al., 2012a). Disruption of CD28 signaling by CTLA-4 may also reduce PI3K/Akt signaling, which inhibits pTreg differentiation (Han et al., 2012). Indeed, CTLA-4 is required for TGFβ-induction of Treg differentiation, but dispensable for tTreg differentiation (Zheng et al., 2006). Furthermore, chronic dosing of foreign antigens in noninflammatory conditions is able to induce a pTreg response that is protective during subsequent, inflammatory challenge (Daniel et al., 2011; Kretschmer et al., 2005).

In addition to signals from the TCR and costimulatory molecules, TGFβ signaling is critical for the development of pTregs. TGFβ signaling activates Smad3, which binds a Smad-NFAT response element located on conserved noncoding sequence 1 in the Foxp3 locus (Foxp3-CNS1) (Tone et al., 2008). TGFβ also reduces cellular proliferation, which inhibits cell-cycle-dependent recruitment of DNA methyltransferase 1 (Dnmt1) to the Foxp3 locus (Josefowicz et al., 2009). Inhibitory signals from TGFβ and CTLA-4 may thus cooperate to keep the Foxp3 locus accessible, enabling stabilization of Foxp3 expression. Interestingly, abrogation of TGFβ signaling also affects tTreg differentiation, but this is likely due to increased negative selection in the thymus, resulting in a smaller precursor pool for tTreg development (Ouyang et al., 2010a). Indeed, Foxp3-CNS1 is dispensable for the generation of tTregs, but absolutely required for pTreg differentiation (Schlenner et al., 2012; Zheng et al., 2010). Much of CNS1 is located on a retrotransposon shared by placental mammals, and CNS1 deletion increases fetal loss, suggesting that pTreg development may have evolved to aid maternal-fetal tolerance (Samstein et al., 2012a). IL-2 also contributes to pTreg differentiation, and appears to
be particularly important in favoring Treg over Th17 differentiation when IL-6 signaling is also active (Laurence et al., 2007). Retinoic acid receptor (RAR) signaling can also induce pTreg differentiation in gut-associated lymphoid tissue and mesenteric lymph nodes through direct binding to CNS1 as well as inhibition of the production of other effector CD4 cytokines (Josefowicz et al., 2012a).

Differences in antigen specificity and TCR repertoire between tTregs and pTregs suggest that these populations may have divergent functions as well. Specifically, tTregs would be expected to be important for preventing autoimmunity due to their recognition of self-antigens, while pTregs might be more active in mucosal tissues where commensal bacteria can induce their differentiation (Plitas and Rudensky, 2016). Thorough study of the relative contribution of tTregs vs. pTregs to observed Treg functions have been severely limited by an inability to readily distinguish these populations. The transcription factor Helios (Thornton et al., 2010) and the surface antigen neuropilin 1 (Nrp1) (Weiss et al., 2012; Yadav et al., 2012) have both been proposed as markers for tTregs. However, both of these markers were found to be expressed by Tregs induced in vitro as well (Akimova et al., 2011; Shevach and Thornton, 2014; Verhagen and Wraith, 2010), suggesting that they are not bona fide markers of tTregs alone. The “gold standard” assay for determining Treg origin is to evaluate the methylation status of the Foxp3 locus conserved noncoding sequence 2 (Foxp3-CNS2), an intronic regulatory element critical for maintenance of Foxp3 expression that is fully methylated in non-Tregs, and de-methylated in tTregs (Feng et al., 2014; Toker et al., 2013; Zheng et al., 2010). pTreg differentiation in vivo is accompanied by progressive demethylation of the region (Polansky et al., 2008),
making it possible to distinguish tTregs from early pTregs, but difficult to distinguish “mature” pTregs from tTregs on the basis of CNS2 methylation. Relying on the unverified assumption that most peripheral Tregs in unperturbed mice are tTregs, some studies have shown that polyclonal tTregs may suppress autoimmunity by preventing effector CD4+ T cells from leaving the lymph node (Kohm et al., 2002). Meanwhile, transferred iTregs (induced \textit{in vitro}) appeared to also be able to suppress autoimmune inflammation, but through IL-10 mediated inhibition of DC function (Chattopadhyay and Shevach, 2013). Given the uncertainty over the origin of isolated Tregs for these experiments, as well as the phenotypic similarity between iTregs and pTregs, it is difficult to attribute specific functions to thymic vs. extrathympically-derived Tregs.

Both tTreg and pTreg populations may have a role to play in infectious or inflammatory states where foreign and self antigens may be released, including cancer. The proportion of tTregs as compared to pTregs is likely to vary with tumor type, tissue context, antigen characteristics, and cytokine milieu. The observation that there was little to no overlap in the TCR repertoires of tumor-infiltrating Tconv and Treg cells in carcinogen-induced tumors in mice have suggested that tTregs are predominant in at least in some tumors (Hindley et al., 2011). A recent study of Treg and Tconv TCR repertoires in human breast cancer similarly found little repertoire overlap between tumor Treg TCRs and both normal breast Treg TCRs and tumor Tconv TCRs, suggesting that tumor Tregs are recruited to the tumor and not induced locally (Plitas et al., 2016). Nevertheless, other studies have demonstrated TGFβ-dependent induction of Treg responses in tumors, and that tumors likely recruit both populations of Tregs to varying degrees (Liu et al., 2007; Zhou and Levitsky, 2007).
Figure 3. Distinct developmental pathways give rise to two populations of Treg cells
CD4 single-positive (SP) thymocytes undergo negative selection in the thymic medulla. Intermediate TCR signal strength instructs regulatory T cell (Treg) development, while low signal strength promotes conventional CD4+ T cell (Tconv) development. Naïve Tconv can be induced to become regulatory T cells by TCR activation in the context of suboptimal priming and TGFβ signaling. As a result of their developmental circumstances, tTreg cells are likely to be reactive to self-antigen, while pTreg cells likely react to commensal bacteria or other foreign antigens. These differing specificities are thought to be reflected in their TCR repertoires and tissue localization, although the lack of good differentiating markers limits the ability to distinguish the populations.

Adapted from (Josefowicz et al., 2012a).
Effector functions of Tregs

The morbidity of mice that have had their Tregs depleted suggests that Tregs play a major role in suppressing autoimmunity. However, the various mechanisms by which Tregs maintain tolerance and restrain immune responses are still under active investigation. Importantly, different Treg populations may employ various combinations immunomodulatory mechanisms depending on the cytokine milieu and tissue residence. Tregs in tumors and tumor-associated lymphoid tissues may adopt any combination of these effector mechanisms, and may also demonstrate functions associated with tissue repair and homeostasis that are just beginning to be understood. The distinct transcriptional profiles of Tregs responding to different types of inflammation and in different tissues, discussed in the next section, provide evidence that Treg functions may be context-dependent. In this section I will review major proposed mechanisms of Treg function (Figure 4).

1. Competition for cytokine and antigen

Tregs require IL-2 for survival (Fontenot et al., 2005b), but are unable to produce IL-2 due Foxp3-mediated silencing at the Il2 promoter (Ono et al., 2007; Wu et al., 2006). This has led to the suggestion that Tregs, which express high levels of IL-2 receptor, can suppress T cell responses by locally depleting IL-2. In in vitro suppression assays, Tregs can induce the apoptosis of Tconv “responder” cells by consuming IL-2, which results in less PI3K/Akt signaling and more pro-apoptotic Bim expression in responder T cells (Pandiyan et al., 2007). IL-2 neutralizing antibody could phenocopy the effect of co-culture with Tregs, and Bim-deficient CD4+ T cells were less susceptible to Treg-mediated immunosuppression in vitro and in an adoptive transfer model of
colitis. In a more recent study using transgenic mice that lack expression of IL-2R but retain Treg development due to constitutive Stat5 expression, IL-2 consumption was essential for suppressing CD8+ T cells, but not Tconv cells, which may reflect greater reliance of CD8+ T cells on IL-2 (Chinen et al., 2016).

It is also possible that Tregs may be able to outcompete Tconv for antigen. Based on their development, tTreg cells tend to have higher affinity TCRs for the same antigen than their Tconv counterparts (Josefowicz et al., 2012a). Thymus-derived Tregs may also have a faster response to TCR activation due to their high expression of the adhesion molecules LFA-1 and ICAM-1, which may prevent Tconv with the same antigen specificity from accessing APCs (Tanaka and Sakaguchi, 2017).

2. **Secretion of immunosuppressive cytokines or metabolites**

Tregs secrete a number of immunomodulatory factors that can contribute to their immunosuppressive function. Since TGFβ plays a critical role in the development of Tregs, and is secreted by many cell types, it has been difficult to determine the effect of Treg-derived TGFβ on other cells. Interestingly, TGFβ is associated with the cell membrane and Tregs may suppress other cells through cell-cell interactions mediated by TGFβ (Nakamura et al., 2001). Consistent with this early finding, subsequent work has suggested that TGFβ on the surface of Tregs can activate immunosuppressive Notch1 signaling on target cells (Ostroukhova et al., 2006). Other studies, however, have shown that soluble TGFβ is responsible for Treg suppression of CD8+ T cell cytotoxic activity (Mempel et al., 2006). It is now known that latent TGFβ is held at the cell surface by GARP, and can be activated and released through integrin
interactions, which may explain the contact-dependence of TGFβ suppressive functions (Tran, 2012).

There are also many sources of IL-10 in inflammatory environments, including cancer. However, mice that have a Treg-specific deficiency in IL-10 production did not have systemic autoimmunity, but developed spontaneous colitis and had stronger hyperreactivity responses in the lung and skin, suggesting that IL-10 may be a particularly important immunosuppressive mechanism for Tregs in mucosal tissues (Rubtsov et al., 2008). Recently, another group has shown that type I IFN signaling can promote IL-10 expression in tumor Tregs, allowing them to suppress Th17 responses (Stewart et al., 2013). In other inflammatory contexts, Tregs may be able to promote production of IL-10 by other cells such that Treg-derived IL-10 may not be necessary for full immunosuppressive potential (Vignali et al., 2008).

IL-35 production has been attributed to both Tregs and an induced population of suppressive, Foxp3- cells (Collison et al., 2007, 2010). IL-35 signals through a heterodimeric receptor expressed by activated NK and T cells, resulting in a much more limited range of action than IL-10. However, a recent report suggests that Treg-specific IL-35 deletion impairs anti-tumor immune responses (Turnis et al., 2016). Furthermore, two distinct effector populations of Tregs have been described that have reciprocal expression of either IL-10 or IL-35 (Wei et al., 2017). IL-35-expressing Tregs also express CCR7 and reside in T cell zones, while IL-10-expressing Tregs express chemokine receptors that direct them to nonlymphoid tissue. Collectively, IL-10 and IL-35 may promote distinct Treg effector programs that facilitate short and long-range immunosuppression.
In addition to secreting immunosuppressive cytokines, Tregs can also promote the conversion of extracellular ATP to adenosine through expression of CD39 and CD73. Adenosine signals through adenosine receptors expressed by many cell types, and can directly suppress the function of APCs and effector T cells (reviewed above).

3. Impairment of APC maturation and function

Tregs express a number of cell-surface molecules that allow them to directly inhibit the activity of APCs. CTLA-4 is highly and ubiquitously expressed on Tregs, and Treg-specific loss of CTLA-4 results in systemic lymphoproliferation and fatal autoimmunity (Wing et al., 2008). Adoptively transferred splenocytes from mice with Treg-specific CTLA-4 deletion are better able to control tumors than splenocytes from wild-type mice. In addition to promoting the developing of pTregs, CTLA-4 is thought to outcompete other T cells for binding to CD80 and CD86 on dendritic cells due to its higher affinity for those receptors in comparison to CD28. CTLA-4 is also able to “capture” CD80 and CD86 from the cell membrane of dendritic cells through a process called trans-endocytosis, which helps further disable priming of effector T cell responses by DCs (Qureshi et al., 2011).

Other “co-inhibitory” receptors may also mediate suppression of DC functions. TIGIT, which is highly expressed on Tregs, can stimulate APCs to secrete IL-10 and TGFβ (Yu et al., 2009). TIM-3 is also highly expressed on Tregs associated with tumors, and Tim3+ Tregs have been shown to be more immunosuppressive than Tim3- Tregs (Anderson et al., 2016). LAG3 is a CD4-like protein that can bind MHC class II on DCs, resulting in inhibitory signaling that impairs maturation and immunostimulatory functions (Liang et al., 2008). However, a recent report demonstrated that Treg-specific deletion
of Lag3 resulted in enhanced Treg proliferation and reduced T cell-mediated autoimmune responses, suggesting that LAG-3 may have an inhibitory effect on Treg function (Zhang et al., 2017b). The effect of LAG-3 and other inhibitory molecules on Treg development and function require further investigation (Anderson et al., 2016).

4. **Direct cell killing**

*In vitro* studies of Treg function have suggested that Tregs can directly kill other immune cells through secretion of granzyme A/B and/or perforin (Gondek et al., 2005; Grossman et al., 2004b; Zhao et al., 2006). In a report using transplant tumor models, tumor cells were found to induce granzyme B (GzmB) expression on Tregs, which was used to suppress NK and CD8+ T cell immune responses to the tumor. Notably, tumors were cleared more effectively in GzmB-deficient animals, suggesting that Tregs are more reliant on GzmB for their function than NK or CD8+ T cells. Treg expression of perforin and GzmB were critical to allow outgrowth of tumors (Cao et al., 2007). More recently, either granzyme A or B expression by Tregs has been shown to be important for their protective role in graft versus host disease, and acute viral infection in the lung (Loebbermann et al., 2012; Velaga et al., 2015)
Figure 4. Effector mechanisms of regulatory T cells

A. Secretion of immunosuppressive cytokines, like TGFβ, IL-10, and IL-35. Tregs can also convert extracellular ATP to adenosine via expression of CD73 and CD39.

B. Competition with effector T cells for IL-2 and other metabolic substrates, as well as antigen.

C. Direct targeting of DC function by trans-endocytosis of CD80, CD86.

D. Direct killing of effector cells through a GzmA/B-dependent mechanism.

E. Mounting evidence suggests Tregs may have a role in tissue repair and regeneration, e.g. through the production of amphiregulin (Areg)

Adapted from (Caridade et al., 2013).
Transcriptional control of Treg differentiation and function

The exquisite control of Foxp3 expression during Treg differentiation and maintenance attests to its importance as a major transcriptional regulator of Treg identity and function. TCR signaling promotes the expression of Foxp3 through induction of NF-κB and NFAT, which have been shown to bind the Foxp3 enhancer elements Foxp3-CNS3 and Foxp3-CNS1, respectively. Binding to CNS3 does not appear to enhance Foxp3 expression, but may alter accessibility to the locus, increasing responsiveness to other Foxp3-inducing signals (Zheng et al., 2010). NR4A1 and CREB/ATF, also induced by TCR signaling, can also transactivate Foxp3, while IL-2-activated STAT5 has also been shown to bind the Foxp3 promoter (van der Veeken et al., 2013). Specific roles have also been identified for the transcription factors Foxo1 and Foxo3, which can bind an intronic region in the Foxp3-CNS2, and thus enhance and maintain Foxp3 expression (Ouyang et al., 2010b).

During pTreg differentiation, half of cells that have induced Foxp3 will subsequently lose expression (Josefowicz et al., 2012b). Stabilization of Foxp3 expression appears to be controlled by demethylation of the CpG island in Foxp3-CNS2, which is regulated by Dnmt1 and the Tet family of DNA demethylases (Toker et al., 2013). Foxp3 repression in non-Treg cells requires Dnmt1 expression (Josefowicz et al., 2009), while it is unclear how Dnmt1 activity is inhibited to allow Treg maturation. Reduced cell proliferation during pTreg differentiation (discussed in the previous section) may help limit Dnmt1 recruitment. Runx, Foxp3, and Stat5 have been shown to bind CNS2 and contribute to stable Foxp3 expression (van der Veeken et al., 2013).
The core Treg transcriptional program consists of upregulation of genes associated with suppressor activity, and reduced expression of genes associated with effector CD4+ T cell function (Josefowicz et al., 2012a). Various components of the Treg transcriptional signature may also be adapted to the tissue or inflammatory context in which the Treg resides (Feuerer et al., 2009a; Panduro et al., 2016). Tregs also demonstrate differential regulation of genes downstream of TCR signaling as compared to Tconv (Li and Rudensky, 2016). Generation of mice expressing a reporter null allele of Foxp3 (Foxp3GFPKO) (Gavin et al., 2007), a truncated Foxp3 tagged with GFP (Lin et al., 2007), or a hypomorphic Foxp3 allele due to insertion of luciferase and GFP in the 3' UTR (Wan and Flavell, 2007) have enabled analyses of Foxp3-dependent Treg functions. Interestingly, GFP+Foxp3KO cells retained some features of Foxp3+ Tregs, including low TCR-induced proliferation and some expression of CD25, GITR, and CTLA-4. But these cells made IL-4 and IL-17, demonstrated features of Th2 cells, and were not suppressive. Treg cells require stable Foxp3 expression to maintain their phenotype, as ablation of Foxp3 in mature Tregs results in loss of suppressor activity and adoption of Tconv functions (Williams and Rudensky, 2007). Chromatin immunoprecipitation (ChIP) studies of Foxp3 have shown that, in activated Tregs, Foxp3 binding to DNA elements is associated with transcriptional repression (Arvey et al., 2014). Consistent with that trend, Foxp3-bound chromatin sites were less accessible and more enriched for H3K27me3 marks, suggesting that Foxp3 coordinates with the PRC complex to repress transcription. Kwon et al. have recently added to this model of Foxp3 transcriptional regulation by using a panel of alanine mutants of Foxp3 to show that transactivation of Foxp3-dependent genes is correlated with the ability of Foxp3 to
form a complex with RELA, IKZF2, EP300, or KAT5, and negatively correlated with its ability to associate with EZH2, YY1, IKZF3, NFAT1, or STAT3 (Kwon et al., 2017). Consequently, Foxp3 can potentiate transcription when associated with the RELA-containing complex, but lead to repression when associated with the EZH2-containing complex. Interestingly, the Foxp3-RELA complex appeared to be better correlated with repression than the Foxp3-EZH2 complex, which may suggest interference with other, activating complexes. Participation in these complexes appears to be mutually exclusive and occur in distinct regions of the nucleus, suggesting that once Foxp3 has bound to an enhancer, its activity is dependent on the availability of other cofactors.

However, cross-referencing of genes differentially expressed in GFP+Foxp3null cells with Foxp3 chIP studies indicated that only 20-30% of Foxp3-regulated genes are actually bound by Foxp3 (Josefowicz et al., 2012a). TCR signaling accounts for 25% of the Treg transcriptional program, as TCR stimulation altered the chromatin accessibility of cells in the absence of Foxp3 expression (Ohkura et al., 2012), and TCR ablation in mature Tregs altered their transcriptional features (Levine et al., 2014). Indeed, Foxp3 has been shown to bind DNA elements that are already occupied by cofactors or open as a result of TCR signaling rather than actively modify chromatin accessibility on its own (Samstein et al., 2012b). Many other signals likely contribute to the Treg transcriptional program, including IL-2, TGFβ, TNF family members, and costimulatory molecules. In fact, mounting evidence suggests that Tregs can adapt their transcriptional phenotype to suit surrounding inflammatory milieu or tissue context (Figure 5). It is likely that Tregs are able to adopt these tailored suppressor programs due to their exposure to the same cytokine or tissue-specific signals as their targets. In
this section I will review some of the functional, “stable” Treg subsets that are thought to
be directed at particular types of inflammation, or confer important tissue homeostatic
functions. It is important to note that these Treg phenotypes are overlapping with each
other and the “core” Treg transcriptional program, and likely represent variations on a
theme rather than discrete categories. I will then address questions concerning Treg
phenotypic stability that necessarily go along with a discussion of Treg plasticity.

Resting ("central") Treg

Resting Tregs (rTregs) are recent thymic emigrants that are
CD44^loCD62L^hiCCR7^hi (CD45RA+ in humans) and recirculate through secondary
lymphoid organs (SLOs) (Smigiel et al., 2014). They are dependent on IL-2 signaling,
and their expression of CCR7 enables them to migrate to T cell zones where they can
receive paracrine IL-2 signaling. Their consumption of IL-2 is thought to suppress
effector T cell priming and differentiation. rTregs are defined in contrast to activated
Tregs (aTregs), which are CD44^hiCD62L^loCCR7^lo (CD45RA- in humans), reside in
nonlymphoid tissues, and are thought to suppress the function of effector T cells.
aTregs do not recirculate from their tissue sites and appear to have shorter half-lives
than rTregs (Luo et al., 2016). Unlike rTregs, aTregs are insensitive to IL-2 blockade,
and instead appear to be maintained by DCs expressing ICOSL, which was shown to
promote aTreg survival. aTregs differentiate from rTregs upon TCR activation and
cytokine stimulation and as a result are sensitive to TCR ablation while rTregs are not
(Levine et al., 2014; Vahl et al., 2014). Remarkably, the Treg transcriptional program of
rTregs post-TCR ablation is well-preserved. Nevertheless, rTregs and aTregs both
express Nr4a1, which is downstream of the TCR, suggesting that both populations
receive continuous TCR signaling. Instead, inflammatory signaling, e.g. by LPS, appears to promote rTreg to aTreg conversion, possibly by activating DCs and providing other differentiating signals. Recently, Foxo1, which is inhibited by strong TCR signaling, has been shown to be critical for the maintenance of resting Tregs. Meanwhile, Foxo1 in aTregs is predominantly cytosolic and phosphorylated, and thus inactive, consistent with greater levels of TCR signaling. Constitutive Foxo1 activity prevents migration of Tregs to nonlymphoid tissue, resulting in CD8+ T cell-mediated autoimmunity (Luo et al., 2016). Collectively, these recent observations suggest that two distinct populations of Tregs have complementary roles in maintaining immune tolerance (Li and Rudensky, 2016). The Treg cell subsets described below can be described as “flavors” of aTregs (Shevach, 2006).

**Th1-directed**

Exposure of Tregs to IFNγ during Th1 inflammatory responses can promote the expression of T-bet, which directly transactivates CXCR3 (Koch et al., 2009). CXCR3+T-bet+ Tregs arise could arise from Foxp3+ but not Foxp3- transferred cells, suggesting that they represent a phenotype adopted by existing Tregs rather than a newly induced CD4+ T cell or converted Th1 cell. T-bet+ Tregs did not express IFNγ, but did express IL-10, GZMB-B, GITR, CTLA-4, and CD103, consistent with an aTreg/effector memory phenotype. T-bet expression appeared to promote the proliferation of Tregs as well as their recruitment to sites of microbial proliferation in a mouse model of mycobacterial disease, while T-bet deficiency led to selective impairment in the Treg response to Th1 inflammation. Another study has confirmed that depletion of T-bet+ Tregs results in severe Th1 autoimmunity (Levine et al., 2017). In spite of their
responsiveness to IFNγ, Tregs do not upregulate IL-12Rb2 in response to T-bet activity because the locus is epigenetically silenced (Koch et al., 2012). Their insensitivity to IL-12 enables them to adopt features of the Th1 program without acquiring its pro-inflammatory potential. IL-27 has also been shown to promote the differentiation of CXCR3+T-bet+ Tregs at mucosal sites (Hall et al., 2012). Notably IL-27-stimulated Tregs appear to differ transcriptionally from IFNγ-stimulated Tregs, suggesting that further specialization may occur in a cytokine or tissue-dependent fashion.

**Th2-directed**

IRF4 promotes Th1 and Th17 differentiation and is a direct Foxp3 target. IRF4 has been shown to associate with Foxp3 and coordinate gene expression in Tregs, which may explain the severe autoimmunity observed in mice with Treg-specific IRF4 deficiency (Zheng et al., 2009). These mice had increased Th2 cytokine production, IgE and IgG1 production, and plasma cell infiltration. Additionally, IRF4-deficient Tregs expressed lower levels of genes associated with Th2 differentiation. GATA3, the lineage-defining transcription factor for Th2 differentiation, has also been found to be a critical regulator of the Treg transcriptional program (Wang et al., 2011; Wohlfert et al., 2011). However, in these studies, the effects of GATA3 and IRF4 ablation are severe, and it is unclear if they cause selective impairment in the response to Th2 inflammation.

**Th17-directed**

STAT3 and RORγT are critical for Th17 differentiation, and both have been found to be expressed by Tregs. Similar to Irf4, Stat3 also cooperates with Foxp3 to drive gene expression in Tregs. Stat3-deficient Tregs have lower expression of IL-1R and IL-
R, which hints that the mechanism for Treg-mediated suppression of Th17 responses may be consumption of these cytokines (Chaudhry et al., 2009). They also express greater levels of Th17-promoting cytokines, and less CCR6, which helps them migrate to sites of Th17 inflammation. Mice with Treg-specific Stat3 deletion developed Th17 autoimmunity, including severe inflammatory bowel disease (IBD), but did not have evidence of increased Th1 or Th2 responses.

Interestingly, RORγT+Tregs (“Tr17”) have also been identified as a stable Treg population capable of suppressing Th17 inflammation (Figure 2). These cells demonstrate demethylation at the promoters of Foxp3 and other Treg-associated genes, suggesting they are mature Tregs (Yang et al., 2016). Upon transfer into mice with colitis, RORγT+ Tregs are superior to RORγT- Tregs in suppressing gut inflammation. The development of this population has been attributed to Stat3 signaling in Tregs downstream of IL-6 signaling, bringing the data on RORγT+ Tregs in concert with earlier studies (Kim et al., 2017). A comparison of RORγT+ Tregs in peripheral LN induced by immunization and the tissue-resident colonic RORγT+ Treg population (discussed below) suggested that both of these populations express genes associated with the Th17 lineage, although the LN RORγT+ Tregs appeared to express a wider range and more Th17-associated genes.

**T follicular regulatory cells (Tfr)**

CXCR5<sup>high</sup>Bcl6+ Tregs have been described in germinal centers and are implicated in the suppression of antibody responses (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). Similar to Tfh, Tfr differentiation also appears to be induced by SLAM-associated protein (SAP), CD28, Bcl-6, and B cells. Depletion of this
population results in unrestrained germinal center reactions and the proliferation of non-
antigen-specific B cells, resulting in fewer antigen-specific B cells. Notably, loss of this 
population does not result in Th1 or Th2 inflammation, suggesting that these cells are 
specialized to function in the B cell follicle.

**Visceral adipose tissue (VAT) Tregs**

Strikingly, 40-80% of VAT CD4+ T cells in lean mice are Tregs (Feuerer et al., 2009b). This population declines precipitously later in life, and their persistence is 
associated with factors including genetic background, diet, obesity, and microbiota 
(Cipolletta et al., 2012). Specifically, obesity also drives a reduction in VAT Tregs. 
Transcriptional profiling of the VAT Treg population revealed major transcriptional shifts 
compared to splenic and lymph node populations of Tregs. The transcriptional regulator 
Ppary was especially highly expressed on VAT Tregs, and was later identified as a 
major driver of the VAT Treg phenotype. Irf4 and Batf have been shown to cooperate 
with Foxp3 to induce expression of the interleukin-33 receptor, ST2, and Ppary, which 
drive much of the VAT Treg phenotype (Vasanthakumar et al., 2015). Indeed, Ppary 
and Foxp3 interact, and transduction of CD4+ T cells with Foxp3 and Ppary recapitulate 
aspects of the VAT Treg program (Cipolletta et al., 2012). Interestingly, ST2+ Tregs 
have also been identified in human omental fat tissue, suggesting that IL-33 signaling 
represents a conserved mechanism of VAT Treg expansion. The role of ST2 in tissue 
Treg biology will be further discussed in the last section.

Consistent with their distinct transcriptional phenotype, the TCR repertoire of 
VAT Tregs demonstrate small clonal expansions and convergent TCRα CDR3 
sequences, suggesting that clones are expanding in response to a few, specific
antigens (Kolodin et al., 2015). There is no repertoire overlap between VAT Tconv and Treg cells, suggesting they are thymus-derived, and cell-labelling studies suggest that they do not recirculate away from VAT. Depletion of VAT Tregs resulted in greater pro-inflammatory signaling in VAT tissue and metabolic derangements, including increased insulin resistance. In spite of their role in maintaining immune homeostasis in adipose tissue, VAT Tregs were not more immunosuppressive than spleen Tregs in in vitro suppression assays (Feuerer et al., 2009b), which highlight the lack of sensitivity of these assays for diverse effector or tissue Treg functions. Treg-specific Ppary deletion resulted in a drastic reduction in VAT Treg numbers. Furthermore, treatment of obese mice with pioglitazone, a Ppary agonist, was able to expand the VAT Treg population, leading to improved insulin sensitivity and lower fasting glucose levels. Collectively, these data point to a unique role for VAT Tregs in maintaining insulin sensitivity by regulating the immune milieu in adipose tissue. Interestingly, VAT Tregs were observed to take up lipids, a characteristic that may be specialized for their tissue context.

**Skeletal Muscle Tregs**

A small resident Treg population was also identified in skeletal muscle that expands dramatically in response to muscle injury (Burzyn et al., 2013). Similar to the VAT Tregs, this population was also found to be clonally expanded. Depletion of Tregs in this model was found to impair tissue regeneration by delaying the transition to a less-inflamed state. Persistent inflammatory infiltrate was associated with more fibrosis and less myogenic potential of satellite cells. Treg-mediated secretion of amphiregulin (Areg), an epidermal growth factor family ligand, was found to be a mediator for their effect on wound healing, as Areg administration rescued the phenotype of Treg-
depleted mice. Since this report, other studies have confirmed the role of Tregs in promoting muscle regeneration after injury (Castiglioni et al., 2015) and in a mouse model of muscular dystrophy (Villalta et al., 2014). Areg+ Tregs have also been shown to be important to promoting tissue repair after influenza infection in the lung (Arpaia et al., 2015), and may play a role in lung tumorigenesis (Green et al., 2017).

Colon Tregs

Two populations of colon Tregs have been described that likely have different developmental origins. Colonic pTregs are RORγT+ (Ohnmacht et al., 2015; Sefik et al., 2015; Yang et al., 2016), while colonic tTregs are GATA3+Helios+ (Schiering et al., 2014). The RORγT+ population is thought to be more sensitive to changes in microbiota and dietary metabolites, and may be responsible for maintaining tolerance to commensals. Interestingly, in spite of their expression of Il17 mRNA, these Tregs do not express IL-17. Meanwhile, the GATA3+Helios+ population is less sensitive to changes in microbiota and may be specialized to prevent autoimmunity in the gut, since ST2+GATA3+ Tregs were shown to be critical for suppressing autoreactive T cell responses in an adoptive transfer model of colitis (Schiering et al., 2014). These observations suggest that other mucosal tissues may also host distinct subsets of tissue Treg populations. Notably, their activity in in vitro suppression assays are the same, suggesting that their phenotypic differences may only be apparent in their tissue context. Furthermore, while there are many overlapping features of Tregs from different nonlymphoid tissues, tissue Treg populations appear to have a small number of unique transcriptional changes that may represent tissue-specific modules that are added to a core “tissue Treg” transcriptional program.
Plasticity or Instability?

Although lineage-mapping studies have shown that the vast majority of mature Tregs do not lose Foxp3 expression, and are thus “stable”, even in an inflamed environment, a small population of cells has consistently been identified to lose expression of Foxp3 (Sawant and Vignali, 2014). Since “exTregs” may have proinflammatory functions and yet be directed at self-antigens, there is active interest in determining whether unstable Tregs are involved in the pathogenesis of autoimmune disease, or can be harnessed in the treatment of cancer. Outside of disease contexts, developmental plasticity between Th17/Treg lineages has been frequently noted in humans and mice, with considerable controversy over whether RORyT+ Tregs are immunosuppressive, or make IL-17 protein (Beriou et al., 2009; Sefik et al., 2015; Voo et al., 2009). It remains unclear whether these populations are cells in transition to a committed Treg phenotype, unstable former Tregs, or a stable phenotype, and it is possible that all three conditions may contribute to this pool. Many of the inflammation-specific specialized subsets of Tregs that have been described maintain strong expression of Foxp3, and are thus likely to represent “stable” states. But a defining feature of these subsets is that they have adopted transcriptional characteristics of effector T cells, often in response to inflammatory cues. Consequently, plasticity of the Treg phenotype may exist on a continuum with lineage instability, with maintenance of Foxp3 expression acting as the critical determinant of function.

A number of studies have recently demonstrated that the stable Treg phenotype can also be subverted by particular “control switches” that can upend the Treg transcriptome. Furthermore, confusion in the Treg phenotype is associated with reduced
tumor immunosuppression and improved anti-tumor immunity. Treg-specific loss of the transcription factor Helios results in an “unstable” phenotype characterized by expression of IFNγ and TNFα, and mice with Helios-deficient Tregs have delayed growth of transplanted tumor cells (Nakagawa et al., 2016). Interestingly, anti-GITR antibodies reduce Helios expression on Tregs, thus providing a link between the anti-tumor efficacy of GITR blockade with the generation of an unstable Treg phenotype. Another recent study showed that a subset of Nrp1-deficient Tregs secrete IFNγ, which abrogates the immunosuppressive activity of neighboring wild-type Tregs (Overacre-Delgoffe et al., 2017b). IFNγ signaling promotes a “fragile” Treg phenotype, which impairs their ability to promote tumor growth. Consistent with the known role of NF-κB signaling in Treg development, Treg-specific c-Rel deletion, but not p65 deletion, results in dramatically slower growth of transplanted tumors. Tumor-infiltrating Tregs in mice with Treg-specific c-Rel deletion were fewer in number and had decreased expression of Treg signature genes, like Ikzf2, and increased expression of effector cytokines and transcription factors, like IFNγ and Tbx21 (Grinberg-Bleyer et al., 2017). These transcriptional changes were similar to those observed in a companion study, which found that ablation of the NF-κB subunits resulted in Tregs adopting features of effector Tconv cells and expressing lower levels of Treg signature genes (Oh et al., 2017). Notably, in all of these instances, unstable or fragile Tregs produce IFNγ, which, based on the findings by Vignali and colleagues, may signal in an autocrine loop and provoke further fragility. As a result, even small increases in IFNγ production by Helios- and c-Rel-deficient Tregs may result in profound impairment of Treg activity. These studies have raised the possibility that Treg instability or fragility may be used as a tool in
dampening tumor immune suppression. These observations also call for further investigation of the relationship between these phenotypes of instability and the phenotypes that the field has recognized as stable demonstrations of Treg heterogeneity. Better insight on the stepwise transitions that stand between instability and specialized Treg function will be critical for harnessing the activity of Tregs.
Figure 5. “Flavors” of effector Treg cells
Inflammatory and tissue context promote different transcriptional programs in Treg cells. Tregs are thought to "adapt" by expressing parts of the transcriptional program of the inflammatory cells they are suppressing. This is likely due to their exposure to the same cytokine cues that other immune cells are responding to. Tissue Tregs represent another branch of specialized Treg populations that have a distinct transcriptional phenotype and may have unique functions. Ppary has been shown to be a regulator of fat Treg cells, but the transcriptional networks promoting the activity of tissue Tregs are under active investigation.

Adapted from (Fan and Rudensky, 2016; Josefowicz et al., 2012a; Panduro et al., 2016)
Role of Tregs in cancer

Tumor-associated Tregs may represent tissue Treg populations that expand in response to the inflammatory insult, are recruited Tregs from other sites, or develop as newly-induced pTregs. Recruitment is likely regulated by the expression of chemokine receptors. CCR4 appears to direct Treg migration to a number of tumors and tissues (Curiel et al., 2004; Faget et al., 2011), while CCR5 has been shown to bring Tregs to the skin and pancreas (Schlecker et al., 2012; Tan et al., 2009). Since chemokine receptors can be associated with a larger program of gene expression, they likely not only direct the migration of Tregs, but may also be indicative of their function. High levels of TGFβ in the tumor microenvironment are also conducive to the induction of pTregs (Adeegbe and Nishikawa, 2013). Treg-derived IL-10 and TGFβ can further induce more Tregs, or convert some naive CD4+ T cells into Tr1 cells. DCs that have become tolerogenic due to their interaction with Tregs can also further promote Treg development by providing suboptimal priming and secreting immunosuppressive factors.

A number of studies using mouse models have provided strong evidence that Tregs promote tumor evasion. Most groups have modelled tumor development in transgenic mice where the diphtheria toxin receptor (DTR) has either been inserted into the 3’UTR of Foxp3, or is under the control of the Foxp3 promoter in a BAC transgene. As a result, temporally and spatially controlled Treg depletion can be achieved through the administration of diphtheria toxin (DT). Using this system, Tregs have been depleted in mouse models of melanoma, colon cancer, lung adenocarcinoma, and breast cancer (Bos et al., 2013; Joshi et al., 2015; Klages et al., 2010; Pastille et al., 2014; Teng et al.,...
Treg depletion invariably results in immune infiltration and regression of established tumors, even in oncogene-driven tumors where there are few, or no, tumor antigens (Bos et al., 2013; Joshi et al., 2015). Polyclonal CD4 and CD4+ T cells infiltrate tumors after Treg depletion, although it may be difficult to interpret immune responses that are occurring to the tumor, and those due to systemic loss of Tregs.

Mouse models first suggested that the efficacy of anti-CTLA-4 may be due in part to depletion of Tregs (Simpson et al., 2013). This hypothesis may be supported by the findings that patients who have responded to CTLA-4 have increased tumoral Tconv:Treg and CD8:Treg ratios (Hodi et al., 2008; Liakou et al., 2008). Low dose cyclophosphamide can also selectively deplete Tregs, raising interest in developing dosing strategies to augment immunotherapy in patients (Ghiringhelli et al., 2004, 2007). Anti-GITR agonistic antibodies also appear to disrupt Treg suppressive function (Ko et al., 2005; McHugh et al., 2002/2; Shimizu et al., 2002; Stephens et al., 2004). Treg depletion has also been achieved through intratumoral injection of anti-CTLA-4 and anti-OX40 along with CpG, suggesting that local, rather than systemic, Treg depletion may be sufficient to eradicate tumors provided there are agonists for an anti-tumor immune response (Marabelle et al., 2013). Attention is now turning to using markers of effector Tregs, like CCR4, to selectively deplete Tregs in tumors (Faget et al., 2011; Sugiyama et al., 2013).

Since tumors are characterized by a particular tissue context and inflammatory milieu (Fridman et al., 2012), tumor Tregs may have distinct transcriptional features that can be used to identify and inhibit this population specifically. For example, a specialized, pro-inflammatory population of RORγT+ Tregs has been shown to promote...
tumor development in the colon in setting of chronic inflammation (Blatner et al., 2012), while CCR4 marks an activated population of Tregs recruited to breast tumors (Gobert et al., 2009). This study and others also point to spatial localization as a determinant of Treg contribution to tumor immunity. Treg association with lymphoid tissue, but not residence in the tumor, was found to have a prognostic effect in breast cancer, which may be consistent with Tregs acting in tertiary lymphoid structures (Joshi et al., 2015). Similarly, another group noted that high tumoral FOXP3+ cell density in colon cancer was a favorable prognostic factor, but high densities of these cells in the normal tissue was associated with worse outcome (Salama et al., 2009). The differences in Treg localization are likely associated with distinct transcriptional programs, which may also confound any potential association of Foxp3 expression with cancer phenotypes. A recent study showed that a subset of colorectal cancer patients had tumors infiltrated by a Foxp3^{low} Treg population that was less suppressive, and perhaps less stable, in addition to a Foxp3^{high} effector Treg population (Saito et al., 2016). While Foxp3 expression was a favorable prognostic marker in this group, it was associated with poor outcome in other patients. Ignoring the functional heterogeneity of the Foxp3+ population in this cohort would have precluded a clear understanding of the role of Tregs in this particular tumor type.

**Profiling Treg cell and other immune cell heterogeneity in cancer**

In recognition of the heterogeneity of Treg and other immune cell populations, and in order to understand better their roles in tumor immune responses, many groups are actively using advances in sequencing technology to profile immune and tumor cell populations. While older single-cell approaches like flow cytometry and
immunofluorescence enable characterization of phenotypic heterogeneity, they are limited by available reagents and the relatively small number of parameters that can be studied at once. RNA sequencing has allowed unbiased transcriptional profiling of bulk tumor and immune cell populations. Bulk tumor transcriptomes have not only provided insight on human cancer biology, but have also been mined for the information they provide on the immune, stromal, and normal epithelial cells that were simultaneously sequenced. For example, normal and stromal cells represent a significant fraction (20-25%) of the bulk tumor samples that have been collected for genomic characterization by The Cancer Genome Atlas (TCGA) and other consortia (Aran et al., 2015), which allows pairing of tumor molecular characteristics and immune infiltrate through application of immune cell gene expression signatures (Charoentong et al., 2017; Rooney et al., 2015; Thorsson et al., 2018). A method has also been developed to reconstruct the immune milieu of tumors by applying known immune cell gene expression signatures (Newman et al., 2015). In some cases, these data have prompted the discovery of tumor-intrinsic factors regulating tumor evasion (Spranger et al., 2015). Meanwhile, the bulk transcriptome of purified populations of tumor-associated immune cells has also provided insight on tumor immune responses. Profiling studies of tumor-infiltrating CD4+ T cells have been able to identify major transcriptional differences between tumor and peripheral blood or LN cells (Gu-Trantien et al., 2013). Recently, transcriptional profiling of Treg and Tconv cells isolated from human breast, lung, and colorectal cancer have revealed that these cells are distinct from peripheral blood Tregs and express various markers that may be associated with clinical outcome (De Simone et al., 2016; Plitas et al., 2016). Since these populations
were sequenced in bulk, however, the heterogeneity of these populations could not be studied.

An appreciation of the diversity and frequency of distinct cell phenotypes is important for understanding the factors that determine a productive anti-tumor immune response. The development of single-cell RNA sequencing (scRNAseq) has allowed unbiased analyses of molecular heterogeneity among immune and other cell populations (Stubbington et al., 2017). Early single-cell profiling of bone marrow derived dendritic cells responding to LPS revealed considerable heterogeneity in a uniform-appearing cell population responding to a stimulus (Shalek et al., 2013). Technical advances allowing greater numbers of cells to be sequenced simultaneously has led to ever more ambitious profiling; bulk tumors from patients may now be sequenced without needing to isolate populations, generating a comprehensive picture of cellular diversity (Macosko et al., 2015). Spatial transcriptomic techniques are also under development that will allow pairing of transcriptional profile with localization data, which would allow resolution correlation of phenotype with physical cellular interactions (Lein et al., 2017).

scRNAseq has proven invaluable in identifying cellular diversity and resolving often subtle shifts in phenotypic state in CD4+ T cell populations (Gaublomme et al., 2015; Lönnberg et al., 2017; Mahata et al., 2014; Patil et al., 2018). These studies have identified rare precursors of CD4+ CTL and steroidogenic Th2 cells (Mahata et al., 2014; Patil et al., 2018), lineage relationships between Th1/Tr1 cells (Lönnberg et al., 2017), and genes governing Th17 pathogenicity (Gaublomme et al., 2015). Comprehensive profiling of human tumors has also revealed considerable diversity in immune populations and tumor cells (Tirosh et al., 2016). A major limitation of this
method, however, is that patient samples represent single snapshots of a tumor in time, which may not capture the temporal evolution of tumor evasion. Profiling of mouse models that allow sampling over the course of tumor development may help contextualize findings from patients. Nevertheless, by combining TCR and expression data, shifts in cell transition can be mapped, which helps build an understanding of the dynamics of the immune tumor environment (Miragaia et al., 2017; Zheng et al., 2017). Furthermore, a recent study of exhausted CD8+ T cells demonstrates how closely-linked phenotypes can be finely distinguished through scRNAseq, perhaps allowing the individual pathways to be targeted (Singer et al., 2017). Recently, mouse Tregs were profiled by scRNAseq, revealing a core Treg transcriptome upon which additional programs are superimposed due to TCR signaling, tissue residence, or other cues (Miragaia et al., 2017; Zemmour et al., 2018). The heterogeneity and plasticity of CD4+ T cell populations have posed great challenges to understanding their role in tumor immunity and identifying tools to target them. scRNAseq may provide the resolution needed to resolve the contributions of particular CD4 phenotypes to tumor evasion.

IV. Role of ST2 in Treg biology and cancer

Overview of ST2/IL-33 biology

ST2 is an interleukin 1 receptor-like molecule encoded by the gene Il1rl1. Two isoforms of ST2 are well-described—a membrane-bound receptor and a soluble receptor lacking the transmembrane domain (sST2) (Bergers et al., 1994; Li et al., 2000) (Figure 6). The only known ligand for ST2 is interleukin-33, which binds ST2 in a complex with IL1RacP, another IL-1R family member that also aids in the binding of IL-
1 to IL-1R (Lingel et al., 2009; Liu et al., 2013; Schmitz et al., 2005). Formation of the heterotrimeric complex results in signaling through MyD88 and the activation of NF-κB. The direct transcriptional targets of ST2 signaling remain unclear, although IL-33 signaling has been shown to induce the expression of IL-4, IL-5, IL-13, and amphiregulin. sST2 acts as a decoy receptor that competes with ST2 for ligand binding (Hayakawa et al., 2007).

Epithelial and endothelial cells are the major sources of IL-33, which is an alarmin that is released during cell injury to signal danger to the immune system (Cayrol and Girard, 2014; Pichery et al., 2012). IL-33 is localized in the nucleus and has been found to associate with histones, although a clear transcriptional role for the protein has not been defined (Carriere et al., 2007). Instead, the nuclear localization of IL-33 is thought to sequester it from the cytosol, preventing accidental release into the extracellular space. In support of that theory, altered localization of IL-33 results in chronic ST2-dependent inflammation (Bessa et al., 2014). While full-length IL-33 is active, it is also the target of multiple proteases that can regulate its function (Liew et al., 2016). Interestingly, mast cell and neutrophil-derived proteases can cleave IL-33 into a shorter form with greater activity, while apoptotic caspases cleave IL-33 into an inactive form. This mechanism may allow cells undergoing apoptosis in noninflammatory settings to avoid release of active IL-33.

ST2 expression has been observed in many cell types, including CD4+ T cells, mast cells, neutrophils, macrophages, innate lymphoid cells type 2 (ILC2s), eosinophils, basophils, NK cells, and NK T cells (Griesenauer and Paczesny, 2017). ST2 was first described as a driver of Th2 responses due to its induction of classical Th2 cytokines
(Meisel et al., 2001). Indeed, antibody-mediated blockade of ST2 results in reduced Th2 inflammation in models of asthma (Byers et al., 2013), allergy (Willart et al., 2012), and lung infection (Chang et al., 2011). ST2 is also expressed by Th9 cells, which respond to IL-33 by making more IL-9 and ST2 (Blom et al., 2011). IL-33 signaling on ILC2s has also been shown to be critical for promoting tissue regeneration after influenza infection in the lung (Monticelli et al., 2011), suggesting that IL-33 may coordinate both allergic inflammation and wound repair in the lung. Curiously, IL-33 has also been shown to augment CD8+ T cell function, particularly in the context of viral infections and vaccination (Bonilla et al., 2012).

**ST2/IL-33 promotes the expansion of tissue Tregs**

Recently, IL-33 has also been described to enhance the proliferation and maintenance of ST2-expressing Tregs in tissue. IL-33 signaling was shown to expand a Treg population that helped promote cardiac allograft survival (Turnquist et al., 2011). ST2+ Tregs residing in the colon lamina propria were critical for preventing adoptive transfer-mediated colitis (Schiering et al., 2014). VAT Tregs also express ST2, and ST2-deficient mice have a dramatic reduction in their VAT Treg population (Kolodin et al., 2015). IL-33-responsive ILCs have also been described in VAT, and it remains unclear whether IL-33 promotes maintenance of the VAT Treg population directly, or through its effects on ILCs (Molofsky et al., 2015). IL-33 can also improve the recruitment of Tregs to skeletal muscle after injury, which is normally impaired in aged mice (Kuswanto et al., 2016). Of note, Tregs have also been shown to traffic to the draining lymph node and site of myocardial infarctions, where they are important for tissue repair (Nahrendorf and Swirski, 2014). It remains unclear whether the recruitment of these cells is ST2-
dependent, although sST2 is under active investigation as a cardiac biomarker because serum sST2 levels increase in response in cardiomyocyte perturbation, including in infarction and congestive heart failure (Griesenauer and Paczesny, 2017).

A number of studies have shown that ST2 signaling can promote wound healing through Treg activity after lung inflammation. In an allergy model, IL-33 prompted mast cells to produce IL-2, which expanded the Treg population, helping to limit inflammation (Morita et al., 2015). Areg+ Tregs were shown to be critical for tissue repair after influenza infection in the lung, and IL-33 and IL-18 could both promote TCR-independent expansion of this Treg population (Arpaia et al., 2015). Recent reports suggest that IL-33-stimulated Tregs upregulate GATA3, and express Th2-associated cytokines (Chen et al., 2017; Siede et al., 2016). ST2+ Tregs were also shown to be more suppressive in vitro than ST2- Tregs isolated from wild-type mice, although this is confounded by the fact that ST2+ Tregs are more likely to be effector aTregs (Siede et al., 2016). In contrast, an earlier study had shown no difference in the in vitro suppressive activity of ST2-deficient Tregs compared to wild-type controls (Schiering et al., 2014).

Interestingly, Treg-specific ablation of IRF4, a regulator of the Treg transcriptional program, results in less expression of ST2 (Zheng et al., 2009). This is consistent with the observation that IRF4 and BATF coordinate with Foxp3 to drive expression of ST2 and Ppary in VAT Tregs (Vasanthakumar et al., 2015). GATA-3 may also play a role in promoting ST2 expression, since the transcription factors GATA-3 and PU.1 appear to be drivers of ST2 expression in Th2, Th9, and ILC2 cells (Griesenauer and Paczesny, 2017).
Role of IL-33 in cancer

Based on its established role in promoting wound repair, one would expect that IL-33 signaling would drive tumor growth. Indeed, in one study of transplantable breast cancer showed that while ST2 deficiency resulted in more infiltration of tumors by proinflammatory cells, administration of IL-33 enhanced immunosuppression by recruiting Tregs and ILC2s (Jovanovic et al., 2014). Recent studies of NSCLC cell lines and xenografts showed that IL-33 recruits type 2 (M2) macrophages and Treg cells (Wang et al., 2016, 2017). In a model of Apc\(^{min}\)-driven colon cancer, IL-33 stimulated myofibroblasts to secrete growth factors, contributing to polyposis (Maywald et al., 2015).

Other studies point to ST2 signaling on tumor cells as a direct driver of transformation and tumorigenesis. IL-33 was shown to promote transformation in breast cancer, as well as directly drive the growth of a myeloproliferative neoplasm (Kim et al., 2015; Mager et al., 2015). In NSCLC lines, IL-33 increased GLUT1 expression, resulting in increased glucose uptake and proliferative ability (Wang et al., 2016). IL-33 was also implicated as a mechanism of resistance to CML; imatinib treatment normalizes ST2 expression on CML progenitor cells, which can then proliferate in response to IL-33 signaling (Levescot et al., 2014).

There are some reports that ST2 may also drive anti-tumor immunity in some contexts. Gao et al. have shown that IL-33 increases the activity of CD8 and NK cells, leading to tumor clearance (Gao et al., 2015). Interestingly, IL-33 administration had a synergistic effect with Treg depletion, suggesting that Treg depletion may head off the pro-immunosuppressive effects of ligand. Exogenous IL-33 was also shown to improve
antigen-specific T cell activity when used as an adjuvant to a cancer vaccine in a human papillomavirus (HPV)-associated tumor model (Villarreal et al., 2014). Another study using exogenous IL-33 demonstrated that IL-33 can stimulate DC expansion and maturation, thus aiding anti-tumor immune responses (Dominguez et al., 2017). In a model of squamous cell lung cancer, tumor cell expression of IL-33 drove expression of sST2, which sequestered extracellular IL-33, preventing IL-33-mediated stimulation of CD8+ T cell responses (Serrels et al., 2017). These conflicting reports on the role of IL-33 in cancer are most likely due its pleiotropic and context-specific functions. Furthermore, many of these studies rely on the use of exogenous, recombinant IL-33, which may far exceed physiological levels and do not undergo the same post-translational modifications (Wasmer and Krebs, 2016).
Figure 6. Overview of the IL-33/ST2 signaling pathway
IL-33 is sequestered in the nucleus, and released upon cell death or stress. Some mast cell and neutrophil-derived extracellular proteases can cleave IL-33, releasing a more active form containing just the cytokine domain. Inactivating proteases can also cleave IL-33 within the cytokine domain, abrogating its signaling function. Full-length or the cytokine domain of IL-33 forms a heterotrimeric complex with ST2 and IL1RAcP, and signaling is initiated through MyD88. Proposed targets of ST2 signaling include ST2, Areg, and Th2 cytokines. Soluble ST2 is an sST2 isoform that lacks the region encoding the transmembrane domain and, as a result, likely functions as a decoy receptor.
V. Conclusions/ Summary

The parallel histories of cancer immunosurveillance and suppressor T cell biology exemplify how science can be heavily influenced by prevailing popular opinion. Both fields went through periods of immense excitement, followed by recoil and skepticism after major setbacks. It is undeniable that the resurgence of both fields came on the heels of technological breakthroughs that could instill confidence in new findings. Nevertheless, both cancer immunology and suppressor T cells were championed by scientists and physicians who remained open-minded while also doggedly pursuing their research interests, however unpopular.

By the time Schreiber and colleagues had established experimental evidence for cancer immunosurveillance in the mouse, decades of clinical observation and trials had suggested that tumor development is associated with an ongoing immune response. Improvements in our understanding of peripheral tolerance and the contribution of inflammation to wound healing provided a means to understand how tumors could not only avoid immune destruction, but adapt immune responses to suit their growth. Mechanisms of tumor evasion are diverse and self-reinforcing, complicating both efforts to model and dismantle strong immunosuppression in cancer. Although aspects of tumor evasion are recapitulated in transplantable models of cancer, autochthonous genetic mouse models of cancer are most likely to capture the breadth and depth of cancer immunosuppression that evolves alongside tumor development. In this thesis, I have used a model of autochthonous, inducible lung adenocarcinoma driven by oncogenic K-ras and loss of p53 (“KP”) to study the role of CD4+ T cells, in particular Tregs, in an endogenous tumor immune response.
Efforts to understand the contribution of CD4+ T cells to tumor immune tolerance are further obscured by their immense phenotypic heterogeneity. The recent development of scRNAseq has enabled unbiased and comprehensive profiling of immune cells in tumors and other contexts. With this technology, researchers have been able to identify rare subpopulations and capture dynamic shifts in cell state that may represent responses to environmental cues or intrinsic stochasticity. In Chapter 2, I will describe efforts to use bulk and scRNAseq to profile CD4+ T cells longitudinally in the KP model. We show that the endogenous CD4+ T cell response is heterogeneous but demonstrates shifts in phenotype over time that could be consistent with strengthening immunosuppression. In Chapter 3, we identify ST2 as a possible driver of Treg function in tumors, and use genetic tools to determine the effect of ST2 deletion on tumor development and Treg phenotype. In Chapter 4, I will discuss ongoing and future work, as well as the implications of our findings.
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CHAPTER 2

Longitudinal mapping of CD4+ T cell heterogeneity in cancer

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ABSTRACT

Various CD4+ T cell populations can impair anti-tumor immune responses and are associated with poor prognosis in multiple cancer types. We show that a gene expression signature derived from lung regulatory T cells (Tregs) in a genetic mouse model of lung adenocarcinoma is associated with clinical prognosis in human cancer. However, other CD4+ T cell populations may contribute to anti-tumor cytotoxicity, or otherwise be required to maintain tissue tolerance. Our limited understanding of the complexity and evolution of CD4+ T cell responses in cancer prevents targeted manipulation of tumor-promoting CD4+ T cells. Here, we use single-cell RNA sequencing to profile conventional CD4+ T (Tconv) and Treg cells longitudinally in our tumor model. We demonstrate that tumor-associated Treg and Tconv cells are highly diverse and undergo major shifts towards increasingly immunosuppressive phenotypes during tumor progression. We propose that longitudinal profiling of CD4+ T cell heterogeneity in tumors enables the identification of specific cell populations responsible for maintaining tumor immune tolerance, and can facilitate their subsequent inhibition.
INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide (Molina et al., 2008). In spite of therapeutic advances, the 5-year total survival rate of patients diagnosed with non-small cell lung cancer (NSCLC), which accounts for ~85% of lung cancer cases, is 21%. The recent clinical success of immune checkpoint inhibitors in the treatment of NSCLC provides proof-of-principle that targeting mechanisms of immunosuppression in the tumor microenvironment may be an effective therapeutic strategy (Makkouk and Weiner, 2015; Soria et al., 2015). Nevertheless, only a subset of patients respond to immune therapies, suggesting that an improved understanding of immunosuppressive mechanisms is needed for effective treatment.

Conventional, Foxp3-negative CD4+ T (Tconv) cells are recruited to the site of tumors and demonstrate both anti-tumor activity and tumor-promoting effects, likely due to their wide diversity of phenotypes and effector functions (Dobrzanski, 2013). Type 17 (Th17) CD4+ T cells directly promote tumor growth, while type 1 (Th1) CD4+ T cells may help clear tumors through their secretion of immunostimulatory interferon gamma (IFNγ). Regulatory T cells, which are critical for maintaining peripheral immune tolerance and preventing autoimmunity (Josefowicz et al., 2012; Sakaguchi, 2011), are the CD4+ T cell subset most implicated in driving tumor immune suppression. Tregs are characterized by their expression of the transcriptional regulator Foxp3 and can inhibit adaptive immune responses through the production of inhibitory cytokines, direct killing of cells, competition with other T cell subsets for antigen or other substrates, and suppression of antigen presentation (Caridade et al., 2013; Savage et al., 2013; Vignali et al., 2008). Recent evidence suggests that Tregs can also promote tumor growth by
suppressing anti-tumor immune responses (Tanaka and Sakaguchi, 2017). Tregs are associated with poor prognosis in a number of cancers, including lung adenocarcinoma, which accounts for 40% of NSCLC (Fridman et al., 2012; Petersen et al., 2006; Shang et al., 2015; Shimizu et al., 2010; Suzuki et al., 2013). Furthermore, mouse models indicate that Treg depletion can enhance anti-tumor immunity (Bos et al., 2013; Joshi et al., 2015; Marabelle et al., 2013), and that antibodies directed against CTLA-4, which is expressed highly on Tregs, act in part by depleting Tregs in the tumor microenvironment (Simpson et al., 2013). Therefore, targeting the function of CD4+ T cells, particularly Treg cells, in tumors may complement other cancer therapies by alleviating tumor immunosuppression and improving the immune response against cancer. Since indiscriminate Treg inhibition would be expected to cause systemic and potentially lethal autoimmunity, an ideal therapeutic strategy targeting this population would be directed specifically toward Tregs in the tumor microenvironment.

CD4+ T cell differentiation is associated with distinct gene expression programs that are dependent on extracellular signals and epigenetic state (Zhu et al., 2010). The transcriptional heterogeneity of this population may thus provide a means for specific targeting of tumor CD4+ T cells that can enhance their activity against tumors and/or inhibit their immunosuppressive functions. Treg cells have also been shown to “adapt” to their surroundings by adopting aspects of the transcriptional program of the proinflammatory cells they are responding to, resulting in an enhanced ability to suppress specific types of immune responses (Chaudhry et al., 2009; Chung et al., 2011; Linterman et al., 2011; Zheng et al., 2009). Populations of Tregs with distinct transcriptional profiles have also been observed in particular tissues that appear to have
functions tailored to their environment (Cipolletta et al., 2012; Feuerer et al., 2009a, 2009b; Kolodin et al., 2015). For example, adipose tissue-resident Tregs help maintain insulin sensitivity (Cipolletta et al., 2012; Feuerer et al., 2009a; Kolodin et al., 2015) and Tregs responding to muscle injury stimulate tissue repair (Burzyn et al., 2013; Kuswanto et al., 2016). Recently, a lung Treg population with a distinct transcriptional program was shown to be important for tissue repair in the lung following influenza infection in mice (Arpaia et al., 2015). Tregs in tumors, which are each characterized by a particular tissue context and inflammatory milieu (Fridman et al., 2012), may thus have unique transcriptional features that can be used to identify and inhibit this population specifically. For example, a recent study showed that Foxp3 expression is associated with better prognosis in colorectal cancer patients whose tumors were simultaneously infiltrated by Foxp3\textsuperscript{low}, poorly-suppressive CD4 cells in addition to Foxp3\textsuperscript{high} effector Treg cells (Saito et al., 2016). Foxp3 expression, however, was associated with poor prognosis in patients with tumors that lack Foxp3\textsuperscript{low} cells, which may be because Foxp3\textsuperscript{low} cells represent an intermediate or unstable Treg phenotype. Therefore, Treg functional heterogeneity must be considered to determine their contribution to clinical outcome in cancer properly. Furthermore, transcriptional profiling of Tregs isolated from human breast, lung, and colorectal cancer have revealed that these cells are distinct from peripheral blood Tregs and express various markers that may be associated with clinical outcome (De Simone et al., 2016; Plitas et al., 2016). However, the expression of single genes may not be a sensitive indicator of effector Treg activity across a wide range of tumors, and it remains unclear whether a core set of pathways is associated with strong Treg immunosuppression in tumors. Understanding the transcriptional
changes that accompany tumor-associated Treg activity is thus essential to improving strategies to block tumor immunosuppression.

Inducible, autochthonous models of cancer are ideal for studying mechanisms of tumor tolerance because they recapitulate the longitudinal development of the tumor in situ and associated immune responses. These tumor models are also more likely to capture the aggressive immunosuppression of the endogenous tumor microenvironment than transplanted tumors, which may inherently appear more “foreign” (Dranoff, 2011). Our laboratory has developed a model of lung adenocarcinoma in which activation of oncogenic K-rasG12D and loss of Trp53 is driven by intratracheal delivery of a lentivirus expressing Cre recombinase (KP: LSL-KrasG12D, p53<sup>fl/fl</sup>) (DuPage et al., 2009; Jackson et al., 2005). By using lentivirus that also expresses firefly luciferase fused to chicken ovalbumin (Ova) and the antigenic peptide SIYRYYGL (Lenti-LucOS), we can program tumors to express known T cell antigens that can be used to monitor tumor-specific T cell responses (DuPage et al., 2011a). Prior studies using this model have shown that T cell infiltration of Ova-expressing tumors delays tumor growth, but the numbers and activity of anti-tumor cytotoxic CD8+ T cells (CTLs) cells decline over time. The development of immune tolerance towards the tumor is due in part to the expansion of lung-resident Treg cells that express various markers of effector activity and terminal differentiation. Treg depletion results in massive infiltration of lungs by CD4+ and CD8+ T cells, suggesting that Tregs actively suppress anti-tumor immune responses. Although Treg-depleted animals succumb to systemic autoimmunity, these findings suggest that a strategy targeting lung tumor-specific Tregs may be able to enhance anti-tumor immune responses while minimizing self-directed cytotoxicity.
In this study, we show that lung tumor-associated Treg cells in the KP model have a distinct transcriptional signature that shares characteristics with previously described tissue-resident Treg cell populations and is associated with survival across multiple cancer types. Using single-cell RNA sequencing (scRNAseq), we have longitudinally mapped transcriptionally diverse subsets of conventional CD4+ T (Tconv) and Treg cells during tumor development. We show that while some Tconv subsets are stable throughout tumor progression, highly activated and differentiated Th1-like populations expand in late tumors. Meanwhile, Treg cells at early timepoints appear less differentiated and express effector CD4+ T cell genes, but tumor progression coincides with the expansion of a terminally-differentiated Treg cell phenotype. We propose that an improved understanding of CD4+ T cell heterogeneity in the tumor microenvironment enables effective and fine targeting of critical mediators of the tumor immune response.

RESULTS

Lung Treg cells in the KP model display an activated, effector phenotype and are similar to other tissue Treg populations

Our group has previously demonstrated that tumor development in the KP model is associated with the expansion of lung-resident Treg cells, a large proportion of which express CD103 (integrin αE) and killer cell lectin-like receptor 1 (KLRG1), which have been associated with Treg effector activity and terminal differentiation, respectively (Beyersdorf et al., 2007; Cheng et al., 2012; Huehn et al., 2004; Lehmann et al., 2002; Sather et al., 2007). Although Treg cells early in tumor development are predominantly CD103-KLRG1- (double-negative, DN), tumor progression is associated with an
expansion of CD103+KLRG1- (single-positive, SP) Tregs and CD103+KLRG1+ (double-positive, DP) Tregs (Figure 1a), suggesting that tumor growth is accompanied by an increase in the proportion of effector Treg cells. DP Treg cells in late-stage tumor-bearing mice have greater expression of various molecules associated with greater Treg cell activity, including GITR, CD39, and PD-1, than SP and DN Treg cells, suggesting that these Treg cell subsets may represent Treg functional heterogeneity (Joshi et al., 2015).

We hypothesized that Treg cells associated with KP tumors may have an altered transcriptional phenotype that reflects both tissue residence and varying expression of markers of strong immunosuppressive activity. We bred KP mice to Foxp3RFP, Foxp3GFP/DTRCD451/1, or Foxp3GFP mice to facilitate isolation and manipulation of Treg cell populations from tumor-bearing mice. We collected DP, SP and DN Tregs from the lungs of tumor-bearing KP, Foxp3RFP mice at week 20 post infection (p.i.) with LentivirUSOS for RNA sequencing (Figure 1b). Systemic populations of Tregs, including SP and DN Tregs from the draining (mediastinal) lymph node (msLN) of the same mice, as well as DN Tregs from the spleen of one tumor-bearing animal, were also sequenced for comparison. Unsupervised hierarchical clustering showed that the Tregs isolated from tumor-bearing lung clustered separately from msLN and splenic Tregs (Figure S1a). Furthermore, independent component analysis (ICA), which elucidates signatures that best explain gene expression differences within this dataset, indicated that the most significant gene signature within the data distinguished lung-resident Tregs from systemic populations of Tregs (Figure S1b). Although ICA also identified a weaker, but significant, gene expression signature distinguishing CD103+ lung and msLN Tregs.
from all other CD103- Treg populations, lung and msLN Tregs did not co-cluster based on CD103 and KLRG1 status (Figure S1a).

We sought to understand better the transcriptional features of lung Treg cells from tumor-bearing KP mice. After filtering for lowly expressed genes, 284 genes were found to be strongly correlated with the signature distinguishing lung Tregs in KP mice (|z-score| > 3), and to have 2-fold or greater expression in lung vs. msLN and spleen Treg cells, or vice versa, hereafter referred to as "KPLungTR signature genes" (Figure 1c). To validate these findings, we analyzed the expression of selected KPLungTR signature genes in Tregs from tumor-bearing KP, Foxp3GFP/DTR mice and found that protein expression of Tim3, Gzmb, and IL-17a were indeed higher in lung-resident Tregs (Figure S1c). Furthermore, quantitative RT-PCR (qPCR) analysis of Tregs isolated from KP, Foxp3GFP/DTR mice confirmed differential expression of a number of transcriptional regulators in lung Tregs as compared to msLN and splenic Tregs, including Pparg1, Nr4a1, and Gata1 (Figure S1d). Although a number of KPLungTR signature genes have been previously associated with Treg immunosuppressive activity, it is notable that Foxp3 expression levels are not significantly altered in lung Tregs from tumor-bearing mice (Figure S1e).

We next asked whether similarities between the KPLungTR signature and the transcriptional profiles of other previously-described Treg populations could inform our understanding of immune responses in the KP tumor model. We performed gene set enrichment analysis (GSEA) (Subramanian et al., 2005) of the KPLungTR signature using a version of the molecular signatures database (MSigDB) updated to include additional Treg-associated gene sets (Supplementary Table 1). This analysis
demonstrated strong enrichment for gene sets associated with Tregs found in other tissues, including visceral adipose tissue (VAT), colonic lamina propria, and wounded muscle (Figure 1d). Although no more than 20% of the genes in any of the three tissue Treg gene sets are shared with another tissue Treg gene set, these tissue Treg gene sets have significantly more shared genes than would be expected by chance (Fisher's exact test, \( p < 2.5\times10^{-6}, \text{OR} > 6 \)), suggesting that tissue Tregs, including lung tumor-associated Tregs, have a core transcriptional phenotype that corresponds with the enhanced effector activity expected of Tregs retained in tissue. Genes upregulated in the KPLungTR signature (KPLungTR_UP) were also enriched for many gene sets associated with activation, differentiation, and possible Treg effector functions (Figure S2). In addition to expressing genes associated with T cell receptor (TCR) signaling, KP lung Treg cells also expressed STAT5a and IRF4 targets, suggestive of lineage commitment, as well as chemokine receptors, integrin signaling targets, and HIF-1α targets indicative of tissue migration and residence. Consistent with prior reports that Treg cells in wounded muscle and influenza-infected lungs can secrete growth factors that are critical for tissue repair (Arpaia et al., 2015; Burzyn et al., 2013), KPLungTR_UP was also enriched for gene sets associated with EGF, IGF, and TGFβ signaling. Thus, lung Treg cells appear to have a common “tissue Treg” cell phenotype that is characterized by effector differentiation and the ability to promote tissue growth in response to injury.

We reasoned that lung Treg cells from healthy mice, and mice bearing less immunogenic tumors, would also demonstrate differential expression of KPLungTR signature genes due to their maintenance in the lung tissue environment. To test this
hypothesis, we performed RNA sequencing on lung and systemic Tregs from naive KP mice, and KP mice infected with a lentivirus expressing only Cre recombinase (Lenti-Cre). Treg cells were also collected from $p53^{\text{floxed}}$ (P-only) mice infected with Lenti-Cre and Lenti-LucOS, which do not develop tumors, to control for changes in Treg phenotype that may be due to inflammation associated with lentiviral infection and/or the expression of strong T cell antigens. ICA signature analysis of lung, msLN, and splenic Tregs from KP mice across various conditions demonstrated that lung-resident Tregs have a distinct transcriptional profile compared to systemic Treg populations regardless of the presence of tumors, or of antigen expression by tumors (Figure S3a). Indeed, GSEA confirms that the gene expression signature describing lung-resident Tregs is highly enriched for KPLungTR signature genes (Figure S3b). To contextualize further the lung Treg population observed in KP mice, we expanded our dataset to include publicly available RNA sequencing data from Tregs from a range of genotypic, tissue, and inflammatory settings (Supplementary Table 1). In spite of differences in sample preparation and sequencing parameters, ICA analysis across this extended dataset again identified a significant gene expression signature distinguishing lung Tregs and, to an intermediate degree, Blimp1-positive splenic Tregs from all other splenic and and lymph node Treg populations (Figure S3c). GSEA of this “pan-lung” Treg signature also revealed strong enrichment for genes found in the KPLungTR signature (Figure S3d).

In order to determine the clinical relevance of the KPLungTR signature, and of using a mouse model of lung adenocarcinoma to study tumor Treg responses, we compared the KPLungTR signature to differentially expressed genes described in Treg
cell populations profiled from human cancers. Interestingly, genes upregulated in colon and NSCLC-associated Tregs (De Simone et al., 2016) were also highly expressed in KP lung Tregs (Figure S4). Treg cells from the KP model and human lung and colon cancer Tregs may therefore have a common program of upregulated genes that confers their effector activity.

The KPLungTR signature is correlated with survival in multiple cancers and marks highly inflamed tumors

Many studies on the link between Tregs and clinical outcome in cancer have focused on the density and location of Tregs and/or their expression of a handful of selected markers, but cell numbers with limited phenotypic information may not be comprehensive or reliable indicators of tumor or tissue-associated Treg activity. Consequently, we examined whether having a gene expression profile correlated with the KPLungTR signature was associated with differential survival in a large dataset of primary human lung adenocarcinomas (LUAD) from The Cancer Genome Atlas (TCGA). In order to limit the influence of tumor cell-intrinsic expression patterns on KPLungTR signature enrichment analyses performed on bulk tumor transcriptomes, we first omitted 7 genes from the signature that are also differentially expressed in lung adenocarcinoma tumors compared to matched normal tissue (Methods). Using single-sample GSEA (Barbie et al., 2009), we scored the bulk transcriptomes of 515 lung adenocarcinomas for their expression of the edited set of KPLungTR signature genes, which included 186 upregulated and 91 downregulated genes in lung Tregs vs. msLN and splenic Tregs. We found that the highest-scoring 15% of patients had significantly
worse 5-year survival (p=0.00023, log-rank test) than the rest of the LUAD cohort (Figure 1e). When applied to other tumor types in TCGA, the KPLungTR signature was also associated with significantly worse 5-year survival in lung squamous cell carcinoma, pancreatic ductal adenocarcinoma, large grade glioma, and head and neck squamous cell carcinoma. Interestingly, the KPLungTR signature was associated with improved survival in colorectal adenocarcinoma (COAD), which is consistent with prior reports that Treg cells are a favorable prognostic factor in this particular tumor type (Xu et al., 2017). These findings suggest that expression of the KPLungTR signature does not simply track with more severe disease, but reflects the presence of a particular, tumor-associated Treg response, which has variable prognostic significance in different tumor types.

To understand further the features of lung adenocarcinomas that score highly for the KPLungTR signature, we compared the highest-scoring 25% (LTR_Hi, n=128), and the lowest-scoring 25% (LTR_Low, n=128) of tumors. The 5-year survival of the LTR_Hi group was significantly lower (p=0.00369, log-rank test) than that of LTR_Low patients (Figure S5a). We reasoned that the worse survival of the LTR_Hi lung adenocarcinomas could be due to other factors, including greater disease severity, lower levels of anti-tumor immune activity, or other molecular features enriched in the tumor subset. Indeed, LTR_Low tumors included a greater fraction of female patients (p=0.0042, hypergeometric test), as well as patients with less advanced T1/T2, N0, or stage I/II disease (Figure S5b-d), while the LTR_Hi group was enriched for patients with T3/T4 disease (p=0.0143, Fisher’s exact test). The LTR_Hi tumors were enriched for patients with recent smoking history (p=0.0037, Fisher’s exact test), while the
LTR_Low group was enriched for non-smokers (p=0.0061, Fisher’s exact test), which is consistent with their higher proportion of patients with a low prevalence of transversion mutations (p=0.0047, Fisher’s exact test) (Figure S5f-g). Nevertheless, the total number of protein-altering mutations and predicted neoepitope count were similar between LTR_Hi and LTRLow tumors, suggesting that neoantigen load is not responsible for the difference in disease severity or immune phenotype (data not shown). To determine whether LTRLow tumors were more heavily infiltrated by immune cells, we used tumor purity estimates from ABSOLUTE, which uses copy number variation to deduce tumor cell fraction (Carter et al., 2012), and ESTIMATE, which applies gene expression profiles to predict immune and stromal cell fractions (Yoshihara et al., 2013), to infer tumor purity in these tumors. Interestingly, LTRLow tumors actually had higher ABSOLUTE scores and lower ESTIMATE scores than all other tumors, suggesting that they are more immunologically “silent” (Figure S6a). Consistent with this finding, even though LTRLow and LTR_Hi tumors expressed similar levels of FOXP3, LTR_Hi tumors expressed greater levels of CD8A, PRF1, and GZMB (Figure S6b). As a result, the FOXP3/CD8A expression ratio was actually significantly lower in LTR_Hi tumors than in other tumors (Figure S6c), suggesting that LTR_Hi tumors may actually have greater CD8+ T cell infiltration.

Since FOXP3 expression was similar between the LTR_Hi and LTRLow tumors, we sought to compare the KPLungTR signature with an existing Treg prognostic signature. A prior report found that a transcriptional module consisting of the 100 genes most positively correlated with FOXP3 expression in immune cells from peripheral blood was associated with clinical outcome in some tumor types (Linsley et al., 2015).
Expression of this FOXP3 transcriptional module was lower in LTR_Hi tumors and higher in LTR_Low tumors (Figure S6d), suggesting that Treg cells are present in similar numbers in both groups, but Treg cells in LTR_Low tumors have an expression profile more characteristic of circulating, systemic Tregs. Taken together, LTR_Hi tumors appear to be characterized by the expression of an effector Treg phenotype as well as greater evidence of CTL activity. The poor prognosis of LTR_Hi tumors suggests that a subpopulation of LUAD patients with highly inflamed tumors may fare worse than even more immunologically inert tumors due to their recruitment of highly active Treg cells and other immunosuppressive mechanisms.

In light of growing evidence for tumor-intrinsic mechanisms for immune evasion, we sought to determine whether correlation with the KPLungTR signature was associated with particular tumor genotypes. LTR_Hi tumors were significantly more enriched for KRAS and KRAS/TP53 mutations, and had fewer EGFR mutations than would be expected by chance in the LUAD cohort (Figure S6e). Moreover, LTR_Low tumors had more EGFR mutations and fewer KRAS and KRAS/TP53 mutations than expected by chance, which is consistent with recent reports that K-ras-mutant lung adenocarcinomas are more heavily infiltrated by immune cells than Egfr-mutant tumors (Busch et al., 2016). We hypothesized that expression of the KPLungTR signature may be inversely related to EGFR pathway activation, and thus could be predictive of a poor response to EGFR inhibitors. Indeed, in a trial of combined bevacizumab/ erlotinib therapy in advanced NSCLC, 12-week disease stabilization was associated with lower KPLungTR signature scores (Figure S6f).
We wondered whether the prognostic significance of the KPLungTR signature could be fully explained by the greater disease severity and KRAS mutation status of LTR_Hi tumors. However, in an expanded multivariate Cox proportional hazard regression analysis of 5-year survival of LUAD patients, the KPLungTR signature remained significantly associated with worse survival even after accounting for many variables, including advanced disease, smoking status, frequency of mutations, tumor purity, and mutation of KRAS or EGFR (Table 1).

CD103 and KLRG1 mark a highly activated subpopulation of lung Tregs that is associated with tumor progression.

Although the KPLungTR signature recapitulates expression patterns observed in human cancer Tregs and is associated with clinical outcome, it appears to be ubiquitously expressed by lung Tregs regardless of the presence of tumors. We thus wondered whether DP Tregs, which increase in proportion during tumor progression, represent a particularly activated subset of the lung Treg population. Tregs have previously been shown to upregulate a large number of genes in response to activation, only a subset of which are maintained in memory Tregs after the resolution of inflammation (van der Veeken et al., 2016). We find that genes upregulated and downregulated transiently in activated Tregs (VdWtrans_UP and VdWtrans_DN, respectively) are similarly differentially expressed in DP Tregs compared to DN Tregs (Figure S7), consistent with chronic antigen exposure of this Treg population in the tumor environment. Furthermore, we identified a subset of genes that are upregulated in DP Tregs compared to all other Tregs in tumor-bearing lungs (Methods, Figure 1f).
Many of the genes upregulated in DP Tregs are associated with T cell activation and putative Treg cell effector functions, including *Nr4a1, Cd69, Il1rl1, Areg, Srgn*, and *Fgl2*. Interestingly, *Cxcr3* was consistently downregulated in DP Tregs compared to other samples, which has been associated with T-bet+ Treg cells that are specialized to counter Th1 inflammation (Koch et al., 2009; Levine et al., 2017). The DP Treg phenotype may thus represent a divergent cell state from Cxcr3+T-bet+ Treg cells. Together these data suggest that heterogeneous populations of lung Treg, and other CD4+ T helper, cells may be particularly active and thus fruitful targets for immunotherapy.
Figure 1. Lung Tregs in the KP model have an activated effector phenotype that is similar to other tissue Treg profiles, and associated with survival

A. Representative flow cytometric analysis of tissue Treg (i.v. PE-neg, CD4+Foxp3+) cells from naive or tumor-bearing mice. Lenti-LucOS-infected mice were analyzed at the timepoints indicated post infection (p.i.).
B. Schematic of RNA sequencing experiment. Briefly, KP, Foxp3-RFP mice were harvested at 20 weeks p.i. RNA sequencing was performed on CD103-KLRG1- (DN, black), CD103+KLRG1- (SP, blue), and CD103+KLRG1+ (DP, red) Treg cells isolated from tumor-bearing lungs. SP and DN Treg cells were collected from the draining mediastinal lymph node. DN Tregs were collected from one spleen for comparison.

C. Heatmap of KPlungTR signature genes ($|z$-score| > 3, |fold change| > 2) illustrating gene expression differences between lung vs. msLN/spleen Tregs.

D. Gene set enrichment analysis (GSEA) reveals the lung signature to be significantly enriched in genes up- and down-regulated in muscle, fat, and colon Tregs. NES = Normalized Enrichment Score, FDR = False Discovery Rate.

E. Survival differences in TCGA patient cohorts between patients with expression profiles highly correlated with the KPlungTR signature (top 15%, red) versus those less correlated (rest of the cohort, blue). Significant differences in survival were observed in diverse cancer types (LUAD: Lung Adenocarcinoma, COAD: Colon Adenocarcinoma, PAAD: Pancreatic Adenocarcinoma, LUSC: Lung Squamous Cell Carcinoma, HNSC: Head and Neck Squamous Cell Carcinoma, LGG: Low Grade Glioma). Kaplan-Meier plots of the 5-year survival and log-rank (Mantel-Cox) p-values are shown.

F. Heatmap of a 45-gene signature (43 up-regulated, 2 down-regulated) found to distinguish DP lung Tregs from all other profiled populations in C.
Table 1: Results of univariate and multivariable Cox proportional hazards model on overall survival in the TCGA LUAD cohort (all patients)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Univariate</th>
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<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p</td>
<td>HR (95% CI)</td>
<td>p</td>
<td>Pinteraction</td>
<td></td>
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<tr>
<td>Signature (KPLungTR-high vs low)</td>
<td>1.25(1.07-1.46)</td>
<td>0.00566</td>
<td>1.38(1.13-1.69)</td>
<td>0.00183</td>
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<tr>
<td>Gender (Male vs Female)</td>
<td>1.08(0.80-1.46)</td>
<td>0.60248</td>
<td>0.99(0.69-1.44)</td>
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<tr>
<td>Age (Years)</td>
<td>1.00(0.99-1.02)</td>
<td>0.57577</td>
<td>1.01(0.99-1.04)</td>
<td>0.1632</td>
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<tr>
<td>TNM Stage (Stage III/IV vs I/II)</td>
<td>2.68(1.95-3.67)</td>
<td>9.38E-10</td>
<td>1.22(0.76-1.55)</td>
<td>0.4046</td>
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<td>T score (T3/T4 vs T1/T2)</td>
<td>2.43(1.66-3.57)</td>
<td>0.00001</td>
<td>1.80(1.11-2.91)</td>
<td>0.01689</td>
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<td>N score (N1/N2 vs NO)</td>
<td>2.52(1.87-3.42)</td>
<td>1.96E-09</td>
<td>2.48(1.60-3.84)</td>
<td>4.42E-05</td>
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<td>Smoking History (reformed &gt; 15yrs vs non-smoker)</td>
<td>0.89(0.53-1.48)</td>
<td>0.647</td>
<td>1.00(0.53-1.92)</td>
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<td>Smoking History (reformed &lt; 15yrs vs non-smoker)</td>
<td>1.10(0.69-1.76)</td>
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<td>1.20(0.62-2.31)</td>
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<td>Smoking History (current smoker vs non-smoker)</td>
<td>0.85(0.50-1.42)</td>
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<td>0.87(0.42-1.78)</td>
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<td>Mutational Load (mutations per coding Mb)</td>
<td>0.99(0.97-1.00)</td>
<td>0.08329</td>
<td>0.98(0.95-1.01)</td>
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<td>Transversion Status (Low vs High)</td>
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<td>0.99736</td>
<td>0.67(0.41-1.11)</td>
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<td>Neoepitope Count</td>
<td>1.00(0.98-1.01)</td>
<td>0.64265</td>
<td>1.01(0.99-1.04)</td>
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<td>Tumor Purity (ABSOLUTE)</td>
<td>1.17(1.01-1.35)</td>
<td>0.03757</td>
<td>1.37(1.07-1.75)</td>
<td>0.01287</td>
<td>0.7317</td>
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<tr>
<td>Tumor Purity (ESTIMATE)</td>
<td>0.85(0.74-0.97)</td>
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<td>0.97(0.77-1.22)</td>
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<td>Kras Mutation (Mutant vs WT)</td>
<td>1.23(0.88-1.72)</td>
<td>0.21716</td>
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<td>EGFR Mutation (Mutant vs WT)</td>
<td>1.32(0.87-2.00)</td>
<td>0.18613</td>
<td>1.56(0.92-2.64)</td>
<td>0.09792</td>
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</table>

HR = Hazard ratio; CI = Confidence Interval; TNM Stage = Stage classification per Union for International Cancer Control (UICC); T score = Primary tumor size/invasiveness; N score = Lymph node metastasis; Pinteraction = p-value of interaction between significant covariates (model comparison; likelihood ratio test)
Heterogeneity and longitudinal population shifts of tumor-associated conventional CD4+ T cells revealed through single-cell RNA sequencing

Previous characterizations of CD4+ T cell heterogeneity have relied on subsetting populations by their expression of specific markers, which may obscure underlying population dynamics. Single cell RNA sequencing (scRNAseq) of human tumors has demonstrated immune cell heterogeneity in cancer, but previous efforts have examined only single timepoints in the natural history of a tumor immune response. We sought to characterize longitudinal patterns of transcriptional heterogeneity in the tumor-associated CD4 population in an unbiased fashion through scRNAseq. By capturing both Tconv and Treg cells, we hoped to expand our understanding of the diversity of CD4+ T cell responses in the tumor microenvironment, as well as the factors that may be promoting their differentiation and function. We single-cell sorted tissue CD4+, Foxp3- and Foxp3+ populations from the lungs and msLN of naive KP, Foxp3GFP mice as well as tumor-bearing mice at weeks 5, 8, 12, and 20 after tumor induction with Lenti-LucOS (Figure 2a). Treg cells from KP mice infected with an adenovirus expressing Cre recombinase under the control of the SPC promoter (Ad-SPC-Cre) were also collected at a late timepoint (week 15-18) to represent Treg cells in a lung tumor environment lacking a strong T cell antigen. A total of 1257 Tconv and 2001 Treg cells were collected across 5 timepoints, with ~4 mice per timepoint. An average of 1600 genes were detected per cell.

We visualized our sorted Tconv and Treg populations together using a two-dimensional projection of t-distributed stochastic neighbor embedding (t-SNE) for dimensionality reduction and for the most part, Tconv and Treg cells segregated into
distinct clusters (Figure 2b). Consistent with our previous findings, we found that both Tconv and Treg cells are transcriptionally distinct from their lymph node counterparts (Figure 2b-c). Overall, the differentially expressed genes in lung CD4+ T cells closely recapitulated the KPLungTR signature (Figure 2d). Lymph node Tconv and Treg cells demonstrated a more naive phenotype, characterized by the expression of Tcf7 and Sell. Notably, a subset of lung Tconv cells (cluster 1) clustered with the lymph node cells, but also expressed genes associated with TCR signaling, including Nr4a1, Nr4a3, and Junb, suggesting that these cells may be recently activated, perhaps in TLS. Although there are many shared genes that distinguish lung Treg and Tconv cells from their LN counterparts, there are some genes upregulated in lung Treg cells that are not similarly upregulated in lung Tconv cells. Specifically, lung Treg cells (cluster 2) have higher expression of genes associated Treg cells, including Areg, Il1rl1, Folr4, Tigit, and Ctla4. The TCR and cytokine signaling that accompany the transition from lymphoid tissue to the lung environment may enhance the expression of these “signature” Treg genes. There is a small group of lung Treg cells that have lower expression of these genes, which may indicate recently-induced or emigrated, and potentially less “committed”, Treg cells in the lung (cluster 4). Interestingly, a subset of lung Tconv cells have particularly high expression of a group of genes that includes Cxcr6, Ccr4, Itgb1, and Cd44, which may represent cells that are particularly tissue-adapted or localized to a particular region of the lung tumor environment (cluster 3). A similar population could not be found among Treg cells, suggesting that this phenotype may be a Tconv-specific program in the lung.
In order to identify similarities between cells within the heterogeneous population of Tconv cells, we used PAGODA to identify groups of coordinately variable genes. Twenty-three gene modules were generated, several of which were able to mark clusters of cells that shared transcriptional similarities (Supplementary Table 2). In some cases, groups of cells expressed overlapping sets of gene modules (Figure 2e), consistent with evidence that Tconv cells have a continuum of phenotypes rather than discrete states. The expression of several modules varied over time (Figure 2f).

Expression of Module 1, which consists of genes associated with a naive phenotype such as Sell, Ccr7, and Tcf7, was strongly inversely correlated with the expression of Module 4, which was expressed by the majority of Tconv cells and included activation and memory-associated genes like Cd44 (Figure 2g). Most Tconv cells in lungs are thus likely to be antigen-experienced. Curiously, increased Module 1 expression at week 12 may represent the development of TLS.

Distinct expression of gene modules marked several populations of non-naive Tconv cells in lungs. Interestingly, Modules 17 and 13 include many genes associated with Th1 CD4 cells, including Tbx21 and Ifng (Figure 2g). However, these modules appear to mark distinct populations of cells (Figure 2e). Module 13 includes Eomes and Gzmk, which may be indicative of a cytolytic activity, as well as Cxcr3 and Ccr5, which are characteristic of Th1 cells and promote antigen-specific CD4+ T cell recruitment to lungs during respiratory virus infection (Kohlmeier et al., 2009). Some Module 13-expressing cells also express Module 11 genes, which include various killer cell lectin-like receptors (e.g. Klrc1, Klrc2, Klrc3, and Klrk1), which may also indicate a cytolytic phenotype. Module 17, however, includes TGFβ1 and Hk2, which have been associated
with immunosuppressive type 1 regulatory (Tr1) cells that are Foxp3-negative but can simultaneously express IFNγ, IL-10, and TGFβ (Mascanfroni et al., 2015; Roncarolo et al., 2001). AA467197 is a gene that has been shown to be upregulated in a Th1-polarized CD4+ T cells in a model of LCMV vaccination and challenge (Penaloza-MacMaster et al., 2015). Interestingly, Module 13 expression increases after week 5 and remains elevated, but Module 17 genes peak late in tumor development (Figure 2f). Together, these data suggest that multiple Th1-like CD4+ T cell lineages are present in naive and tumor-bearing lungs, and advanced disease may be accompanied by an expansion of a Th/ Tr1-like population of cells.

A Th17-like population is defined by Module 5, which includes Rorc, Il17a, and Il1r1 (Figure 2g). Many Module 5-expressing cells also express Module 21, which includes the Th17-associated genes Maf, Ikzf3, and Rora. Expression of these modules is already apparent at week 5, and these genes remain expressed throughout tumor development, suggesting that a Th17 response is established early, consistent with its reported role in promoting tumorigenesis.

Interestingly, we noticed that a subset of Module 5-expressing cells also expressed Module 10 genes and were CD4-negative, likely due to contamination during single-cell sorting. Module 10 includes Zbtb16, which encodes the transcription factor PLZF, which is characteristic of natural killer T (NKT) cells. These cells express Il23r and Lingo4 in addition to Rorc and Il17a, indicating that they are NKT17 cells in particular, which have been shown to reside in the lung. Module 10 expression is elevated at both week 5 and late in tumor development, suggesting that sufficient
glycolipid antigen may be present in KP tumors to promote an expansion of the tissue NKT17 population.

We were surprised to find that there was little evidence of a type 2 (Th2)-like population in spite of the role this population might be expected to play in lung inflammation. Instead, we observed that Module 22 marked a small population of cells that expressed *Il9r, Il4*, and *Il1rl1*, which is indicative of a type 9 (Th9) CD4+ T helper population. Module 22 genes are most highly expressed at week 5 and then lowly expressed throughout tumor development, suggesting that their development and activity may be inhibited during the development of an immunosuppressive tumor environment, and consistent with the reported role of Th9 cells in driving anti-tumor immune responses (Végran et al., 2015).
Figure 2. Single-cell RNA sequencing reveals longitudinal shifts in heterogeneity of conventional CD4 T cells during KP tumor development
A. Schematic of longitudinal profiling experiment. Briefly, mice were harvested at the indicated weeks after tumor induction with Lenti-LucOS. Tconv (i.v.neg,Thyl.2+CD4+Foxp3-) and Treg (i.v.neg,Thyl.2+CD4+Foxp3+) cells from lung and msLN were single-cell sorted and sequenced.

B. A tSNE visualization of the 1257 Tconv and 2001 Treg cells analyzed from all timepoints colored by tissue of origin (left) and Treg/Tconv identity (right).

C. A biclustering heatmap correlating cell identity and origin with expression of genes that are significantly differentially expressed between lung and msLN in Treg and/or Tconv cells. Genes identified as being high in LN cells (UPinLN) are delineated with a blue line, while genes identified as being high in lung cells (UPinLung) are marked with a red line. Numbers note cell clusters of interest.

D. Empirical cumulative distribution function plot comparing Lung vs LN log2-transformed FC in expression values of UPinLN (blue), UPinLung (red), and all other expressed (gray) genes from C. LungvsLN FC values were calculated from the bulk RNAseq data collected from bulk lung and msLN Tregs in Figure 1. pUPvsDN = 9.35e-11, pUPvsBkgd = 0, pDNvBkgd = 1.38e-8, two-sided Kolmogorov–Smirnov test.

E. Biclustering heatmap showing average gene module expression by cells. Average module expression was z-scored and capped at (-2,2). Modules were identified using PAGODA (see Methods).

F. Heatmap demonstrating changes in expression of gene modules over time. Colors indicate the coefficient for each time point in a linear model when naive lung was taken as reference. The multiple hypothesis adjusted likelihood ratio test (LRT.fdr) was computed when comparing the full model to a reduced model that lacked time (see Methods).

G. tSNE visualizations colored by expression of gene modules of interest (where indicated), and single genes from those modules.
A terminally differentiated, effector Treg phenotype stabilizes and becomes dominant during tumor development.

Given the diversity of phenotypes observed in Tconv cells, and the reported ability of Treg cell populations to adapt to inflammatory responses, we wondered whether corresponding Treg cells would be observed in the KP model. We used PAGODA to identify twenty-nine modules of covarying genes expressed by Treg cells, and found clusters of cells that expressed multiple modules, suggesting that groups of gene modules converge on cell phenotypes that are overlapping and heterogenous rather than discrete (Figure 3a). Several of these gene modules also varied in expression over time, consistent with the longitudinal shifts in the Treg cell population we had observed (Figure 1a, 3b). Correlating module expression with previously published Treg transcriptional signatures revealed significant overlap between some gene modules and described Treg cell subsets (Figure 3d, Supplementary Table 2).

Module 5 includes Sell, Ccr7, and Tcf7, several genes that denote a resting, or central, Treg (rTreg) cell phenotype (Campbell, 2015; Li and Rudensky, 2016). Notably this rTreg-associated module peaks at week 8, while other modules increase in expression at later timepoints (Figure 3b-c). Module 5 genes were best correlated with genes upregulated in mouse lymphoid tissue and human peripheral blood Treg cells (Figure 3d). An influx of rTreg cells into the tumor microenvironment early in tumor development could be in response to the large degree of inflammation observed in the KP model at week 5, particularly that mediated by CD8+ T cells (Canner, Li, Herbst et al. in preparation). Cxcr5 expression in a subset of Module 5-expressing Tregs may also represent a T follicular regulatory (Tfr) cell population, which may reside in TLS.
In addition to rTreg cells, two major populations of Treg cells could be identified based on correlated expression of gene modules; one group was characterized by the expression of Modules 7, 8, 12, and 28, while the other group demonstrated overlapping expression of Modules 6, 29, 4, and 10. Modules 7 and 8 were strongly enriched for genes in the DP signature, as well as genes upregulated in mouse non-lymphoid tissues and human Treg cells in breast cancer, NSCLC, and CRC, confirming the distinct gene expression profile we had previously identified in this Treg subpopulation (Figure 3d). Module 7 and 8 genes also increased in expression throughout tumor progression, consistent with the observation that DP Tregs expand over time (Figure 3b). Interestingly, Modules 12 and 28 were also expressed by many of the activated Treg cells marked by Modules 7 and 8, and also demonstrated increased expression over during tumor growth. While some Module 12 and 28 genes have been associated with Treg cell function, including Havcr2, Lag3, and Cx3cr1, other genes have not been well-described in Treg cells, including Tff1, Mt1, Ky, and Plek. Notably, Modules 12 and 28 did not include any of the 43 DP signature genes. Looking back at the bulk Treg RNAseq data, we found that Module 12 genes were highly expressed in DP vs DN Tregs, suggesting that our bulk DP Treg signature lacked the sensitivity to identify Module 12 genes (Figure S8a). Meanwhile, Module 28 genes were not significantly differentially expressed been lung vs. LN, or DP vs. DN, Treg cells, indicating that the module may not be associated with the expression of CD103 and KLRG1, and was only uncovered through unbiased single cell profiling (Figure S8b).

Although many Treg cells have overlapping expression of genes in Modules 6, 29, 4, and 10, Module 29 genes are the most widely expressed across these cells.
Module 29 includes many genes associated with Treg cell differentiation, including Foxo1 and a number of Foxp3 targets such as Klf2, Lef1, and S1pr1. Interestingly, Lef1 has been shown to coordinate with Foxp3 to control Treg cell lineage commitment, but is later repressed after stabilization of the gene expression program associated with Treg identity (Fu et al., 2012). Meanwhile, S1pr1 has been shown to impair the development, egress, and terminal differentiation of Treg cells, but recently has also been shown to promote Treg cell recruitment to tumors (Eken et al., 2017; Liu et al., 2009; Priceman et al., 2014). Klf2 has been reported to have a role in the induction of peripherally-derived Treg (pTreg) cells and has been shown to direct Treg cells to secondary lymphoid organs (SLO), where they are able to maintain immune tolerance (Pabbisetty et al., 2016). Indeed, some Module 29-expressing cells also express Sell, and Module 29 is correlated with genes upregulated in mouse lymphoid tissues. Taken together, Module 29 genes may mark recently-arrived, activated Treg cells that may retain the expression of rTreg genes associated with early stages of Treg cell differentiation and residence in SLO. Alternatively, cells expressing these modules may be resident in TLS, and thus have a features of a rTreg-like phenotype.

Consistent with the model that Module 29-expressing Treg cells may be in an intermediate differentiation state, a subset of these cells also express Module 6, which includes Rorc and Il1r1, suggesting that these cells share features with Th17 Tconv cells. A recent report has suggested that RORγT+ Treg cells may represent a distinct subset of thymus-derived Tregs that develop in response to Th17-inducing cues, dubbed "Tr17" cells (Kim et al., 2017). Indeed, Module 6 is highly correlated with genes upregulated in Tr17 cells (Figure 3d). Module 6 expression is relatively low throughout
tumor development, but is lowest at early timepoints, which may be consistent with Treg cells in the early tumor environment being exposed to Th17-inducing cues.

A larger group of Treg cells expressed Module 29 genes as well as Module 4 and 10 genes, many of which are interferon-stimulated genes (ISGs). These Treg cells demonstrated evidence of both type I and type II interferon (IFN) signaling, as they expressed genes common to both pathways, such as Stat1 and various guanylate binding protein (GBP) family members, as well as type I interferon-specific genes (e.g. oligoadenylate synthetase family members) and IFNγ-specific genes (e.g. Irf1) (Der et al., 1998). Interestingly, Ifngr1 is widely and highly expressed across most lung Treg cells, while the expression of Ifnar1 is more limited to cells expressing Module 4 and 10 genes (Figure S9a), although no interferon receptor subunits are members of either module. Ifngr2 and Ifnar2 expression are still more limited, consistent with the higher expression of various ISGs observed in these cells. Expression of Stat2 and Irf9, both components of interferon-stimulated gene factor 3 (ISGF3), which drives the expression of many ISGs, is also more restricted to Module 4 and 10-expressing cells. IFNγ has been reported to promote a Tbet+CXCR3+ Th1-like Treg cell population that can suppress Th1 responses (Hall et al., 2012; Koch et al., 2009, 2012), and indeed some Module 4/10-expressing cells also express Tbx21 and Cxcr3 (Figure S9b). Furthermore, Module 4 and 10 expression is highest at week 5 and declines thereafter, which coincides with increased expression of Module 7/8/12/28 genes, corroborating our earlier finding that Cxcr3 is downregulated in DP Tregs cells. In line with that observation, module 4 genes are positively correlated with genes upregulated in mouse lymphoid tissue Tregs, which may suggest a phenotype of intermediate differentiation.
Taken together, our data suggest that tumor progression may be associated with a shift from a more flexible, less-differentiated group of Treg cells that may be actively suppressing Th17 and Th1 inflammation to a terminally-differentiated, effector Treg cell population.
Figure 3. A terminally differentiated, effector Treg phenotype becomes dominant during tumor development
A. Biclustering heatmap showing average gene module expression by cells. Average module expression was z-scored and capped at (-2,2). Modules were identified using PAGODA (see methods).

B. Heatmap demonstrating changes in expression of gene modules over time. Colors indicate the coefficient for each time point in a linear model when naive lung was taken as reference. The multiple hypothesis adjusted likelihood ratio test (LRT.fdr) was computed when comparing the full model to a reduced model that lacked time (see methods).

C. tSNE visualizations colored by expression of gene modules of interest (where indicated), and single genes from those modules.

D. Heatmap representing pearson correlations between average expression of Treg gene modules and previously-identified Treg gene expression signatures.
Immunogenic tumor development recruits a large polyclonal T cell population, but heterogeneous CD4+ T cell phenotypes are associated with clonal expansion. The identification of transcriptionally-distinct CD4+ T cell subsets during tumor development led us to ask whether these cells may have shared transcriptional features due to clonal expansion. We used TraCeR (Stubbington et al., 2016) to reconstruct TCR sequences from our scRNAseq data, and were able to identify clonal expansions in ~25% of Treg and Tconv cells at all timepoints (Figure S10a-b). Clonally expanded cells were more transcriptionally similar to each other than non-clonally expanded cells across all mice at all timepoints (Figure S10c), suggesting that phenotypically similar groups of CD4+ T cell may arise as a result of the activation and proliferation of a few precursor cells. Consistent with that hypothesis, the expression of some, but not all, Treg and Tconv modules was higher in clonally expanded cells (Figure 4a). As expected, expression of Module 1, or naive phenotype genes, was lower in clonally-expanded Tconv cells. Module 13 (CD4 CTL-like) and Module 22 (Th9-like) genes were equivalently expressed between clonally-expanded and non-expanded Tconv cells, which may suggest that these phenotypes are associated with less proliferation, or represent a common set of traits that cells can adopt from various states. On the other hand, expression of Module 5 (Th17-like) and, to a lesser degree, Module 17 (Th1/Tr1-like) genes was higher in clonally-expanded Tconv cells, suggesting that both of these pathways are associated with substantial clonal proliferation. Similarly, in Treg cells the expression of Module 5 (rTreg-like) genes was slightly lower in clonally-expanded Treg cells, consistent with reduced TCR signaling and a less proliferative state in rTregs. Interestingly, clonally-expanded and non-expanded Treg cells have similar expression
of modules associated with a Tr17 or IFN-inducible/ Cxcr3+ phenotype. This may reflect the fact that Tregs with these phenotypes may be less stable, or more “fragile”, and have less proliferative potential as a result of receiving many conflicting cytokine cues. However, the expression of genes in Modules 7, 8, 12, and 28, which are all associated with an effector Treg phenotype, was higher in clonally-expanded Treg cells. The prevalence of cells bearing the effector Treg phenotype in clonally-expanded Tregs is indicative of their terminally differentiated state, which likely follows substantial proliferation of their precursor cells. To determine if effector Tregs are more clonally-expanded, we extracted and analyzed TCRβ chain sequences from the bulk RNA sequencing data generated from DP, SP, and DN Treg cells in Figure 1 using MiXCR and VDJTools software (Bolotin et al., 2015; Shugay et al., 2015). Though not statistically significant, there was a trend toward DP Treg cells having less clonal diversity than SP and DN Tregs (Figure 4b). Furthermore, the overlap in the SP and DP Treg cell repertoires was significantly higher than that of the DN and DP Treg cell repertoires (Figure 4c). The fact that SP and DP Treg populations have more shared TCRβ chains than DN and DP Tregs suggests that DP Tregs represent an outgrowth of SP Tregs that develops as some highly activated clones expand and become terminally differentiated. Based on these data, we wondered whether overall lung Tconv and Treg clonal diversity is altered during tumor progression. We performed TCRβ chain sequencing on sorted bulk populations of Tconv, Treg, and CD8+ T cells from naive and late-stage Ad-SPC-Cre and Lenti-LucOS-infected mice (Figure 4d). We found that tumor development was associated with increases in the clonal diversity of all three T cell populations, suggesting that tumor development is associated with a major influx of
new cells that adds to the diversity of the tissue-resident population. In particular, T cells from Lenti-LucOS mice were far more clonally diverse than T cells from Ad-SPC-Cre-infected animals. The greater immunogenicity of the LucOS model, in which tumors may express Cre recombinase and luciferase in addition to Ova and SIY, likely account for this increase in diversity.

**Shared clones between Tconv and Treg may highlight considerable cytokine-induced CD4+ T cell heterogeneity**

In spite of their increased overall diversity, a significant fraction of both Treg and Tconv cells in tumors are clonally expanded. This led us to ask whether Treg and Tconv have an overlapping repertoire that may hint at the developmental origin of the Treg population. One LucOS tumor-bearing mouse had three clones that consisted of both Treg and Tconv cells, but otherwise there was no overlap in repertoire observed between Treg and Tconv cells (Figure S10d). To determine whether the limited repertoire overlap in Lenti-LucOS mice was due to insufficient sampling, we performed TCRβ chain sequencing on Treg and Tconv cells with 10X greater read count on a second group of LucOS tumor-bearing mice at week 20 p.i. (Figure 4e). Interestingly, about 5-10% of the unique TCRβ chains identified in the Treg cells were shared with Tconv cells, which is several fold higher than the percentage of repertoire overlap that has been reported in CD4+ T cells infiltrating carcinogen-induced tumors (Hindley et al., 2011). Indeed, we were able to identify several families of Treg and Tconv cells that had identical paired-chain TCR sequences (Figure 4f). These clonotypic families were small, mostly consisting of several cells, making statistical analyses difficult. However, the largest shared clone is Clone 74, consisting of 4 Tconv and 5 Treg cells.
Interestingly, the Tconv cells within this clone had a Th1 or Th17-like phenotype, while the Treg cells within this clone appeared to have a Tr17-like phenotype. This may suggest that these cells arose from a common precursor cell subject to subtly different differentiation signals, resulting in a branching of phenotypes. Alternatively, Treg and Tconv cells derived from the same precursor may initially have divergent phenotypes, but converge in the presence of overwhelming cytokine cues.
Figure 4. TCR repertoire analyses correlate longitudinal shifts in phenotypic diversity with clonal expansion
A. Notched boxplot comparing the average module expression in clonally expanded (red) vs. non-expanded (teal) Tconv (top) or Treg (bottom) cells. Average module expression refers to the mean of all genes in a module after centering and scaling each gene across all cells. Modules of interest from Figures 2 and 3 are labelled in red. Note that “non-expanded” cells had TCRα or β chain reconstructed, but had no clonal partner(s).

B. Barplot showing unique TCRβ chains per 1000 TCRβ-encoding reads from whole-transcriptome RNAseq of DN, SP, and DP Treg populations.

C. Barplot showing F2 repertoire overlap metric (Izraelson et al., 2018) between DN, SP, and DP lung Treg populations within a given mouse. *p<0.05, Tukey’s multiple comparisons test.

D. Barplot showing unique unique TCRβ chains per 1000 reads from b-chain sequencing of lung-resident T cell populations from Lenti-LucOS, Ad-SPC-Cre, or naive mice.

E. Venn diagrams representing clonal overlap between Treg (green) and Tconv (blue) cells in mice (n=3) at week 20 p.i. with Lenti-LucOS. Numbers represent total number of unique TCRβ chains, with population overlap indicated at the intersections.

F. tSNE visualizations of Treg (left) and Tconv (right) cells. In grey are all cells, in black are clonally expanded cells and in color are shared clones between Treg and Tconv. Numbers refer to unique shared clones, which are colored distinctly, and clone size is denoted by dot size.
DISCUSSION

Here, we used single-cell RNA sequencing of sorted T cells from a genetic mouse model of lung adenocarcinoma to construct a comprehensive portrait of the evolution of CD4+ T cell heterogeneity in the lung tumor environment. Our data suggest that a wide array of CD4+ T cell populations with different, likely opposing, effector functions are recruited to tumors, and that tumor progression is associated with the rise of Treg and Tconv cell populations that may be highly immunosuppressive. Although we were able to derive a clinically prognostic lung Treg signature using population-level profiling of Treg cells in the KP model, scRNA-seq allowed us to map diverse subsets of CD4+ T cells that would otherwise be missed during analyses of bulk populations. Importantly, longitudinal profiling of Tconv and Treg cells gave us a window into the natural history of the development of T cell-mediated immunosuppression that is not currently possible to achieve using patient samples. Furthermore, the transcriptional similarities between our profiled Treg cells and human NSCLC Treg cells, as well as the observation that the KPLungTR signature preferentially marks KRAS-mutant tumors, suggests that the KP model is able to recapitulate major aspects of the immune response in KRAS-driven lung adenocarcinoma.

Our data point to a strong lung-specific gene expression signature for both Tconv and Treg cells, which is consistent with a number of profiling studies of CD4+ T cells. Given the similarities between T cell populations profiled from diverse tissue sites, it is possible that much of the lung CD4+ T cell signature that we have described represents a core program shared by all tissue-resident T cells. T cells prompted to enter and remain in tissue environments are likely to be antigen-experienced and will have
transcriptional features of activated and differentiating cells. Adaptation to tissue
environments also poses similar challenges for all cells, resulting in shared expression
of chemokine receptors and integrins to migrate properly, as well as growth and survival
factors to contend with an altered metabolic environment. In line with this, we
consistently observed enrichment of genes associated with non-lymphoid tissue CD4+ T
cells in the genes upregulated in KP lung Treg and Tconv cells. However, subtle
differences may still exist among CD4+ T cells in different tissues, since lung Treg cells
do not perfectly match their counterparts in other tissues. The tissue effector Treg
phenotype is also not just a signature of activated Treg cells, since the genes transiently
upregulated in activated splenic Tregs (MouseActivatedTreg_Trans.UP) only partially
recapitulate their expression profile (Figure 3d). Timepoint-dependent shifts in CD4+ T
cell phenotype, and the presence of “intermediate” phenotypes, indicate that lung CD4+
T cell phenotypes dynamically shift along a continuum, with most phenotypes
represented at different timepoints to varying degrees. We therefore propose that
transcriptional differences observed between tissue and tumor-associated T cell
populations are likely to represent shifts in cellular heterogeneity, perhaps due to
variable cytokine milieus and antigen availability/ quality, rather than uniform adoption of
a tissue-specific program. Further scRNA-seq of CD4+ T cell populations in a variety of
tissues and inflammatory contexts will bring clarity to this question.

Although we observed heterogeneity in the CD4+ T cell population throughout
tumor development, we were struck by the preponderance of immunosuppressive and
potentially pro-tumorigenic phenotypes even at early stages of tumor development.
Th17-like and NKT17 cells were already established in the tumor microenvironment at
week 5 after tumor induction, while advanced disease was associated with the expansion of terminally differentiated Treg and Tr1-like cells. A number of CD4+ T cell subsets may contribute to anti-tumor immune responses, including CD4 CTLs, Th9-like cells, and RORγT+ Treg cells that may be able to suppress Th17 responses. However, cells with these phenotypes are either stable or decline over time, suggesting that the balance of tumor immune responses progressively shifts toward immunosuppression in coordination with tumor growth.

While Th17 cells and IL-17 signaling have been described as promoting anti-tumor inflammation in some contexts, their pro-tumorigenic effect is well-described in KRAS-driven lung adenocarcinoma (Akbay et al., 2017; Chang et al., 2014). In those models, IL-17 has been shown to directly promote tumor cell proliferation, angiogenesis, and the recruitment of immunosuppressive neutrophils that exclude anti-tumor T cell responses. Consistent with those reports, we observed that Th17-like and NKT17 cells represent a stable source of IL-17 throughout tumor development. More comprehensive, longitudinal profiling of IL-17-producing cells is needed, however, to determine whether Th17-like and NKT17 cells represent the dominant source of IL-17 at any timepoint. A companion study has identified a population of IL-17+ CD8 T (Tc17) cells early in tumor development, while work done by our lab and others have noted that gamma delta T cells represent a major source of IL-17 in KRAS-driven lung adenocarcinomas (Busch et al., 2016). It is also unclear whether IL-17 is “purely” pro-tumorigenic throughout tumor development, and it is plausible that some sources of IL-17 may simultaneously secrete other factors that aid anti-tumor immunity. The diverse
and shifting array of IL-17-producing cells is a challenge that will require novel, combinatorial strategies for inhibiting IL-17 function in tumors.

Th9-like and CD4 CTL cells have been associated with anti-tumor activity and may thus balance the pro-tumorigenic effects of IL-17 signaling early in tumor development. Th9 differentiation is stimulated by both TGFβ and IL-4, suggesting that there is an early source of IL-4 in spite of the noticeable paucity of Th2-like cells in naive and tumor-bearing lungs, which has also been noted by others working with the KP model (Busch et al., 2016). IL-9 has been shown to promote anti-tumor CD8+ T cell responses by activating dendritic cells (DCs) in transplant tumor models and in mice with spontaneous expression of oncogenic K-ras (Kim et al., 2015). Their differentiation and expansion may also be enhanced by signaling through ST2, which is the receptor for interleukin-33 (IL-33), an IL-1 family member that is ubiquitously expressed by lung epithelial cells. The decline of Th9 cells during later stages of tumor development, however, may be associated with the expansion of a terminally differentiated population of Treg cells, which express high levels of membrane-bound and soluble ST2, possibly limiting ligand for further Th9 activity. Manipulation of Treg or other CD4+ T cells may promote a more sustained Th9 response that may impair tumor growth.

A diverse population of Th1-like cells is present early in tumor development, including Tbet+Eomes+ CD4 CTL and Tgfb1+Hk2+Tr1 cells, and certain phenotypes peak late in tumor development. The functional contributions of these cells remain unclear. CD4 CTL and Tr1 cells have both been described as arising from Th1 cells, and it has been suggested that these polarized phenotypes represent terminally-differentiated Th1 cells with overlapping features (Cope et al., 2011). Indeed, CD4 CTL
have been described to express IL-10, while Tr1 cells may express GzmB. The shared transcriptional features of these cells have led to a model wherein Th1 cells can readily shift between the “classic” IFNγ-producing, CD4 CTL, and Tr1 phenotypes. Other studies have indicated that CD4 CTL and Tr1 cells can be derived from other T helper cell lineages, which may represent another source of these cells in tumors.

Our data indicate that CD4 CTL are present early and maintained throughout tumor development. A number of cancer vaccine and adoptive cell transfer (ACT) studies have demonstrated that Th1-like, CD4 CTLs can have potent anti-tumor activity. They can indirectly kill tumor cells via IFNγ secretion, or directly kill MHC class II-expressing tumor cells through release of perforin and granzyme (Zanetti, 2015).

Reflecting their close relationship with Tr1 cells, CD4 CTLs may also direct their cytolytic activity towards other immune cells, such as antigen presenting cells (APCs), and thus it is possible that CD4 CTLs may have dual roles in tumor immunity. Nevertheless, their maintenance throughout tumor progression suggests that the cytokine milieu and antigen load in the tumor environment are sufficient to promote differentiation of these cells, raising the possibility that transferred antigen-specific CD4 CTLs may have efficacy, as a recent report has shown (Tran et al., 2014).

Meanwhile, the expansion of Tr1-like cells in advanced tumors may serve to reinforce an immune-tolerant tumor microenvironment. Treg cell recruitment may contribute to the expansion of Tr1 cells by secreting TGFβ and inducing APCs to adopt a tolerogenic phenotype (Adeegbe and Nishikawa, 2013). This may explain the delay in peak expression of this module, since early timepoint Tregs have a less differentiated phenotype, and they expand over time. Although best described for their
immunosuppressive effects, Tr1 cells may have variable effects on tumor development because TGFβ and IL-10 are both pleiotropic cytokines that have been shown to have both tumor-promoting and anti-tumor functions (Mannino et al., 2015; Sanjabi et al., 2017). Further investigation of the role of Tr1 cells in advanced tumors is needed to clarify the function of these cells.

Our scRNA-seq analysis of tumor Treg cells confirmed and extended upon our earlier observations that tumor development is associated with an increase in terminally differentiated Treg cells. We independently identified two gene modules that were enriched for DP signature genes, and highly expressed by Treg cell in advanced tumors. Consistent with a role in tissue repair recently ascribed to tissue Treg cell populations, terminally-differentiated Treg cells in late-stage KP mice express high levels of *Areg* (Arpaia et al., 2015; Burzyn et al., 2013; Green et al., 2017). Moreover, our analysis identified additional genes, including *Tff1* and *Ky*, that are associated with this population that may provide further clues to their function in tissue regrowth. *Tff1* encodes trefoil factor 1 (TFF1), a protein secreted by gastric epithelial cells that is thought to be tissue-protective (Playford et al., 1996). *Ky* encodes kyphoscoliosis peptidase, which stabilizes neuromuscular junctions and is important for muscle growth (Blanco et al., 2001). The expression of these genes by late-stage Treg cells may signal a shift toward a role in tissue repair and homeostasis.

We were also intrigued to find that while activated Treg cells are present in naive lungs and throughout tumor development, Treg cells at early timepoints have a less-differentiated, more flexible phenotype characterized by continued expression of various genes that are usually repressed in mature Treg populations, as well as expression of
genes associated with Tconv effector functions. Rorc+ Treg cells may suppress Th17 responses, providing a counterbalance to pro-tumorigenic IL-17 signaling (Kim et al., 2017). Furthermore, many early-timepoint Tregs express Cxcr3, Tbx21, and a strong signature of IFN signaling, which may indicate their exposure to Th1-polarizing cytokines and Th1-like cells, including CD4 CTLs. The functional consequences of IFN signaling on Treg cells are unclear. There is evidence that type I and II IFN signaling can impair Treg cell development and function in chronic viral infections and tumors (Overacre-Delgoffe et al., 2017; Srivastava et al., 2014). However, many components of antigen presentation and processing machinery are upregulated in these Treg cells, which may be in line with evidence that MHC class I is a target of Foxp3, and that class I and II expression are associated with improved suppressor function (Baecher-Allan et al., 2006; Mu et al., 2014). Others have reported that type I IFN signaling promotes Treg cell differentiation in tumors and activity in controlling autoimmunity (Metidji et al., 2015; Stewart et al., 2013), while IFNγ signaling is thought to promote specialized Treg cell responses directed at Th1 inflammation (Koch et al., 2009, 2012; Levine et al., 2017). It is possible that the early Treg cell response to tumors is characterized by the expression of effector CD4+ T cell genes, likely due to exposure to the same cytokine stimuli, which may confer suppressor activity specialized for Th1 or Th17 inflammation. Meanwhile, in advanced tumors, terminally-differentiated effector Treg cells are also able to promote tumor growth through other mechanisms, including the secretion of growth factors like amphiregulin or other proteins associated with mucosal integrity like TFF1.
We found that expression of some gene modules for both Treg and Tconv cells was associated with clonal expansion. Outgrowth of a clone expressing a certain gene module may explain why clonal expansions enrich for the expression of some modules. Indeed, some expanded clones do appear transcriptionally similar. However, some clones appear disparate in phenotype, suggesting that a highly proliferative precursor cell could give rise to cells of diverse phenotypes, presumably due to exposure to different stimuli. Indeed, other studies of CD4+ T cells have demonstrated that cells can embark on divergent trajectories from a common path (DuPage and Bluestone, 2016; Lönnberg et al., 2017). Isolation and profiling of antigen-specific CD4+ T cell populations with more limited clonal diversity, perhaps using mice with a restricted TCR repertoire, may allow better tracking of phenotypic branchpoints in the differentiation of CD4+ T cells.

We were surprised to find that the overlap in Treg and Tconv cell TCRβ chain usage was 2-5X higher than what has been previously published (Hindley et al., 2011). This was especially remarkable because the previous study was performed using highly mutated MCA-induced sarcomas, thus increasing the likelihood of neoantigen-associated induction of pTreg cells. It is possible that the site of tumor development dramatically altered the generation and thus frequency of pTreg cells. Based on our lab’s previous work comparing KP sarcomas in the hindlimb to KP lung adenocarcinomas in the lung, muscle appears to foster much stronger anti-tumor immune responses, likely because tolerogenic mechanisms are not active in skeletal muscle (DuPage et al., 2012). Meanwhile, the lung environment may be more immunosuppressive at baseline to avoid life-threatening pneumonitis during
inflammatory insults, and to tolerate commensal bacteria in the airways. Indeed, the colon is characterized by a RORγT+ Treg population that is thought to be distinct from thymus-derived colon Tregs, which may be similar to the organization of Treg cells in normal and/or inflamed lungs (Sefik et al., 2015). As a result, inflammation in the lung may promote the induction of some pTreg cells in addition to recruitment of tTreg cells, while muscle inflammation may be more robust and thus fail to generate pTregs.

Our profiling thus demonstrates that the complexity and interrelatedness of CD4+ T cell responses in tumors are further complicated by longitudinal phenotypic shifts that may drastically change their net effect on tumor growth. While the heterogeneity of tumor CD4+ T cells makes them a challenging therapeutic target, these data also suggest that manipulations that can skew towards and away from particular CD4+ T cell populations may be sufficient to favorably alter the balance between immunosuppressive and anti-tumor effects.
Supplementary Figure 1. Validation of KPLungTR signature genes
A. Unsupervised hierarchical clustering of DN, SP, and DP lung Treg cells, DN and SP msLN Treg cells, and spleen DN Treg cells.

B. Heatmap illustrating two significant gene expression signatures detected in the dataset using ICA. The color-map represents positive or inverse correlation with the ranked gene list within each signature. The first signature distinguishes lung populations (DP, DN, SP) from spleen and LN samples. The second signature distinguishes CD103+ Tregs from CD103- populations. P-values for each signature (Kruskal-Wallis test) are shown.

C. Flow cytometric analyses of Tim3, Gzmb, and IL-17 expression in lung, msLN, and splenic Tregs from tumor-bearing (week 20, Lenti-LucOS) mice.

D. qRT-PCR analysis of expression of Pparg, Nr4a1, Gata1, and Areg1 in DP, SP, and DN lung Treg cells as well as in SP and DN msLN Treg cells. Shown are $2^{\triangle \Delta C_{T}}$ values, with splenic Treg expression as control.

E. Barplot of log2-transformed Foxp3 counts from RNA sequencing data of lung and msLN Tregs in KP mice.
Supplementary Figure 2. Enrichment map of KPLungTR signature
Network representation of GSEA gene sets from the curated collection (c2) enriched in the KPLungTR signature (p < 0.05, FDR < 0.05). Each circle is a gene set, where size denotes size of the gene set, and color denotes whether upregulated or downregulated genes are enriched. Note that in this analysis, all significant gene sets were enriched in genes upregulated in the KPLungTR signature. Thickness of the interconnecting lines represent degree of overlap between gene sets, with a threshold set at minimum 50% overlap. Related pathways have been manually annotated.
Supplementary Figure 3. Expanded lung Treg signatures
A. Heatmap of signature genes ($|z\text{-score}| < 3, |\text{fold-change}| >2$) distinguishing lung Tregs from lymphoid tissue Tregs derived from an expanded set of sequenced Treg populations.

B. Enrichment plots from GSEA analyses of the expanded lung Treg signature (A) showing enrichment of genes up and downregulated in the original KPLungTR signature.

C. Heatmap of signature genes ($|z\text{-score}| > 3, |\text{fold-change}| >2$) distinguishing lung Tregs from lymphoid tissue Tregs derived from a compiled set of Treg RNA sequencing data (Supplementary Table 1).

D. Same as in B, for GSEA enrichment of the signature described in C.
Supplementary Figure 4
Empirical cumulative distribution function (ECDF) plot comparing log2-transformed Lung vs LN fold-change values of genes found to be up-regulated in CRC and NSCLC Treg cells (DeSimone_UP) (De Simone et al., 2016) and all other expressed genes (gray). p = 1.137e-4, two-sided Kolmogorov–Smirnov test.
Supplementary Figure 5. Features of KPLungTR signature-associated tumors

A. Kaplan-Meier plot of the 5-year survival of the 25% highest-scoring (LTR_Hi, red) vs. 25% lowest-scoring (LTR_Low, blue) TCGA lung adenocarcinoma patients for correlation with the KPLungTR signature (\(|z\text{-score}| > 3\)). Log-rank (Mantel-Cox) p-values are shown.

The following analyses compare top and bottom 25% of TCGA LUAD patients after scoring with KPLungTR signature (\(|z\text{-score}| > 3\)).

B. Barplot of gender distribution of LTR_Hi and LTR_Low lung adenocarcinomas. LTR_Low was enriched for female patients; \(p = 0.0042\); hypergeometric test.

C-E. Barplot of T score (C), N score (D), and staging (E) of LTR_Hi and LTR_Low lung adenocarcinomas. LTR_Low was enriched for lower T score (\(p=0.0346\)), N0 status (\(p=0.0089\)), and stage I/II (\(p=0.0099\)). LTR_Hi was enriched for higher T score (\(p=0.0143\)); hypergeometric test.
F. Barplot of smoking status of LTR_Hi and LTR_Low lung adenocarcinomas. LTR_Low was enriched for non-smokers (p=0.0061), while LTR_Hi was enriched for smokers reformed for <= 15 yrs (p=0.0037); hypergeometric test.

G. Barplot of prevalence of transversion mutations of LTR_Hi and LTR_Low lung adenocarcinomas. LTR_Low was enriched for patients with low numbers of transversions (p=0.0047); hypergeometric test.
Supplementary Figure 6. KPLungTR signature-associated tumors are inflamed and enriched for KRAS-mutant tumors

A. ECDF plots comparing the ESTIMATE (left) and ABSOLUTE (right) scores of LTR_Hi (red) and LTR_Low (blue) lung adenocarcinomas to all other lung adenocarcinomas (gray). P-values indicated are from two-sided Kolmogorov–Smirnov tests between LTR_Hi vs. LTR_Low (h vs l), LTR_Hi vs. All Other LUAD (h vs o), or LTR_Low vs All Other LUAD (l vs o).
B. ECDF plots comparing *FOXP3*, *CD8A*, *PRF1*, and *GZMB* expression of LTR_Hi and LTR_Low lung adenocarcinomas to all other lung adenocarcinomas (gray).

C. ECDF plots comparing *FOXP3*:CD8A Log2 ratio of expression (left) and score for expression of Foxp3 Signature Module (right) (Linsley et al., 2015) of LTR_Hi and LTR_Low lung adenocarcinomas to all other lung adenocarcinomas (gray).

D. Barplot of prevalence of *KRAS* (left) and *EGFR* (right) mutations in LTR_Hi and LTR_Low lung adenocarcinomas. LTR_High was enriched for *KRAS*-mutant tumors (*p*=0.0007, hypergeometric test), while LTR_Low was enriched for *KRAS-*wild-type tumors (*p*=3.48e-06, hypergeometric test). LTR_High was enriched for *EGFR*-wild-type tumors (*p*=0.0006, hypergeometric test), while LTR_Low was enriched for *EGFR*-mutant tumors (*p*=0.0082, hypergeometric test).

E. Comparison of KPLungTR signature scores of responders and non-responders in a clinical trial of combined bevacizumab and erlotinib treatment for advanced NSCLC, dataset accession GSE37138 (Baty et al., 2013). "Responders" refers to patients whose disease had stabilized by week 12 of treatment. *p*= 0.0349; one-sided Mann-Whitney test
Supplementary Figure 7. DP Tregs express higher levels of genes upregulated in activated Tregs

Supplementary Figure 8. Module 28 genes are not differentially expressed in lung vs. LN or DP vs DN Tregs
A. ECDF plot comparing log2-transformed DP vs DN Treg (left) or Lung vs LN (right) fold-change in gene expression values of Module 12 genes. $p = 1.57 \times 10^{-5}$ for DP vs. DN, $p = 2.69 \times 10^{-11}$ for Lung vs LN; two-sided Kolmogorov–Smirnov test.
B. Same as above, but for Module 28 genes. $p = 1.306 \times 10^{-1}$ for DP vs. DN, $p = 1.531 \times 10^{-1}$ for Lung vs LN; two-sided Kolmogorov–Smirnov test.
Supplementary Figure 9. Expression of IFNγ receptor subunits and Th1-associated genes

A. tSNE visualization of Treg cells colored by expression of the indicated genes.
B. Same as above.
**Supplementary Figure 10**

**A.** Barplots of fraction of clonally expanded Treg (top) and Tconv (bottom) cells identified in individual mice; each bar represents a mouse. Data are organized by timepoint and tissue or origin.

**B.** tSNE visualization of Treg (left) and Tconv (right) cells colored by membership in a TCR clone, with color denoting size of the clone. Only clones with shared TCRα and β chains were included.
C. Barplots of correlation in gene expression in Treg (top) and Tconv (bottom) cells between cells that are clonally expanded (pink) vs. between cells that are not members of a clone (dark gray) vs. between cells without a TCR reconstructed (light gray).

D. Table comparing the numbers of unique TCRβ chains identified in β chain sequencing of lung-resident T cell populations from different Lenti-LucOS, Ad-SPC-Cre, or naive mice.
**Supplementary Table 1. Datasets**

Supplementary table 1, which includes the full KPLungTR signature and other gene lists, can be found at the link below.

https://www.dropbox.com/s/uyj87z9mrp1ahf1/ALTThesis_Chapter2_SuppTable1.xlsx?dl=0

**Supplementary Table 2. qRT-PCR primer sets and FACS antibodies**

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<td>Fisher</td>
<td>1:200</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

Mice

KP, Foxp3\textsuperscript{GFP}, Foxp3\textsuperscript{RFP}, and Foxp3\textsuperscript{GFP/DTR} mice have been previously described (Bettelli et al., 2006; DuPage et al., 2011b; Kim et al., 2007; Wan and Flavell, 2005). All studies were performed under an animal protocol approved by the Massachusetts Institute of Technology (MIT) Committee on Animal Care. Mice were assessed for morbidity according to MIT Division of Comparative Medicine guidelines and humanely sacrificed prior to natural expiration.

For in vivo labelling of circulating immune cells, anti-CD4-PE (eBioscience, RM4-4, 1:400) and anti-CD8\textsuperscript{p}-PE (eBioescience, 1:400) were diluted in PBS and administered by IV injection 5 minutes before harvest (Anderson et al., 2012).

Lentiviral Production and Tumor Induction

The lentiviral backbones Lenti-LucOS and Lenti-Cre have been described previously (DuPage et al., 2011b). Lentiviral plasmids and packaging vectors were prepared using endo-free maxiprep kits (Qiagen). Lentiviruses were produced by co-transfection of 293FS* cells with Lenti-LucOS or Lenti-Cre, psPAX2 (gag/pol), and VSV-G vectors at a 4:2:1 ratio with Mirus TransIT LT1 (Mirus Bio, LLC). Virus-containing supernatant was collected 48 and 72h after transfection and filtered through 0.45um filters before concentration by ultracentrifugation (25,000 RPM for 2 hours with low decel). Virus was then resuspended in 1:1 Opti-MEM (Gibco) - HBSS. Aliquots of virus were stored at -80C and titered using the GreenGo 3TZ cell line (Sánchez-Rivera et al., 2014).
For tumor induction, mice between 8-15 weeks of age received 2.5 x10^4 PFU of Lenti-Cre or Lenti-LucOS intratracheally as described previously (DuPage et al., 2009).

For Ad-SPC-Cre infection, 1.8 x 10^8 PFU of Ad-SPC-Cre was administered to mice between 8-15 weeks of age for tumor induction as previously described (Sutherland et al., 2011).

**Tissue Isolation and Preparation of Single Cell Suspensions**

After sacrifice, lungs were placed in 2.5mL collagenase/ DNAse buffer (Joshi et al., 2015) in gentleMACS C tubes (Miltenyi) and processed using program m_impTumor_01.01. Lungs were then incubated at 37°C for 30 minutes with gentle agitation. The tissue suspension was filtered through a 100 um cell strainer and centrifuged at 1700 RPM for 10 minutes. Red blood cell lysis was performed by incubation with ACK Lysis Buffer (Life Technologies) for 3 minutes. Samples were filtered and centrifuged again, followed by resuspension in RPMI 1640 (VWR) supplemented with 1% heat-inactivated FBS and 1X penicillin-streptomycin (Gibco), and 1X L-glutamine (Gibco).

Spleens and lymph nodes were dissociated using the frosted ends of microscope slides into RPMI 1640 supplemented with 1% heat-inactivated FBS and 1X penicillin-streptomycin (Gibco), and 1X L-glutamine (Gibco). Spleen cell suspensions were spun down at 1500 RPM for 5”, and red blood cell lysis with ACK Lysis Buffer was performed for 5”. Cells were filtered through 40 um nylon mesh and, after centrifugation, resuspended in supplemented RPMI 1640. Lymph node suspensions were filtered through a 40 um nylon mesh, spun down at 1500 RPM for 5”, and resuspended in supplemented RPMI 1640.
For ex vivo T cell stimulation experiments to detect intracellular cytokines, 0.5 x 10^5 cells were plated in a 96-well U-bottom plate (BD Biosciences) in RPMI 1640 (VWR) supplemented with 10% heat-inactivated FBS, 1X penicillin-streptomycin (Gibco), 1X L-glutamine (Gibco), 1X HEPES (Gibco), 1X GlutaMAX (Gibco), 1mM sodium pyruvate (Thermo Fisher), 1X MEM non-essential amino acids (Sigma), 50uM beta-mercaptoethanol (Gibco), 1X Cell Stimulation Cocktail (eBioscience), 1X monensin (BioLegend), and 1X brefeldin A (BioLegend). Cells were incubated in a tissue culture incubator at 37C with 5% CO2 for 4 hours.

**Staining for Flow Cytometric Analysis**

Approximately 0.5-1 x 10^6 cells were stained for 15-30 minutes at 4C in 96-well U-bottom plates (BD Biosciences) with directly conjugated antibodies (Supplementary Table 3). After staining, cells were fixed with Cytofix/ Cytoperm Buffer (BD). Samples that were destined for Foxp3 or other transcription factor staining were fixed with the Foxp3 Transcription Factor Staining Buffer Kit (eBioscience). Intracellular cytokine and transcription factor staining were performed right before analysis using either the BD Perm/Wash Buffer (BD) or the Foxp3 Transcription Factor Staining Buffer Kit (eBioscience); staining was performed for 45” at 4C. Analysis was performed on an LSR II (BD) with 405, 488, 561, and 635 lasers. Data analysis was performed using FlowJo software.

**Quantitative PCR for validation of RNAseq experiments**

Quantitative PCR was performed using various primer sets (Supplementary Table 3). 1ng of cDNA generated using Smart-SEQ2 was included in a reaction with 1uL of each primer (2uM stock) and 5uL of KAPA SYBR Fast LightCycler 480 (KAPA Biosystems).
Cp values were measured using a LightCycler 480 Real-Time PCR System (Roche). Relative fold-change in expression values were calculated using the following formula:

\[ 2^{(\Delta C_p(\text{Sample}) - \Delta C_p(\text{Spleen}))} \text{, where } \Delta C_p(\text{Sample}) = \text{Sample } C_p \text{Gene of Interest} - \text{Sample } C_p \text{GAPDH}, \]

and \[ \Delta C_p(\text{Spleen}) = \text{Spleen } C_p \text{Gene of Interest} - \text{Spleen } C_p \text{GAPDH}. \]

**Isolation of bulk Treg populations for RNA sequencing**

100-200 DP, SP, and DN Treg cells from four LucOS-infected, KP, Foxp3-RFP mice were sorted into Buffer TCL (Qiagen) plus 1% B-mercatoethanol using a MoFlo Astrios cell sorter. SMART-Seq2 (Picelli et al., 2013) was then used as previously described to generate cDNA, with with some modifications: RNA was purified using 2.2X RNAclean SPRI beads (Beckman Coulter) without final elution, after which beads were air-dried and immediately resuspended with water and oligoDT for annealing, and 18 cycles of preamplification were used for cDNA. cDNA was then mechanically sheared and prepared into sequencing libraries using the Thru-Plex-FD Kit (Rubicon Genomics). Sequencing was performed on an Illumina HiSeq 2000 instrument to obtain 50 nt paired-end reads.

cDNA from bulk Treg cell populations from Lenti-Cre-infected, p53\(^{5/6}\), or naive mice were prepared as described, but libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and subsequently sequenced on an Illumina NextSeq instrument.

**RNA-seq data processing and signature analyses:**

Reads that passed quality metrics were mapped to the UCSC mm9 mouse genome build (http://genome.ucsc.edu/) using RSEM(v1.2.12) (http://deweylab.github.io/RSEM/) (Li and Dewey, 2011). Mapped read counts estimated
from RSEM were upper-quartile normalized to a count of 1000 per sample (Bullard et al., 2010). Genes with normalized counts less than an upper-quartile threshold of 20 across all samples were considered lowly expressed and excluded from further analyses. The dataset was log2 transformed before subsequent analysis. All RNA-Seq analyses were conducted in the statistical programming language R (http://www.r-project.org/).

Unsupervised clustering of samples was performed using a Pearson correlation-based pairwise distance measure.

High-resolution signature analyses between bulk Treg cell populations were performed using a blind source separation methodology based on ICA (Hyvärinen and Oja, 2000). The R implementation of the core JADE algorithm (Joint Approximate Diagonalization of Eigenmatrices) (Biton et al., 2014; Nordhausen et al., 2014; Rutledge and Jouan-Rimbaud Bouveresse, 2013) was used along with custom R utilities. In order to detect biologically relevant signatures, they were visualized using the sample-to-signature correspondence schematic afforded by Hinton plots, where colors represent relative directionality of gene expression (red relatively upregulated, blue relatively downregulated within each signature) and the size of each rectangle quantifies the strength of a signature (column) in a given sample (row). Each signature is two-sided, allowing for identification of upregulated and downregulated genes for each signature within each sample. Biologically relevant and statistically significant signatures were identified using the Mann–Whitney U-test for assessing statistical significance between a pair of conditions and the Kruskal-Wallis test for greater than two conditions.
Signature correlation scores (z-scores) for each gene are included in supplementary table 1.

**External datasets used to generate pan-lung signature**

For the generation of the pan-lung Treg signature, previously published RNA-seq datasets (accessions GSE58998, GSE62535, GSE71588) were downloaded from the sequence read archive (SRA, www.ncbi.nlm.nih.gov/sra) and converted to fastq format using the SRA toolkit for use in pan-lung signature analysis. Reads were aligned to the mouse genome (mm9) using RSEM, as before. Sample “Ezh2 Het CD62Lhi rep3” was dropped from GSE58998 due to poor coverage. Samples from the current study were re-processed as single-ended fastq files to match external samples. The combined dataset was filtered to drop lowly expressed genes and analyzed for signatures, as described above.

**Heatmaps**

Heat maps for signatures were plotted with genes with standardized signature correlation scores \(|z| > 3\) or otherwise indicated. Heat maps were generated using the Heatplus package in R.

**Gene set enrichment analyses (GSEA)**

Gene set enrichment analyses were carried out using the pre-ranked mode in GSEA with standardized signature correlation scores for the KPLungTR signature and default settings using gene-sets from MSigDB (Subramanian et al., 2005) and a custom immunologic signatures library of gene sets (included in supplementary materials). These custom gene sets were added to the existing MSigDB immunologic collection (c7) to produce a custom immunologic library. Enrichment statistics (Normalized
Enrichment Score – NES, p-value, FDR) for the custom immunologic gene-sets were calculated in the context of the combined c7 MSigDB collection (original MSigDB c7 set plus custom gene-sets).

Network representations of GSEA results were generated using EnrichmentMap (http://www.baderlab.org/Software/EnrichmentMap) for Cytoscape v3.3.0 (http://www.cytoscape.org) with FDR < 0.05 and an overlap parameter value of 0.5. Each circle in the enrichment map represents a gene set where diameter corresponds to gene set size. Connecting lines correspond to minimum 50% mutual overlap with line thickness corresponding to the number of overlapping genes. Red circles are upregulated gene sets while blue circles are downregulated gene sets. Pathway identification was manually annotated.

Clinical Data Analyses

RNA-seq gene expression profiles of primary tumors and relevant clinical data of 515 lung adenocarcinoma (LUAD) patients were obtained from the Cancer Genome Atlas (TCGA; cancergenome.nih.gov).

From the set of TCGA LUAD normal tissue samples with matched tumor samples, a random set of five normal tissue samples was selected. These, together with corresponding matched tumor samples, were used to create an RNA-seq expression subset from the TCGA repository (total n=10 samples). This dataset was analyzed for expression signatures using ICA, as described earlier. The most significant signature (p=0.00397, Mann-Whitney test) separating tumor samples versus normal samples was chosen to identify genes differentially expressed in LUAD tumor tissue compared to normal lung tissue (|z| > 3). 117 genes were identified as up-regulated in tumor tissue.
and 172 genes were identified as down-regulated in tumor tissue. The KPLungTR signature, which represents genes (|z| > 3) distinguishing lung and lymph node/spleen Treg populations genes, was depleted of these LUAD tumor-normal differentially expressed genes prior to downstream clinical data analyses. Differentially expressed genes in LUAD tumor tissue compared to normal tissue are included in Supplementary Table 1.

This modified KPLungTR signature was used to score individual TCGA tumor expression profiles using ssGSEA (Barbie et al., 2009). Patients were stratified based on standardized ssGSEA scores and Kaplan-Meier survival analyses were conducted to compare high-scoring patients with wither low-scoring patients (equal sized patient groups) or with the rest of the patient cohort and significance was assessed using the log-rank test. A similar strategy was followed for various other cancer types using expression and clinical datasets from TCGA: Colon Adenocarcinoma (COAD), Pancreatic Adenocarcinoma (PAAD), Lung Squamous Cell Carcinoma (LUSC), Head and Neck Squamous Cell Carcinoma (HNSC), and Low Grade Glioma (LGG).

Cox proportional hazards analysis was conducted across all patients in the TCGA LUAD cohort to assess the prognostic significance of the signature while controlling for various clinical covariates. Gender, T-score, N-score, Stage covariate data were obtained from TCGA clinical files. KRAS and EGFR mutational status and mutational load per patient were inferred using protein altering mutational calls from MuTect (Cibulskis et al., 2013) and an exome size of 30Mb. Data on prevalence of transversions, neoepitope count, smoking status, and tumor purity estimates (ABSOLUTE) (Carter et al., 2012) scores were obtained from (Campbell et al., 2016).
ESTIMATE tumor purity scores were calculated as described (Yoshihara et al., 2013). FOXP3/CD8A log2 expression ratio was calculated from TCGA LUAD RNA-seq data. Foxp3 transcriptional module scoring was done with ssGSEA using the Foxp3 module described in (Linsley et al., 2015). The CYT metric was derived using the geometric mean of TCGA expression levels of GZMA and PRF1 (Rooney et al., 2015). Empirical Cumulative Distribution Function (ECDF) plots were used to analyze difference in values of continuous covariates between top and bottom 25th percentiles of patients (LTR_Hi and LTR_Low) ranked by the KPLungTR standardized ssGSEA signature score. Significance was assessed using the Kolmogorov-Smirnov test. Stacked barcharts were used to visualize fractions of discrete covariate values within the top and bottom patient buckets and significance was assessed using the hypergeometric test.

All survival analyses were conducted in the R statistical programming language using the survival package.

Scoring GSE37138 (responders/non-responders) for KPLungTR signature

Affymetrix CEL files for GSE37138 were downloaded from the GEO repository (Gene Expression Omnibus) and processed (rma-sketch) with Affymetrix Power Tools (APT) using array annotation for HuEx_1_0_STv2. Probesets were collapsed to the maximum value per gene and the resulting expression set was scored with the KPLungTR signature (|z-score| >3, |fold-change| > 2) using ssGSEA. Significance between scores of responders and non-responders was assessed using a 1-sided Mann-Whitney test.

Identification of DP Signature
In order to detect signatures with lower amplitudes of gene expression changes, we employed ICA on a non-log-transformed version of the dataset. In general, the log transform stabilizes the variance in the dataset, and helps the signature analysis approach handle low-level noise better. However, even in the absence of this variance stabilizing transformation, we are able to detect biologically relevant gene expression changes at a finer level in this dataset. We detected a signature separating CD103+KLRG1+ lung Tregs from other populations. Genes in this signature with \(|z\text{-score}| > 3\) were selected for downstream analysis. Given the absence of a variance stabilizing transform in this dataset, an additional expression level filter was implemented to select the final list of signature genes. For each gene, its expression in all CD103+KLRG1+ lung Treg samples had to be greater (for up-regulated genes) than all but a maximum of 3 other samples (3 out of a total 8 other samples). A similar filtering scheme was employed in the other direction for down-regulated genes. This yielded a total of 43 up-regulated and 2 down-regulated genes in CD103+KLRG1+ lung Tregs. This set of genes was used to illustrate gene expression level changes in a heatmap (Figure 1f).

**Single-cell sorting of Tconv and Treg populations for RNA sequencing**

Tconv (DAPIneg, i.v. neg, Thy1.2+CD4+Foxp3-GFPneg) and Treg (DAPIneg, i.v. neg, Thy1.2+CD4+Foxp3-GFP-positive) cells were single-cell sorted into Buffer TCL (Qiagen) plus 1% B-mercaptoethanol in 96-well plates using a MoFlo Astrios cell sorter. Each plate had 30-100 cell population well and an empty well as controls. Following sorting, plates were spun down for 1" at 2000 RPM and frozen immediately at -80C.

**Preparation of scRNAseq libraries**
Plates were thawed and RNA was purified using 2.2X RNAclean SPRI beads (Beckman Coulter) without final elution (Shalek et al., 2013). SMART-seq2 and Nextera library preparation was performed as previously described (Picelli et al., 2013), with some modifications noted in a previous study (Singer et al., 2017). Plates were pooled into 384 single-cell libraries, and sequenced 50 x 25 paired end reads using a single kit on the NextSeq500 5 instrument.

**Pre-processing of SMART-Seq2 scRNA-seq data**

BAM files were converted to de-multiplexed FASTQs using the Illumina-provided Bcl2Fastq software package v2.17.1.14. Paired-end reads were mapped to the UCSC mm10 mouse transcriptome using Bowtie with parameters ‘-n 0 -m 10’, which allows alignment of sequences with zero mismatches and allows for multi-mapping of a maximum of ten times.

Expression levels of genes were quantified using TPM values calculated by RSEM v1.2.8 in paired-end mode. For each cell, the number of detected genes (TPM > 0) was calculated and cells with less than 600 or more than 4000 genes detected were excluded as well as cells that had a mapping rate to the transcriptome below 15%.

Furthermore, to remove doublets which we saw came from mostly B cells and epithelial cells, we calculated the sum log2(TPM+1) over Cd79a, Cd19, Lyz1, Lyz2 and Sftpc, and excluded any cell that scored higher than 3. To remove lowly expressed genes, a gene had to be expressed above log2TPM of 3 in at least five cells. Some Tconv cells came from mice that did not have the Foxp3-GFP reporter allele, therefore we had to infer which cells were Tregs from the data. In order to do so, we assigned each cell a Treg signature score by centering and scaling the log2TPM values and averaging over
Foxp3, Ikzf2, Areg, If1r1, Folr4, Wls, Tnfrsf9, Klrq1. Cells with a higher Treg signature score of -1 were labeled as Treg cells.

**Batch effect**

Some samples were expressing a group of genes that were highly correlated (identified by pagoda, see below). The only confounder that we could identify with this signature was the plate processing. Since these genes have no biological relevance based on gene annotation, we think that these genes come from some contamination, hence affecting some batches of plates that were processed together.

**Dimensionality reduction using SVD and tSNE**

We performed dimensionality reduction using singular value decomposition (SVD) using the irlba function implemented in the irlba package in R. SVD was performed on the full expression matrix except for the batch genes after centering and scaling gene expression across cells. The left singular vectors were multiplied with the singular values. Significant dimensions were picked by looking for the elbow in the distribution of singular values as we as comparing the distribution of singular values to a null distribution when shuffling the gene expression table. Shuffling the gene expression table was performed by keeping the number of expressed genes per cell the same, but rather shuffling the expression values detected, and by that randomizing the gene x cell association without deleting the complexity distribution of the cells. Further dimensionality reduction to two dimensions for visualization was then performed using t-Distributed stochastic neighbor embedding (tSNE) (Maaten and Hinton, 2008) on the significant weighted singular vectors using the Barnes-Hut implementation in R (Rtsne function from Rtsne package, with $10^4$ iterations and a perplexity of 30).
Identifying gene modules and timepoint-dependent changes

Gene modules were identified using pagoda (Fan et al., 2016) using the scde R package. This was done on the counts table from rsem after cleaning the data using the clean.counts function (min.lib.size=1000,min.detected=50). The knn.error.model function was run using a k of 30, which is much lower than default. But the modules were not significantly different when running ok default k (# cells / 4). We then ran the pagoda.varnorm and the pagoda.subtract.aspect function which then allowed us to run pagoda.gene.clusters which identifies de-novo correlated genes in the dataset. We forced pagoda to return 100 modules. We identified modules with a significance z.score above 1.96 and that contained more than 5 genes. Mean module expression was calculated by averaging over the genes in each module of the centered and scaled gene expression table. When testing whether module expression changes over the course of tumor development, we ran a linear model for each module and compared a full model: module.activity ~ detected genes + gender + time point to a reduced model: module.activity ~ detected genes + gender. For the time point covariate, healthy lung was the reference. Likelihood ratio test p-values were then corrected for multiple hypothesis testing for the number of modules using the p.adjust function computing the false discovery rate in the stats package.

Comparison of bulk and single cell RNAseq signatures to published signatures

Lists of differentially expressed genes in human cancer Tregs, mouse tissue Tregs, Tr17 cells from mice, and mouse activated Tregs were collected either from the supplementary tables of the publications, or generously provided by the authors (De
Simone et al., 2016; Kim et al., 2017; Miragaia et al., 2017; Plitas et al., 2016; van der Veeken et al., 2016). See Supplementary Table 1.

**T cell receptor (TCR) reconstruction from scRNAseq**

TCR were reconstructed using tracer. Because our reads were 20-50 paired end, we ran tracer in a short read mode with the following settings ‘--inchworm_only=T --trinity_kmer_length=17’.

**Population-level TCR Beta chain sequencing and analysis**

To extract TCR beta chains from RNA sequencing data of DP, SP, and DP Tregs, we used MiXCR software with library version repseqio.v1.3 for each population (Bolotin et al., 2015). Populations were then analyzed and compared with VDJtools software (Shugay et al., 2015).

For bulk TCR beta chain sequencing, T cells were sorted directly into 250μl RNAprotect buffer (Qiagen), spun down for 1" at 2000 RPM, and immediately frozen at -80°C. Samples were sent to iRepertoire (Huntsville, AL) for library preparation and sequencing. TCR sequences were analyzed and compared with VDJtools software as described above.
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REFERENCES


CHAPTER 3

The role of IL-33 in promoting regulatory T cell expansion
and function in lung adenocarcinoma

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ABSTRACT

Regulatory T (Treg) cells can promote tumor development by suppressing anti-tumor immunity through a variety of mechanisms. Depletion of Treg cells can invigorate tumor immune responses, but carries significant risk of morbidity due to the importance of Treg cells in maintaining immune tolerance. Using transcriptional profiling of Treg cells in a genetic mouse model of lung adenocarcinoma, we recently identified ST2 as a marker of highly activated Treg cells in advanced tumors. ST2, which is the receptor for interleukin 33 (IL-33), has an established role in promoting tissue Treg cell proliferation and function, but a controversial role in tumor development. Here, we show that ST2 may be required for increased numbers of terminally-differentiated Treg cells in lung cancer. Furthermore, preliminary data suggests that Treg-specific ablation of ST2 may enhance CD8+ T cell infiltration of tumors and have a modest effect on tumor burden. We therefore show that IL-33 regulates Treg cell-mediated tumor immune suppression, and may thus be an attractive therapeutic target in cancer.
INTRODUCTION

Lung cancer accounted for 12.9% of all new cancer diagnoses in 2012, and is the leading malignancy and cause of cancer-related deaths worldwide (Wong et al., 2017). Non-small cell lung cancer (NSCLC), which accounts for 80-85% of lung cancer cases, presents as advanced disease in over half of new cases (Cetin et al., 2011). 40% of NSCLC patients have the lung adenocarcinoma histologic subtype, which has a 5-year survival rate of 1-4% for patients diagnosed with stage IV disease. The advent of immune checkpoint inhibitors may drastically change the prognosis for NSCLC; the longest-running trials show that patients treated with anti-PD-1 therapy have a 5-year survival rate of 16%, which is four-fold greater than the 5-year survival rate on standard of care (Brahmer et al., 2017). Nevertheless, only a subset of patients will achieve durable responses on current immune therapies, indicating a strong need for therapeutic strategies that can inhibit additional immunosuppressive mechanisms in lung cancer.

A suppressive population of T lymphocytes called regulatory T cells (Tregs) may play a dominant role in impairing anti-tumor immune responses (Tanaka and Sakaguchi, 2017). Treg cells are a subset of CD4+ helper T cells that maintain immune tolerance and prevent autoimmunity (Josefowicz et al., 2012; Sakaguchi, 2011). They are characterized by their expression of the transcriptional regulator FOXP3, which drives much of the Treg cell transcriptional program. Treg cells can suppress inflammation through a variety of mechanisms, including the production of inhibitory cytokines, direct killing of cells, competition with other T cell subsets for antigen or other substrates, and suppression of antigen presentation (Caridade et al., 2013; Savage et al., 2013; Vignali...
et al., 2008). In a number of mouse models, Treg cell depletion can enhance anti-tumor immunity (Bos et al., 2013; Joshi et al., 2015; Marabelle et al., 2013). Furthermore, preclinical models suggest that antibodies directed against CTLA-4, which is expressed on Tregs and other T cells, act in part by depleting Treg cells in the tumor microenvironment (Simpson et al., 2013). Therefore, inhibiting the function of Treg cells in tumors may complement other cancer therapies by alleviating tumor immunosuppression and improving the immune response against cancer. To avoid systemic and potentially lethal autoimmune reactions, therapeutic strategies should be aimed specifically at targeting the tumor Treg cell population.

Inducible, autochthonous models of cancer recapitulate the longitudinal development of tumors and associated immune responses. They are therefore more likely to recapitulate a highly immunosuppressive tumor microenvironment than transplanted tumors, which may inherently appear more “foreign” (Dranoff, 2011). Our lab has developed a model of lung adenocarcinoma where activation of an oncogenic allele of K-ras and loss of Trp53 is driven by intratracheal delivery of a lentivirus expressing Cre recombinase (KP: LSL-KrasG12D, p53f/f) (DuPage et al., 2009). These mice allow us to study endogenous tumor immune responses, and through expression of the known T cell antigens chicken ovalbumin (Ova) and the peptide SIYRYYGL (Lenti-LucOS) in tumor cells we can also monitor tumor-specific cytotoxic T cell (CTL) responses (DuPage et al., 2011a). Tumor growth in the KP model is accompanied by an expansion of Treg cells (DuPage et al., 2011a; Joshi et al., 2015) that express CD103 (Huehn et al., 2004; Lehmann et al., 2002; Sather et al., 2007) and KLRG1 (Beyersdorf et al., 2007; Cheng et al., 2012), surface molecules associated with terminal
differentiation. Although Tregs early in tumor development are predominantly CD103-KLRG1- (double-negative, or DN), tumor progression is associated with an expansion of CD103+KLRG1- (single-positive, or SP) Tregs and CD103+KLRG1+ (double-positive, or DP) Tregs. DP Tregs have a gene expression signature associated with TCR signaling and Treg effector functions, suggesting that tumor growth is accompanied by enhanced Treg activity (Chapter 2). Furthermore, Treg depletion results in massive infiltration of lungs by CD4+ and CD8+ T cells, suggesting that Tregs actively suppress anti-tumor immune responses (Joshi et al., 2015).

We recently identified /IL1R1/, the gene encoding growth stimulation expressed gene 2 (ST2), as a gene upregulated in lung Treg cells from tumor-bearing KP mice (Chapter 2). ST2 is an interleukin 1 receptor (IL-1R)-like molecule expressed by mast cells, innate lymphoid cells, and several lymphocytic populations, including type 2 (Th2) and Treg CD4+ T cells (Griesenauer and Paczesny, 2017). ST2 has one known ligand, interleukin 33 (IL-33), which is an alarmin thought to recruit immune cells to sites of tissue damage. Signaling through ST2 promotes Th2 responses in models of asthma and allergic inflammation. Recently, ST2 has also been shown to drive Treg cell suppressive activity in mouse models of colitis (Schiering et al., 2014) and graft-versus-host disease (GVHD) (Matta et al., 2016). The recruitment and function of tissue Treg cells appears to be particularly dependent on ST2 signaling; ST2-deficient mice have fewer adipose tissue Treg cells (Kolodin et al., 2015; Vasanthakumar et al., 2015), and impaired tissue regeneration after muscle injury and hepatic viral infection (Kuswanto et al., 2016; Popovic et al., 2017). IL-33 may also stimulate Treg cells in influenza-infected lung to produce amphiregulin, which aids in tissue repair (Arpaia et al., 2015). We
therefore hypothesized that ST2 may be a critical mediator of Treg cell function in the lung tumor environment.

In this study, we show that ST2 is expressed preferentially on lung effector Treg cells associated with advanced lung adenocarcinoma. IL-33 is highly expressed by normal lung epithelial and tumor cells, and recombinant IL-33 is sufficient to promote the expansion of terminally-differentiated Treg cells. Using IL-33-deficient mice we also demonstrate that IL-33 signaling is necessary for the observed increase in effector Treg cells in the tumor microenvironment. Early data suggests that Treg cell-specific ablation of ST2 results in greater CD8+ T cell infiltration of tumors and a subtle reduction in tumor size. Taken together, we show that ST2 regulates Treg cell-mediated tumor immune suppression, and disruption of ST2 signaling may thus be an attractive therapeutic target in cancer.

RESULTS

ST2 is upregulated on terminally-differentiated Tregs in KP tumor-bearing mice

We have previously shown that tumor development in the KP model is associated with the longitudinal expansion of terminally-differentiated DP Treg cells in tumor-bearing mice (Chapter 2). We hypothesized that DP Treg cells are major contributors to tumor immune suppression and therefore sought to impair the development of this population by targeting genes that may regulate the Treg effector phenotype. ST2 is expressed by nearly 40% of lung Treg cells as compared to ~10% of Treg cells in the mediastinal (draining) lymph node (Figure 1a). In contrast, <5% of Tconv cells in the lung express ST2. Moreover, ST2 expression is highest in the DP subset of Treg cells, suggesting that ST2 levels track with the effector Treg phenotype
(Figure 1b). Treg cells from tumor-bearing KP, LucOS-infected mice express the membrane-bound and soluble isoforms of ST2 (Figure 1c). Soluble ST2 (sST2) is thought to mitigate ST2 signaling through sequestration of IL-33.
Figure 1. ST2 is upregulated in terminally differentiated Tregs in lung tumor-bearing mice

A. Barplot showing %ST2-positive of lung and msLN Tregs (i.v.neg,CD4+Foxp3+) and Tconv cells (i.v.neg,CD4+Foxp3-) from tumor-bearing LucOS mice week 20 p.i. as measured by flow cytometry.

B. Flow cytometric analysis of ST2 expression on CD103-KLRG1- (DN, black), CD103+KLRG1- (SP, blue), and CD103+KLRG1+ (DP, red) Treg cells isolated from tumor-bearing lungs.

C. qRT-PCR analysis of expression of NM_001025602.3 (Ili1rl1 transcript variant 1 encoding membrane-bound ST2) and NM_010743.3 (variant 2 encoding soluble ST2) in DP, SP, and DN lung Treg cells as well as in SP and DN msLN Treg cells. Values represent $2^{\Delta\Delta CT}$, with control being splenic Treg expression.
IL-33 is sufficient to promote an increase in terminally-differentiated Tregs in tumor-bearing lungs

We were further motivated by the observation that IL-33 is highly expressed in normal lung and in early and late lung adenocarcinomas in the KP model (Figure 2a). In normal lung, we found that IL-33 expression was predominantly found on surfactant protein C (SPC)-expressing type II epithelial cells (Figure 2b). Indeed, while tumor cells at all timepoints express IL33 transcript variant 2 (NM_133775), tumor development is associated with an increase in expression of IL33 transcript variant 1 (NM_001164724) (Figure 2c), which has been shown to be upregulated following inflammatory signaling.

In order to determine the effect of IL-33 on tumor immune responses, we intratracheally administered recombinant mouse IL-33 (rIL-33) to tumor-bearing KP, Lenti-LucOS-infected mice (Figure 2d). Consistent with prior reports, we observed significant inflammatory infiltration and epithelial thickening in IL-33-treated mice compared to controls (Figure 2e). Immune infiltration was found in tumors and throughout the lung, and we observed similar inflammation in naive wild-type mice treated with IL-33 (data not shown). Flow cytometric analysis revealed a striking increase in eosinophils (Figure 2f), as well as an 8 to 9-fold increase in CD4+ and CD8+ T cells in the lung (Figure 2g). A greater percentage of CD4+ T cells were Treg cells, of which 64% of cells were DP compared to 34% in PBS-treated controls (Figure 5d-i). Correspondingly, rIL-33 treated mice had fewer SP and DN Treg cells. The proportion of Tregs and their phenotype were unchanged in ST2-deficient mice treated with rIL33 (data not shown), suggesting that the observed effects on Treg numbers are dependent on IL-33 signaling through ST2. Taken together, rIL-33 administration is
sufficient to drive not only a major expansion of the lung Treg population, but also to promote a shift towards the effector Treg phenotype.
Figure 2. IL-33 is sufficient to promote Treg cell differentiation in tumor-bearing lungs
A. Immunohistochemical staining of tumor-bearing lungs from KP mice at weeks 12 and 22 p.i. with Lenti-LucOS. Two representative images are shown per timepoint.

B. Representative immunofluorescent staining of lung from naive (un-infected) lung.

C. Heatmap of expression of $\beta 33$ isoforms in Lenti-LucOS tumors at indicated timepoints after tumor induction. Expression values are shown as log(TPM-1), where TPM stands for transcripts per million.

D. Schematic of experiment administering recombinant IL-33 (rIL-33) or PBS control to late-stage, tumor-bearing KP mice. All rIL-33 experiments are representative of 2-3 separate experiments, each with n=4-5 mice/group.

E. Representative H&E-stained histological images of control and rIL-33-treated lungs.

F. Barplot of proportion of eosinophils (i.v.neg, CD45.2+CD11c-/low, SiglecF+) in i.v.neg CD45+ lung cells from control and rIL-33-treated mice.

G. Barplots of proportions of CD8 and CD4 lung cells of i.v. neg cells in control and rIL-33-treated mice. Absolute numbers of cells are plotted below.

H. Barplot of %SIINFEKL/Kb tetramer-positive i.v.neg, CD8+ lung T cells in control and rIL-33-treated mice.

I. Barplot of proportion of Treg cells of i.v. neg, CD4+ lung T cells in control and rIL-33-treated mice. Absolute numbers of cells are plotted below. *p < 0.05, two-tailed, unpaired Student’s t test.

J. Barplot of %CD103-KLRG1- (DN, black), CD103+KLRG1- (SP, gray), and CD103+KLRG1+ (DP, red) Treg cells isolated from the tumor-bearing lungs of control and rIL-33-treated mice. ****p < 0.0001, Sidak’s multiple comparisons test.
IL-33 is required for the observed increase in terminally-differentiated lung tumor Treg cells

We next asked whether IL-33 was necessary for the development of the Treg effector phenotype. We crossed Il33-/- mice to KP mice to generate “KP, IL33KO” mice that we could use to study tumor development and associated immune responses in an IL-33-deficient context. We observed no difference in the proportion of Treg cells between tumor-bearing KP, IL33KO and KP mice (Figure 3a). However, 8% of Tregs in KP, IL33KO mice were DP, as compared to 27% of Tregs in KP mice (Figure 3b). Meanwhile, KP, IL33KO mice had a 2.4-fold greater proportion of DN Treg cells than KP controls, resulting in a ~60% reduction in DP:SP ratio. We wondered whether the primary source of IL-33 was other immune cells or epithelial cells, and therefore transferred IL-33-deficient bone marrow into lethally irradiated KP mice (“IL33KO>KP BM chimeras”) to study tumor development in the context of loss of hematopoietic cell-derived IL-33. IL33KO>KP BM chimeras, however, did not demonstrate the same reduction in DP Tregs as seen in KP, IL33KO animals (Figure 3c), suggesting that the majority of IL-33 available for signaling in the lung tumor microenvironment is epithelial or stromal cell-derived.

Immunohistochemical staining confirmed that tumors in KP, IL33KO animals were IL-33-negative (Figure 3d). Histological analysis of tumor-bearing lungs revealed that KP, IL33KO mice had 50% fewer lesions normalized to total lung area compared to KP control mice (Figure 3e). Interestingly, IL-33-deficient KP mice demonstrated a slight trend toward having larger tumors that failed to reach statistical significance. Furthermore, no difference was observed in the degree of tumor infiltration by CD8+ T
or Foxp3+ Treg cells. These data demonstrate that IL-33 may be required for full
differentiation to, or maintenance of, the Treg cell effector phenotype, but loss of IL-33 is
insufficient to boost anti-tumor immune responses to strongly affect tumor burden.
Figure 3. IL-33 is necessary for complete differentiation of lung tumor Treg cells
All experiments with KP, IL33KO mice are representative of 2-3 separate experiments, each with n=4-5 mice/group.

A. Barplot comparing %Foxp3+ of i.v.neg, CD4+ lung T cells from KP vs. KP, IL33KO mice.

B. Barplot of %CD103-KLRG1- (DN, black), CD103+KLRG1- (SP, gray), and CD103+KLRG1+ (DP, red) Treg cells isolated from the tumor-bearing lungs of
KP vs. KP, IL33KO mice. ***p < 0.0001, *p<0.05, Sidak’s multiple comparisons test.

C. Same as in B, but comparing KP mice grafted with wild-type (WT) or IL-33-deficient bone marrow (IL-33 KO). Mice were harvested at week 20-24 p.i. with Lenti-LucOS.

D. Barplot of average number of IL-33+ cells normalized to tumor area per mouse. IL-33 was measured by immunohistochemical (IHC) staining of histological cross-sections of tumor-bearing lungs. ****p < 0.0001, unpaired, two-tailed Student’s t test.

E. Barplot of total number of lung tumors normalized to total lung area of KP vs KP, IL33KO mice as measured in histological cross-sections of tumor-bearing lungs. **p < 0.01, unpaired, two-tailed Student’s t test.

F. Barplot of average tumor size (um²) of KP vs KP, IL33KO mice as measured in histological cross-sections of tumor-bearing lungs.

G. Same as in D, but showing average number of Foxp3+ cells normalized to tumor area per mouse.

H. Same as in D, but showing average number of CD8+ cells normalized to tumor area per mouse.
Treg-specific ST2 ablation results in impaired expansion of terminally-differentiated Treg cells and enhanced CD8+ T cell infiltration of tumors

Given some reports of IL-33 in promoting CD8+ T cell memory responses and antigen presentation in the tumor microenvironment (Dominguez et al., 2017; Gao et al., 2015), we reasoned that KP, IL33KO mice may not have a reduction in tumor burden because both Treg and other anti-tumor immune responses may be dependent on IL-33 signaling. To determine the effect of Treg-specific loss of IL-33 signaling on the development of tumors, we used a modified version of the KP model wherein FlpO recombinase drives expression of oncogenic K-ras and loss of p53 (KPfrt: FSF-Kras<sup>G12D</sup>, p53<sup>frt/frt</sup>), which allows use of the Cre-lox system to study the effects of Treg-specific Il1rl1 deletion. We crossed KPfrt mice to Foxp3<sup>YFP-Cre</sup> and Il1rl1<sup>fr/fl</sup> mice to model lung adenocarcinoma development in the setting of Treg-specific ST2 deficiency (Figure 4a). We infected these mice with a lentivirus expressing FlpO recombinase and GFP fused to Ova and SIYRGYYL (FlpO-GFP-OS) in order to induce tumors that would express the same strong T cell antigens as those in the Lenti-LucOS model. Similar to IL-33-deficient mice, KPfrt, Foxp3<sup>YFP-Cre</sup>, Il1rl1<sup>fr/fl</sup> mice did not differ from KPfrt, Foxp3<sup>YFP-Cre</sup> mice in their percentage of CD4+ T cells that were Tconv or Treg cells (Figure 4b-c). KPfrt, Foxp3<sup>YFP-Cre</sup>, Il1rl1<sup>fr/fl</sup> mice had a statistically insignificant trend towards a lower proportion of DP Treg cells, and a higher proportion of DN cells, resulting in a DP:SP ratio that was ~50% lower than in control mice (Figure 4d). Taken together, KPfrt, Foxp3<sup>YFP-Cre</sup>, Il1rl1<sup>fr/fl</sup> mice recapitulate the reduction in the number of Tregs with the DP phenotype observed in KP, IL33KO mice.
We next looked at whether KPft, Foxp3^{YFP-Cre}, Il1rl1^{f/f} mice differ in tumor burden or immune cell infiltration from control KPft, Foxp3^{YFP-Cre} mice. Immunohistochemical staining revealed that tumors from KPft, Foxp3^{YFP-Cre}, Il1rl1^{f/f} mice had over 50% greater CD8+ T cell infiltration than tumors from control mice (Figure 4e). Interestingly, tumors from KPft, Foxp3^{YFP-Cre}, Il1rl1^{f/f} mice also had nearly 40% greater Foxp3+ cell infiltration than tumors from control KPft, Foxp3^{YFP-Cre} mice, consistent with Treg-specific ST2 ablation resulting in more inflamed tumor lesions on average. Although not statistically significant, KPft, Foxp3^{YFP-Cre}, Il1rl1^{f/f} mice also have a slight trend towards reduced total tumor burden and average tumor size compared to control mice, suggesting that greater immune infiltration of tumors may result in better inhibition of tumor growth. Perhaps consistent with Treg-specific ST2 loss promoting a less immunosuppressive environment, in our preliminary data we observe a higher percentage of TNFα-producing CD4+ T cells and fewer neutrophils in KPft, Foxp3^{YFP-Cre}, Il1rl1^{f/f} mice. Our studies suggest that Treg-specific inhibition of ST2 signaling may result in a less immunosuppressive tumor microenvironment characterized by increased immune infiltration of tumors and a trend towards lower tumor burden.
Figure 4. Treg-specific ST2 ablation results in impaired Treg cell terminal differentiation and enhanced CD8 T cell infiltration of tumors
All experiments with KPft, Foxp3<sup>YFP-Cre</sup>, Il1rl<sup>fl/fl</sup> mice are representative of 2-3 separate experiments, each with n=3-5 mice/group.

A. Schematic of Lenti-FlpO-GFP-OS used to infect KPft, Foxp3<sup>YFP-Cre</sup> and KPft, Foxp3<sup>YFP-Cre</sup>, Il1rl<sup>fl/fl</sup> mice.

B-C. Barplot of %Foxp3+ (B) and %Foxp3- (C) of i.v. neg CD4+ lung cells in KPft, Foxp3<sup>YFP-Cre</sup> vs. KPft, Foxp3<sup>YFP-Cre</sup>, Il1rl<sup>fl/fl</sup> mice.

D. Barplot of %CD103-KLRG1- (DN, black), CD103+KLRG1- (SP, gray), and CD103+KLRG1+ (DP, red) Treg cells isolated from the tumor-bearing lungs of KPft, Foxp3<sup>YFP-Cre</sup> vs. KPft, Foxp3<sup>YFP-Cre</sup>, Il1rl<sup>fl/fl</sup> mice. Ratio of DP to SP Tregs per mouse is shown on the right. ***p<0.001, two-tailed, unpaired Student’s t test.

E. Barplot of number of CD8+ cells in pooled tumors normalized to tumor area from KPft, Foxp3<sup>YFP-Cre</sup> vs. KPft, Foxp3<sup>YFP-Cre</sup>, Il1rl<sup>fl/fl</sup> mice. CD8 was measured by immunohistochemical (IHC) staining of histological cross-sections of tumor-bearing lungs.

F. Same as above, but showing number of Foxp3+ cells.

G. Barplot comparing total percentage of lung occupied by tumor in KPft, Foxp3<sup>YFP-Cre</sup> vs. KPft, Foxp3<sup>YFP-Cre</sup>, Il1rl<sup>fl/fl</sup> mice in histological cross-sections of tumor-bearing lungs.

H. Barplot comparing average tumor size (um2) in KPft, Foxp3<sup>YFP-Cre</sup> vs. KPft, Foxp3<sup>YFP-Cre</sup>, Il1rl<sup>fl/fl</sup> mice in histological cross-sections of tumor-bearing lungs.

I. Barplot comparing %IFNγ+TNFα+ (DP, red), %IFNγ+TNFα- (IFNγ SP, gray), %IFNγ-TNFα+ (TNFα SP, black), and %IFNγ-TNFα-, white) of CD4+ T cells in KPft, Foxp3<sup>YFP-Cre</sup> vs. KPft, Foxp3<sup>YFP-Cre</sup>, Il1rl<sup>fl/fl</sup> mice. ***p < 0.001, *p<0.05, Sidak’s multiple comparisons test.

J. Barplot comparing % neutrophils (i.v.neg,CD45.2+CD11c-SiglecF-Ly-6G+CD11b+) of tissue CD45.2+ cells. *p<0.05, two-tailed, unpaired Student’s t test.
DISCUSSION

A variety of strategies for inhibiting tumor Treg cells are currently under investigation. Many efforts attempt to deplete Treg cells by exploiting their high expression of various markers, which may also affect populations of Treg cells in lymphoid organs and other tissue sites. Recent reports have shown that genetic deletion of various transcription factors and signaling molecules critical to Treg cell identity, including neuropilin 1 (Nrp1), Nr4a1, c-Rel, and Helios, result in unstable or “fragile” Treg cells that are pro-inflammatory and can promote anti-tumor activity (Grinberg-Bleyer et al., 2017; Hibino et al., 2018; Nakagawa et al., 2016; Overacre-Delgoffe et al., 2017). Here, we use genetic mouse models to determine the role of ST2 in regulating the expansion and maintenance of terminally-differentiated Tregs in lung adenocarcinoma. IL-33 signaling was both sufficient and necessary to promote an increase in the number of terminally-differentiated Tregs in tumor-bearing mice. Remarkably, disruption of ST2 signaling on Treg cells may have impact tumor immunosuppression while sparing total Treg cell numbers, as tumors were more infiltrated by CD8+ T cells and slightly smaller in size. We therefore show that manipulation of IL-33 signaling may impair aspects of the Treg effector program without abrogating Treg identity or promoting an effector CD4+ T cell phenotype, which may be useful in avoiding immune adverse effects.

The role of IL-33 in cancer is controversial, most likely due its pleiotropic and context-specific functions (Wasmer and Krebs, 2016). IL-33 has been implicated in promoting tumor immunosuppression through the recruitment of type 2 innate lymphoid cells (ILCs) and Treg cells in a model of breast cancer (Jovanovic et al., 2014). A recent
study of NSCLC xenografts showed that IL-33 recruits type 2 (M2) macrophages and Treg cells (Wang et al., 2016, 2017), though we are the first to show in an immune-competent, genetically-defined mouse model of lung adenocarcinoma that IL-33 signaling may contribute to an expansion of differentiated Tregs and, consequently, tumor immunosuppression. Some studies point to ST2 signaling on tumor cells as a direct driver of transformation and tumorigenesis (Kim et al., 2015; Mager et al., 2015; Wang et al., 2016). Membrane-bound and, to a lesser degree, soluble ST2 are expressed at low levels on KP tumors (data not shown). KP, IL33KO animals do have fewer tumors, but they also have a slight trend toward having larger tumors, which complicates our interpretation of the effect of systemic loss of IL-33 signaling. Tumor-specific expression of ST2 is spared in KPfrt, Foxp3^{YFP-Cre}, Il1rl1^{fli} mice, which have a trend toward slightly smaller tumors, but we cannot rule out the possibility that tumor-specific ST2 deficiency would result in further impairment of tumor growth.

Other reports have demonstrated an immunostimulatory effect of IL-33 on dendritic cells, CD8+ T cells, and NK cells that can promote anti-tumor cytotoxicity (Dominguez et al., 2017; Gao et al., 2015; Villarreal et al., 2014). These findings are consistent with the role IL-33 has been shown to play in promoting CD8+ T cell memory responses and Th1 inflammation (Bonilla et al., 2012; Yang et al., 2011). These conflicting findings are likely due to the wide array of cell types that can express ST2, which may have opposing functions and varying degrees of relevance based on the tissue and inflammatory context. The interpretation of experiments performed in IL-33- or ST2-deficient models is thus complicated. Our data show that, compared to wild-type controls, KP, IL33KO mice have a more profound reduction in the number of terminally-
differentiated Treg cells, but less prominent CD8+ T cell infiltration of tumors than do KPfrt, Foxp3YFP-Cre, Il1r1fl/fl mice. Cells involved in the anti-tumor immune response, like CD8+ T cells and NK cells, may not be sufficiently activated in IL-33-deficient mice, which could account for a failure to recruit a stronger CD8+ T cell response in spite of having a muted Treg cell population. Conversely, IL-33 has been shown to indirectly promote Treg activity through its effects on dendritic cells and mast cells (Matta et al., 2014; Morita et al., 2015), which may blunt the cell-intrinsic effect of Treg-specific ST2 ablation. A comparison of Treg cells in KP, IL33KO mice and KPfrt, Foxp3YFP-Cre, Il1r1fl/fl mice may provide further insight on direct role of IL-33 in Treg cell function in tumors.

We were struck by the uniform high expression of IL-33 on KP tumors. Increased expression of the inducible isoform of IL33 during tumor progression suggests that tumor cells are undergoing active Toll-like receptor (TLR) signaling (Polumuri et al., 2012; Talabot-Ayer et al., 2012). Indeed, normal airway epithelial cells and lung tumors have been shown to express a wide variety of TLRs (Pinto et al., 2011). In spite of the large number of alveolar macrophages and other immune cell populations present in the lung, the major sources of IL-33 in the tumor microenvironment appear to be tumor and normal epithelial cells. This observation is consistent with other reports that IL-33 is expressed predominantly by epithelial and endothelial cells (Chen et al., 2015; Mager et al., 2015; Nakanishi et al., 2013). A number of shorter isoforms of IL-33 have also been described in human cells that demonstrate different half-lives and functional capabilities (Gordon et al., 2016; Hong et al., 2011). Further investigation is needed to determine
whether the expression of these IL-33 variants contribute to the regulation of ST2 signaling and Treg cell activity in the tumor microenvironment.

Although it has been well-established that IL-33 can promote and maintain tissue Treg cell populations, the effect of ST2 signaling on Treg cell function remains unclear. Previously published in vitro suppression assays suggest that ST2-deficient Tregs are just as immunosuppressive as their wild-type counterparts (Schiering et al., 2014). In contrast to studies showing that IL-33 can stimulate TCR-independent expansion of Treg cells (Arpaia et al., 2015; Kolodin et al., 2015), we did not observe a consistent reduction in lung Treg cell numbers as a result of IL-33 or Treg-specific ST2 deficiency, suggesting that ST2 is not required for Treg cell proliferation and maintenance in the lung. IL-33 has been shown to increase expression of Foxp3 and GATA-3 (Kolodin et al., 2015; Vasanthakumar et al., 2015), transcription factors integral for Treg cell terminal differentiation, which may explain the phenotypic changes observed in Treg cells deprived of ST2 signaling. A recent report identified amphiregulin as a target of IL-33 signaling (Arpaia et al., 2015), but administration of amphiregulin to KPfrt, Foxp3YFP-Cre, Il1r1<sup>1<sup>W/11</sup> </sup> mice with advanced disease did not revert the effect of ST2 deficiency on tumors (data not shown). Nevertheless, it is possible that earlier administration of amphiregulin during tumor development may complement the phenotypic changes observed in those mice. Meanwhile, we are currently investigating the transcriptional changes in Treg cells from KPfrt, Foxp3<sup>YFP-Cre</sup>, Il1r1<sup>1<sup>W/11</sup> </sup> mice that may underlie the observed shift in effector Treg phenotype.

Roughly 30-50% all ST2+ cells are Treg cells (data not shown), but their dual expression of the soluble and membrane-bound forms of ST2 may allow them to
receive ST2 signaling and prevent IL-33 signaling in other cell populations. Alternatively, sST2 secretion may also help restrain overactive ST2 signaling on Treg cells. The availability and activity of endogenous IL-33 in the tumor microenvironment is unclear. Since IL-33 has no signal sequence and is thus not actively secreted, ligand release is presumably dependent on cell death, although there has been a report of mechanical stress-induced secretion by living cells (Kakkar et al., 2012). Studies have shown that cytosolic mislocalization or overexpression of full-length IL-33 result in severe inflammation, suggesting that extracellular pools of IL-33 must be highly regulated (Bessa et al., 2014; Talabot-Ayer et al., 2015). Once released, full-length IL-33 can be processed into more active or inactive forms by different myeloid cell-derived proteases. Consequently, levels of active IL-33 are also dependent on the presence and activation status of diverse immune cell populations (Liew et al., 2016). We propose that ST2 expression on tissue Treg populations not only directs Treg recruitment, expansion, and/or differentiation in sites of tissue damage, but may also restrain local inflammation by cornering a limited supply of IL-33. This model may explain why we observe an influx of CD8+ T cells into tumors but a relatively modest effect on Treg cell phenotype.

A number of therapeutic antibodies directed against ST2 and IL-33 are in preclinical development, mostly for the treatment of allergy and asthma. As this pathway is increasingly considered for its immunomodulatory effects in cancer, our data point to the value of disrupting ST2 signaling on Treg cells, but also highlight the potential pitfalls of widespread inhibition of ST2 signaling. More preclinical studies using well-defined, immunocompetent tumor models may provide insight on which tumor environments would respond best to ST2-targeted treatment. New therapeutic antibody
modalities, such as bispecific antibodies, can also achieve cell-type-specific inhibition of particular targets (Lee et al., 2018), which could enable Treg-directed ST2 blockade in patients. Although we have not explored the effect of checkpoint blockade in our model of Treg-specific ST2 ablation, we predict that PD-1 and/or CTLA-4 blockade may enhance the function of tumor-infiltrating CD8+ T cells and thus further reduce tumor burden. Our findings thus support further investigation into the use of ST2 disruption, especially targeted to Treg cells, in combination with other immunotherapies in the treatment of cancer.
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MATERIALS AND METHODS

Mice

KP, KPfrt, Foxp3\textsuperscript{GFP}, and Il1r\textsuperscript{f/f} mice have been previously described (Bettelli et al., 2006; Chen et al., 2015; DuPage et al., 2011b; Young et al., 2011). Il33\textsuperscript{-/-} frozen sperm was purchased from the Knockout Mouse Project Repository (KOMP, Il33\textsuperscript{tm1.1(KOMP)v'cg}) and used to in vitro fertilize eggs from C57BL6/J females before implantation. Foxp3\textsuperscript{YFP/Cre} and Rosa26\textsuperscript{LSL-tdTomato} mice were purchased from Jackson Laboratory (Stock Nos. 007914 and 016959). All studies were performed under an animal protocol approved by the Massachusetts Institute of Technology (MIT) Committee on Animal Care. Mice were assessed for morbidity according to MIT Division of Comparative Medicine guidelines and humanely sacrificed by CO2 asphyxiation prior to natural expiration.

For in vivo labeling of circulating immune cells, anti-CD45-PE-CF594 (30-F11, BD Biosciences, 1:200) was diluted in PBS and administered by IV injection 2 minutes before harvest (Anderson et al., 2012).

For IL-33 treatment studies, 200ng of recombinant mouse IL-33 (BioLegend) was diluted in 50uL of PBS and administered intratracheally to mice as described previously (Li et al., 2014). Control mice received PBS only.

Lentiviral Production and Tumor Induction

The lentiviral backbone Lenti-LucOS has been described previously (DuPage et al., 2011b). The pGK::GFP-LucOS::EFS::FlpO lentiviral plasmid was cloned using Gibson assembly (Akama-Garren et al., 2016; Gibson et al., 2009). Briefly, GFP-OS was created as a protein fusion of GFP and ovalbumin\textsubscript{257-383}, which includes the
SIINFEKL and AAHAELNEA epitopes, and SIYRYYGL antigen. Lentiviral plasmids and packaging vectors were prepared using endo-free maxiprep kits (Qiagen). Lentiviruses were produced by co-transfection of 293FS* cells with Lenti-LucOS or Lenti-Cre, psPAX2 (gag/pol), and VSV-G vectors at a 4:2:1 ratio with Mirus TransIT LT1 (Mirus Bio, LLC). Virus-containing supernatant was collected 48 and 72h after transfection and filtered through 0.45um filters before concentration by ultracentrifugation (25,000 RPM for 2 hours with low decel). Virus was then resuspended in 1:1 Opti-MEM (Gibco) - HBSS. Aliquots of virus were stored at -80C and titered using the GreenGo 3TZ cell line (Sánchez-Rivera et al., 2014).

For tumor induction, mice between 8-15 weeks of age received 2.5 x10^4 PFU of Lenti-LucOS or 4.5 x 10^4 PFU of FlpO-GFP-OS intratracheally as described previously (DuPage et al., 2009).

**Tissue Isolation and Preparation of Single Cell Suspensions**

After sacrifice, lung lobes were allocated for histological analysis (usually lower right lobe) and/ or flow cytometric analyses. Lungs were placed in 2.5mL collagenase/ DNAse buffer (Joshi et al., 2015) in gentleMACS C tubes (Miltenyi) and processed using program m_impTumor_01.01. Lungs were then incubated at 37C for 30 minutes with gentle agitation. The tissue suspension was filtered through a 100 um cell strainer and centrifuged at 1700 RPM for 10 minutes. Red blood cell lysis was performed by incubation with ACK Lysis Buffer (Life Technologies) for 3 minutes. Samples were filtered and centrifuged again, followed by resuspension in RPMI 1640 (VWR) supplemented with 1% heat-inactivated FBS and 1X penicillin-streptomycin (Gibco), and 1X L-glutamine (Gibco).
Spleens and lymph nodes were dissociated using the frosted ends of microscope slides into RPMI 1640 supplemented with 1% heat-inactivated FBS and 1X penicillin-streptomycin (Gibco), and 1X L-glutamine (Gibco). Spleen cell suspensions were spun down at 1500 RPM for 5", and red blood cell lysis with ACK Lysis Buffer (Gibco) was performed for 5". Cells were filtered through 40 um nylon mesh and, after centrifugation, resuspended in supplemented RPMI 1640. Lymph node suspensions were filtered through a 40 um nylon mesh, spun down at 1500 RPM for 5", and resuspended in supplemented RPMI 1640.

For ex vivo T cell stimulation experiments to detect intracellular cytokines, 0.5 x 10^5 cells were plated in a 96-well U-bottom plate (BD Biosciences) in RPMI 1640 (VWR) supplemented with 10% heat-inactivated FBS, 1X penicillin-streptomycin (Gibco), 1X L-glutamine (Gibco), 1X HEPES (Gibco), 1X GlutaMAX (Gibco), 1mM sodium pyruvate (Thermo Fisher), 1X MEM non-essential amino acids (Sigma), 50uM beta-mercaptoethanol (Gibco), 1X Cell Stimulation Cocktail (eBioscience), 1X monensin (BioLegend), and 1X brefeldin A (BioLegend). Cells were incubated in a tissue culture incubator at 37C with 5% CO_2 for 4 hours.

**Staining for Flow Cytometric Analysis**

Approximately 0.5-1 x 10^6 cells were stained for 15-30 minutes at 4C in 96-well U-bottom plates (BD Biosciences) with directly conjugated antibodies. After staining, cells were fixed with Cytofix/ Cytoperm Buffer (BD). Samples that were destined for Foxp3 or other transcription factor staining were fixed with the Foxp3 Transcription Factor Staining Buffer Kit (eBioscience). Intracellular cytokine and transcription factor staining were performed right before analysis using either the BD Perm/Wash Buffer
(BD) or the Foxp3 Transcription Factor Staining Buffer Kit (eBioscience); staining was performed for 45" at 4C. Analysis was performed on an LSR II (BD) with 405, 488, 561, and 635 lasers. Data analysis was performed using FlowJo software (BD). Directly-conjugated antibodies are listed in Supplementary Table 1. SIINFEKL-Kb tetramer was prepared using streptavidin-APC (Prozyme) and SIINFEKL-Kb monomer from the NIH Tetramer Core.

**Quantitative PCR for validation of RNAseq experiments**

1 ng of SMART-Seq2-generated cDNA from sorted Treg populations were used for each qPCR reaction (Chapter 2). Quantitative PCR to detect Il1rl1 isoform expression was performed with a common forward primer: 5' - GATGTCCTGTGGCAGATTAACA-3'. Transcript variant 1 (NM_001025602.3) was detected with reverse primer 5'-AGCAACCTCAATCCAGAACACT-3' and transcript variant 2 (NM_010743.3) was detected with reverse primer 5'-TGGAAGACAGAAACATTCTGGA-3'. 1 ng of cDNA generated using Smart-SEQ2 was included in a reaction with 1 uL of each primer (2 uM stock) and 5 uL of KAPA SYBR Fast LightCycler 480 (KAPA Biosystems). Cp values were measured using a LightCycler 480 Real-Time PCR System (Roche). Relative fold-change in expression values were calculated using the following formula: \(2^{(dCp(Sample) - dCp(Spleen))}\), where \(dCp(Sample) = Sample \ Cp_{Gene \ of \ Interest} - Sample \ Cp_{GAPDH}\) and \(dCp(Spleen) = Spleen \ Cp_{Gene \ of \ Interest} - Spleen \ Cp_{GAPDH}\).

**Immunohistochemical (IHC) and Immunofluorescence Staining**

Lung lobes and spleens allocated for IHC and IF were perfused with 4% paraformaldehyde in PBS and fixed overnight at 4C. Lung lobes and/ or spleen were
transferred to histology cassettes and stored in 70% ethanol until paraffin embedding and sectioning (KI Histology Facility). H&E stains were performed by the core facility using standard methods. Tumor grading was done by A.L. and C.D. under consultation with R.T. Bronson.

For IHC, 5 um unstained slides were dewaxed, boiled in citrate buffer (1 g NaOH, 2.1 g citric acid in 1L H2O, pH 6), for 5 minutes at 125C in a decloaking chamber (Biocare Medical), washed with 3X with 0.1% Tween-20 (Sigma) in TBS, and blocked and stained in Sequenza slide racks (Thermo Fisher). Slides were blocked with Dual Endogenous Peroxidase and Alkaline Phosphatase Block (Dako) and then with 2.5% Horse Serum (Vector Labs). Slides were incubated in primary antibody overnight, following by washing and incubation in HRP-polymer-conjugated secondary antibodies (ImmPRESS HRP mouse-adsorbed anti-rat and anti-goat, Vector Laboratories). Slides were developed with ImmPACT DAB (Vector Laboratories). Primary antibodies used were goat anti-IL-33 (R&D, AF3626), rat anti-Foxp3 (Thermo Fisher, FJK-16s, 1:100, 30 minutes develop), and rat anti-CD8a (Thermo Fisher, 4SM16). Stains were counterstained with hematoxylin using standard methods before dehydrating and mounting.

After fixation, lung lobes and spleen allocated for IF were perfused with 30% sucrose in PBS for cryoprotection for 6-8h at 4C. Tissues were then perfused with 30% optimum cutting temperature (O.C.T.) compound (Tissue-Tek) in PBS and frozen in 100% O.C.T in cryomolds on dry ice. 6 um sections were cut using a CryoStar NX70 cryostat (Thermo), and air-dried for 60-90 minutes at room temperature. Sections were incubated in ice-cold acetone (Sigma) for 10 minutes at -20C and then washed 3 x 5
minutes with PBS. Samples were permeabilized with 0.1% Triton-X-100 (Sigma) in PBS followed by blocking with 0.5% PNB in PBS (Perkin Elmer). Primary antibodies were incubated overnight. Primary antibodies used were rabbit anti-prosurfactant protein C (SPC) (Millipore, AB3786, 1:500) and goat anti-IL-33 (R&D, AF3626, 1:200). After washing 3 x 5", samples were incubated in species-specific secondary antibodies conjugated to Alexa Fluor 568 and Alexa Fluor 488, respectively, at 1:500. Sections were then fixed in 1% PFA and mounted using Vectashield mounting media with DAPI (Vector Laboratories).

Immunohistochemistry and immunofluorescence tissue section images were acquired using a Nikon 80 Eclipse 80i fluorescence microscope using 10x and 20x objectives and an attached Andor camera.

**Analysis of IHC Images**

Stained IHC slides were scanned using the Aperio ScanScope AT2 at 20X magnification. Aperio's ImageScope software was used to annotate tumor and lobe areas, and the built-in Aperio Nuclear Algorithm was used to classify intensity of staining for Foxp3, CD8, or IL-33. Note that the nuclear algorithm could be used for CD8 because of the small size and scant cytoplasm of lymphocytes. Nuclei were scored from 0 to 3+; only nuclei with intermediate to strong nuclear staining (2-3+) were classified as positive. Scored cells were normalized to tumor area.

**RNA Sequencing of Lenti-LucOS tumors**

KP, Rosa26<sup>LSL-tdTomato</sup> mice infected with Lenti-LucOS were sacrificed 5, 8, or 12 weeks post tumor induction. Micro-dissected Tomato-positive tumors were processed as described for lung lobes, but manual dissociation with scissors. tdTomato-positive,
i.v. CD45neg, CD31neg, Ter119neg tumor cells were FACS sorted into 200-cell samples in Buffer TCL. Library preparation and RNA sequencing were performed using SMART-Seq2 and Nextera Tagmentation as described in Chapter 2 for bulk T cell populations.

**Statistical Analyses**

Unpaired, two-tailed Student’s t tests, Tukey’s multiple comparisons tests, and Sidak’s multiple comparisons tests were used for all statistical comparisons.
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CHAPTER 4

DISCUSSION
In this thesis, we characterized the longitudinal progression of the endogenous CD4+ T cell response in an autochthonous lung adenocarcinoma model, and studied the role of ST2 in particular in helping to control Treg cell function in tumors. We found that tumor development was associated with shifts in cellular heterogeneity that likely strengthen and reinforce tumor immunosuppression over time. Furthermore, Treg-specific ST2 ablation appears to alter the phenotypic diversity of tumor-associated Treg cells, which may contribute to impaired Treg function and enhanced anti-tumor immunity. In this section I will discuss the implications of this work on our understanding of tumor evasion and the development of novel approaches for cancer immunotherapy.

**Longitudinal profiling offers insight on the evolution of tumor immune responses**

Although major efforts to characterize immune cell populations in patient tumors are ongoing, we have a limited understanding of the natural history of immune responses to tumors that arise from single, transformed cells in their native tissue. Samples from human cancers represent a temporally, and often spatially, isolated snapshot of a tumor immune response that can only hint at underlying mechanisms of tumor immune evasion. Profiling studies that have been done highlight the immense inter- and intratumoral diversity of human tumors, which adds additional complexity to the study of the heterogeneity of T cell responses in human cancer (McGranahan and Swanton, 2017; Tirosh et al., 2016).

Longitudinal immune profiling has been performed in mouse models of acute and chronic infection, revealing distinct CD4+ T cell responses to different pathogens, and the molecular features of memory and exhausted T cells (Crawford et al., 2014; Phetsouphanh et al., 2017). Mouse modeling thus offers the opportunity to follow
changes in the immune characteristics of tumors from initiation to late-stage disease. Most studies of tumor immune tolerance in mice have used transplantable models of cancer, however, which often employ large doses of cells that have already adapted to optimal growth in vivo and in vitro. The drastically shortened timeframe of tumor development suggests that transplantable tumor models would appear to capture the immune response raised by a completely naive mouse suddenly exposed to a stage 4 tumor. Such a mismatch in the timing of immune-tumor interactions is unlikely to capture the full range of complex phenotypic changes that occur in patients over the course of months to years. Indeed, we observe shifts in T cell phenotype during tumor development that are likely the result of dynamic changes in tumor-immune interactions. Increased IL-33 expression in tumors, for example, probably occurs as a result of strong inflammatory signaling, and subsequent release of IL-33 into the environment may provoke an altered CD4+ T cell response and/or set the stage for Treg expansion and activity. Similarly, the IFN-signaling signature observed in Treg cells at earlier timepoints implies elevated IFN signaling throughout the tumor environment, which appears to decline as tumors progress. The slower growth of KP lung adenocarcinomas, which arise from single transformed cells, is thus more amenable to longitudinal profiling of immune cell populations.

Other advantages of using inducible GEM models of cancer for long-term profiling studies include the ability to study tumors with defined genetic and antigenic characteristics that arose in their native tissue. Our data point to the importance of tissue context in T cell phenotypes. We consistently observed major transcriptional differences between lymphoid tissue and lung CD4+ T cells, which is in agreement with
human studies of tumor Treg cells. It is clear from our study that identification of cell types with simple lineage-distinguishing markers may fail to capture functional phenotypes of cells. In fact, expression of the KPLungTR signature, derived from KP lung Tregs, in human primary lung adenocarcinomas is poorly correlated with the expression of a signature generated from circulating Tregs. The blood Treg-derived signature may pick up the presence of Tregs, but likely preferentially identifies rTregs. In contrast, the KPLungTR signature is likely to identify tumors that have more effector Tregs.

It has been suggested that tissue Tregs may have a generalizable phenotype, and lung Tregs do share many transcriptional features with previously characterized tissue Treg populations. Nevertheless, particular tissue environments may add to the core transcriptional program of tissue-resident cells, and the functional impact of tissue CD4+ T cells may still vary due to interactions with other cells or pathways that are tissue or context-specific. The phenotype of tissue CD4+ T cells is apparent even in naive mice, suggesting that immune responses to tumors are already pre-programmed by the inflammatory state of the surrounding tissue, which includes the immunostimulatory properties of the tumor cell of origin. This is consistent with previous work in our lab showing that tumors bearing the same genetic alterations are met with drastically different immune responses in the lung as compared to the hindlimb (DuPage et al., 2012). Moreover, the effector Treg phenotype is correlated with improved survival in colon cancer, which may reflect immune regulation unique to the gut. Due to ease of collection, many studies of human tumor immunology profile circulating immune cells collected from patients, which may not reflect the phenotype of
tissue-resident, tumor-associated cells. However, scRNAseq and insight from studies of mouse tissue Tregs may allow identification of circulating cells that are more likely to have been associated with the tumor environment.

The KP model may also facilitate modeling of tumor immune responses that are particular to lung adenocarcinomas driven by oncogenic K-ras and the loss of p53. Notably, the greater prevalence of KRAS and TP53 mutations in tumors highly correlated with the KPLungTR signature is consistent with the genetic perturbations used to induce KP tumors. Our finding that these tumors were more inflamed is also in line with recent observations that Kras-driven and EGFR-driven mouse models of lung adenocarcinoma have distinct immunological features (Busch et al., 2016), and that oncogenic Ras signaling can induce Treg conversion (Zdanov et al., 2016). Looking beyond Treg cell phenotypes, the predominance of IL-17-producing cells in early KP mice corroborate the tumorigenic role of IL-17 recently described in oncogenic K-ras-driven tumors (Chang et al., 2014). Our data thus reiterate that genetic mouse models can be used to identify and study tumor-intrinsic determinants of cancer-associated immune responses.

Studies of human cancers have suggested that the characteristics and availability of antigen can strongly influence tumor immunity. Although an oft-cited disadvantage of GEM models of cancer is their low mutational burden, programmed antigen expression can facilitate the study of antigen-specific T cell responses in the context of strong or weak tumor antigens. Indeed, we identified clonally-expanded CD4+ T cells in our model, in spite of the fact that Lenti-LucOS encodes soluble Ova, which is poorly presented and thus fails to invoke an OT-II response (data not shown). However,
CD4+ T cells in the KP model may recognize other antigens encoded by the lentivirus, such as Cre recombinase or luciferase, or weakly-expressed self-antigens. Indeed, Ad-SPC-Cre-infected tumors, which do not have stable expression of Cre recombinase, are still able to recruit a diverse CD4+ T cell response, although their repertoire diversity is less than that observed in cells from LucOS tumors. Future studies using antigens of varying strength, mutations generated as a result of DNA repair defects, or partially-tolerized self-antigens may add further insight into the effect of antigen characteristics on CD4+ T cell responses to tumors.

Although the KP model appears to recapitulate many features of tumor immunosuppression in Kras-driven lung adenocarcinoma, it is important to note important caveats to the model that may impact our analysis of the longitudinal immune response. First, there are likely to be key differences in tumor immune responses mounted in mice and humans, though our findings indicate that there are nevertheless strong similarities between mouse and human lung cancer Tregs. Second, tumor induction involves viral administration of lentivirus, which may prime a strong initial inflammatory reaction that may have lasting effects on the recruitment and phenotypes of immune cells. The tissue CD4+ T cell phenotype is apparent in naive mice, and is therefore unlikely to be the result of viral induction, but we cannot rule out the effect of an early antiviral response on CD4+ T and Treg cell heterogeneity throughout tumor development. It is possible that the inflammation associated with viral induction may mimic inflammation associated with a transforming event, e.g. a sunburn, or the chronic inflammation associated with smoking or acid reflux in the lung and esophagus, respectively. However, studies using spontaneous tumor models or models induced
using less immunogenic mechanisms, like tamoxifen administration, may clarify the role of viral infection in our findings. Furthermore, KP tumors arise from single transformed cells but a number of cells are transformed, resulting in the formation of multiple tumors in tumor-bearing mice. Local tumor immunosuppression may vary slightly among tumors, and it is likely that any single tumor of sufficient size is influenced by the growth and immunosuppressive characteristics of surrounding tumors. Using a lower viral titer may reduce the influence of other tumors on immune responses by lowering the number of lesions. Finally, two major oncogenic events are introduced simultaneously in the KP model, which bypasses the stepwise acquisition of mutations and adaptive signaling pathways that characterizes other tumors. Future studies may temporally separate the induction of oncogenic K-ras from the loss of p53, which could provide insight on how progressive genetic alterations affect tumor immune responses.

Previous work done in our lab had shown that tumor progression is associated with changes in the phenotype and localization of CD8+ T cells, as well as an expansion of Treg cells and the development of TLS (DuPage et al., 2011; Joshi et al., 2015). Our data add to our understanding of the natural history of immune responses in the KP lung tumor model. A number of CD4+ T cell phenotypes can be identified in tumor-bearing lungs that vary over time, including a Treg population that demonstrates more flexibility early in tumor development but progressively becomes more dominated by terminally-differentiated effector Treg cells. Furthermore, longitudinal phenotypic shifts are likely to be regulated by multiple pathways, including ST2. Diversity of CD4+ T cells arises from interactions with varied cytokine and immune cell milieus, suggesting that the shifts in heterogeneity we have observed belie dynamic changes in their interactions.
with tumor and other cells in the microenvironment. Although our study provides just one piece of the puzzle, we can infer the events that contribute to changes in CD4+ T cell responses over time. An early proinflammatory response, perhaps due in part to lentiviral infection, may provoke the recruitment of CD4+ and CD8+ CTL responses, but also induce Th1-directed Treg responses. Other CD4+ T helper responses may arise in direct response to transformation events due to inflammatory programs driven by K-ras\textsuperscript{G12D} or loss of p53. A resident population of NKT17 cells and an induced population of Th17 cells may then facilitate tumor growth and angiogenesis directly through production of IL-17. Secretion of TGF\(\beta\) and IL-10 by tumor, endothelial cell, or macrophage populations may further promote the peripheral conversion of Treg cells and the promotion of a Tr1 phenotype in Th1 cells. Alarmin release by stressed or dying tumor or normal epithelial cells may simultaneously recruit anti-tumor T cell and Treg responses. Many factors may thus contribute to Treg cells expressing a progressively more differentiated phenotype, which may make Tregs that are responsive to IFN or other Th1 or Th17-inducing cues more rare. Tumor immune tolerance is further reinforced by the activity of Tr1-like cells and tumor-associated monocyte and granulocyte populations that have similarly been influenced by immunosuppressive signaling.

It remains unclear from our analysis whether the observed phenotypic changes occur in tissue-resident CD4+ T cells during the length of tumor development, or if they reflect shifting characteristics of immune cells recruited to tumors at early vs. late timepoints. Circulation of cells from the tumor microenvironment and the recruitment of new cells may also vary with tumor development, as tumor development has been
associated with myelopoiesis in the bone marrow and spleen (Cortez-Retamozo et al., 2012; Engblom et al., 2017), which may be suggestive of the far-reaching effects of tumor development on systemic immune responses. Lineage tracing and cell transfer experiments may provide insight on the numbers and phenotypes of newly-arrived cells at various timepoints. Similarly, the spatial orientation of CD4+ T cells may also contribute to observed longitudinal changes in heterogeneity. Early tumors may be more heavily infiltrated by immune cells, while late tumors have acquired stromal changes, including the recruitment and activation of fibroblasts, that enable exclusion of immune cells. The development of TLS may also impact the functions of CD4+ T cells, as our late-stage profiling may capture the phenotypes of cells in TLS, normal lung parenchyma, and within and surrounding tumors. Disruption of the epithelial cell barrier and obstruction of airways by growing tumors may also perturb the lung microbiome, affecting CD4+ T cell responses.

Longitudinal studies of patients enrolled in clinical trials of immune checkpoint blockade have provided valuable insight into the determinants of response as well as possible resistance mechanisms. A more complete understanding of the natural history of tumor immune responses, however, would provide insight on the myriad ways by which patient tumors arrive at favorable and less favorable immune states, as well as the factors that will further promote tumor immune tolerance. A better understanding of the progression of tumor immune responses could be incorporated into our cancer staging schemes. Patient tumors would be evaluated not only for their proliferation and invasiveness, but also their immunosuppressive characteristics, thus providing an “Immunosuppression Stage” that may be both prognostic and diagnostic. A map of
immunosuppressive trajectories could also inform the design of combinatorial strategies aiming to break tolerance in tumors where it is well-established, and prevent further tumor immune evasion in lesions where immunosuppression is less advanced. For example, treatment of KRAS-mutant lung adenocarcinoma with checkpoint inhibitors would be expected to be effective due to the presence of tumor-associated CD8 T cells, but tumors in advanced stages of immune suppression may only respond if Treg-mediated and other immunosuppressive mechanisms are simultaneously inhibited. Similarly, treatments that disrupt IL-17 signaling may inadvertently increase pTreg conversion or the efficiency of terminal differentiation of Treg cells, which may require additional therapeutic manipulation. Additional, and more comprehensive, longitudinal profiling studies of tumor immune responses in cancer will refine the observations we have described here, and hopefully build a foundation for improving the precision and scope of cancer immunotherapies.

**Plasticity of CD4+ T cells and implications for CD4-targeted therapies**

Our data highlight the wide phenotypic diversity of Treg and Tconv cells, even when they arise from the same clones. We observed considerable phenotypic overlap, but the assortment of transcriptional features adopted by any given cell was highly variable. For example, features of Th1-like cells, including the expression of Tbx21, Cxcr3, or IFNγ could be found in cells with a broad range of phenotypes, including CD4 CTL and Tr1-like cells, Treg cells, and even some Rorgt+ Th17-like cells. The heterogeneity of observed CD4+ T cell populations suggests that cells expressing lineage-defining transcriptional factors still retain plasticity in the face of diverse, often conflicting, cues. The epigenetic landscape of CD4+ T cells may be responsible for
flexibility in CD4+ T cell phenotypes, as a more tightly-regulated epigenetic state may be associated with less responsiveness to cytokine signaling. For example, effector Treg cells with the DP phenotype may not respond as readily to IFN signaling due to epigenetic silencing associated with terminal differentiation. Conversely, the closely linked Th1 and Tr1 phenotypes may reflect an epigenetic state where IFNγ and IL-10 can be made simultaneously such that cells can slip easily from one phenotype to another. Varying degrees of epigenetic regulation could be responsible for the ability of CD4+ T cells to express features of a transcriptional program without complete conversion into another phenotype. The chromatin state of T-bet+ Treg cells, for example, permits the expression of some T-bet targets, but these cells remain unresponsive to IL-12 and do not secrete IFNγ due to epigenetic silencing of Il12rb2, which encodes an IL-12 receptor chain (Koch et al., 2012). Inheritance of epigenetic marks may also explain why clonally-expanded populations can diverge phenotypically, but on average, intraclonal gene expression is more correlated than interclonal gene expression.

The factors that determine epigenetic state in CD4+ T cells are not clear, especially in the context of chronic inflammation and/ or tumor immune responses. Evidence suggests that lineage-specifying transcription factors like T-bet and Foxp3 act within a predetermined enhancer landscape that is dictated by earlier events, including TCR and cytokine signaling (van der Veeken et al., 2013). Early in tumor development, a confluence of different signals may promote a relatively open epigenetic landscape in Treg cells that results in relative permissiveness to express targets of RORγT or T-bet under certain circumstances. Indeed, STAT1 and STAT4 have been shown to promote
chromatin remodeling that allows responsiveness to T-bet (Vahedi et al., 2012), which may explain why Treg cells with an IFN-signaling signature also tend to express Cxcr3 and Tbx21. It is possible that the epigenetic landscape of CD4+ T cells is also heterogeneous, which, in addition to variable expression of transcriptional regulators, may account for observed CD4+ T cell heterogeneity. To add further complexity, remodelling of chromatin accessibility may also be dynamic. For example, the TCR repertoire overlap of DP and SP Treg cells suggests that DP Treg cells may arise from SP progenitors, which may themselves arise from more flexible DN Tregs. Lineage-tracing experiments are needed to determine whether maturation of the effector Treg phenotype follows this sequence, and future studies should explore whether terminal differentiation in Treg cells is driven by progressive changes in chromatin accessibility.

The plasticity of CD4+ T cell populations may complicate therapeutic interventions targeted at CD4+ T cell functions, as well as efforts to adoptively transfer CD4+ T cell populations. Both endogenous and transferred populations of CD4+ T cells are quite heterogeneous, and selection for cells with a particular phenotype may not guarantee that the population will not acquire additional or alternate phenotypes, especially in highly inflamed settings. Nevertheless, mouse models and a few case reports of autologous transfers of heterogeneous CD4+ T cells have demonstrated anti-tumor efficacy (Muranski and Restifo, 2009; Tran et al., 2014; Zanetti, 2015), suggesting CD4+ T cells may be a powerful therapeutic tool provided the right balance can be struck. Understanding the factors that regulate CD4+ T cell plasticity may help guide the heterogeneity of endogenous and adoptively transferred populations to best target
tumors without simultaneously turning on programs of immunosuppression or instigating autoimmunity.

**Distinct functions of Tregs in tissues and tumors**

The transcriptional phenotype of effector Tregs in the KP model are suggestive of possible effector mechanisms, but it remains unclear what functions are employed by Tregs tumor-bearing lung tissue, TLS, or the draining lymph node. The delineation of a lymphoid tissue-resident, "central" population of rTregs and activated Tregs in nonlymphoid tissues has raised the possibility that Treg effector functions in nonlymphoid tissue environments may be distinct. Indeed, rTregs are thought suppress immune responses in lymphoid tissues by consuming IL-2 and suppressing APC maturation, while aTregs in inflamed tissue environments may have a more diverse array of functions, including direct killing of effector cells, or the promotion of tissue repair pathways (Li and Rudensky, 2016; Smigiel et al., 2014). Though few in number, rTregs are present in tumor-bearing KP mice, predominantly at early timepoints, which may represent newly-arrived Tregs in the tissue, or Tregs in TLS. The expression of Cxcr5 by a subset of these cells suggest that that Tregs may be suppressing B cell responses in KP lung tumors. Interestingly, most msLN Tregs have an rTreg phenotype, suggesting that tissue Treg cells do not regularly circulate away from tumor tissue.

It is unclear how the transcriptional heterogeneity of effector Tregs in early and late-stage KP tumor-bearing mice impacts their function. The secretion of IL-10 and TGFβ is typical of *in vitro* cultured Treg cells, but it is unclear if Tregs are actively secreting either cytokine *in vivo*. pTregs that are clonally related to Tconv cells may be able to outcompete their effector T cell counterparts for antigen and disrupt their
costimulation. The high expression of Il2ra by DP Tregs suggests that IL-2 depletion may be an effector mechanism employed by both rTregs and aTregs. Furthermore, the expression of Gzmb and Srgn by effector Tregs suggests that Tregs may be actively killing effector T cells in the tumor microenvironment. The expression of Areg and Tff1 is also consistent with a tissue-protective and reparative role for terminally differentiated Tregs. Further work is needed to determine whether Tregs bearing the intermediate, flexible phenotype observed earlier in tumor development are functionally distinct from the DP-like, terminally differentiated Tregs present at later timepoints.

**Role of IL-33 in tumor immunosuppression**

In this thesis, we have explored IL-33 as a driver of Treg function in KP tumors. Our data suggest that IL-33 signaling may promote terminal differentiation of Tregs, and may thus be essential for the full realization of Treg immunosuppressive potential. A number of studies have pointed to a pro-tumorigenic role of IL-33, either by promoting immunosuppressive immune cell function, or directly driving tumor cell proliferation (Wasmer and Krebs, 2016). This function of IL-33 may be an extension of its role as an alarmin, as its release into the extracellular environment would be suggestive of tissue damage and thus a need to activate tissue repair pathways. However, a role for IL-33 in driving Th2 and ILC2 responses has also been well-described, and earlier reports also suggested that IL-33 promotes the generation of CD8 T cell memory (Bonilla et al., 2012; Liew et al., 2016).

In our study, treatment with IL-33 resulted in profound inflammation that included the expansion of many cell lineages, including CD8+ and CD4+ T cells. It is therefore possible that ST2 expression on Tregs may function primarily to allow them to limit the
responses of other cells to IL-33. It remains puzzling to us that we have not identified Th2 cells in KP tumors in spite of their lung-localized inflammation and presumed IL-33 availability. A population of Th9-like cells has been identified that may be IL-33-dependent but they are rare and predominantly found at early timepoints. Perhaps IL-33 drives Th2 inflammation very early in tumor development, such that by week 5 the Th2 response has waned, perhaps due to competition with Tregs for ligand. Further analysis may identify other IL-33-responsive cells in the tumor microenvironment that have improved function or expansion due to Treg-specific ST2 deletion. Indeed, preliminary data suggests that much of the inflammatory response to IL-33 is maintained when IL-33 is administered to mice with Treg-specific ST2 deficiency. It is therefore possible that Tregs primarily limit the effect of IL-33 on other populations, rather than produce a robust, cell-intrinsic response to IL-33.

The nuclear localization of IL-33 has also led to the suggestion that IL-33 may have a transcriptional regulatory role. IL-33 has been shown to associate with chromatin, and some studies have suggested that it can act as a transcriptional repressor (Ali et al., 2011; Carriere et al., 2007; Roussel et al., 2008). However, a recent report found that loss of nuclear IL-33 had no effect on the proteome of cells (Gautier et al., 2016). We have used CRISPR/Cas9-mediated genome editing to delete IL-33 selectively in transformed cells, but have not observed defects in tumor size or grade (data not shown). An evaluation of the effect of tumor-specific loss of IL-33 has been complicated by low efficiency of in vivo Cas9-mediated editing, which leaves IL-33 expression intact in most tumors. Future studies using more efficient Cas9 systems are needed to assess a cell-intrinsic role of IL-33 in tumor development.
It remains unclear how IL-33 signaling directly affects Treg function. Previous groups have shown that both ST2 and IL-33 deficiency result in drastic reductions in fat Treg recruitment, and that anti-ST2 antibody can deplete VAT Tregs (Bapat et al., 2015; Kolodin et al., 2015; Vasanthakumar et al., 2015), but we have seen no change in Treg numbers as a result of IL-33 or ST2 deficiency in tumor-bearing lungs (data not shown). It is possible that a defect in lung Treg numbers is more noticeable in naive mice, or redundant pathways may exist in the lung to promote Treg recruitment and expansion. ST2 has been reported to drive the expression of Th2 cytokines and amphiregulin in other tissue Tregs, and we are currently investigating whether genetic ablation of IL-33 signaling alters the transcriptional phenotype of KP tumor Tregs.

Surprisingly, preliminary data suggests that ST2-deficient mice do not phenocopy IL-33-deficient mice (data not shown); we have not observed an alteration in DP Treg numbers or a change in tumor burden as a result of ST2-deficiency. Interestingly, another study reported that ST2-deficiency, but not IL-33 deficiency, had reduced disease severity in an experimental model of arthritis (Kamradt and Drube, 2013). We are currently validating this finding, which could suggest that a second functional receptor exists for IL-33, or that ST2 has an alternate function that can indirectly counteract its own signaling. The former hypothesis appears unlikely, since IL-33 administration in ST2-deficient mice fails to generate the strong inflammatory response we observe in wild-type mice. Meanwhile, it has been well-established that the signaling of IL-1 receptor family members can be regulated through the expression of soluble decoy receptors and the formation of alternate receptor complexes (Garlanda et al., 2013). As a result, ST2 deletion may alter the regulation of other IL-1 receptor family
members or TLRs, resulting in effects that mitigate the loss of cell-intrinsic ST2 signaling.

The increased CD8 T cell infiltration and trend toward reduced tumor burden observed in mice with Treg-specific ST2 deletion suggests that ST2 may be a valuable therapeutic target that can alter Treg differentiation without affecting systemic tolerance. However, the discrepancy between the effects of ST2 and IL-33 deficiency suggest that systemic inhibition of ST2 may affect a wide range of cells and may not ultimately improve outcomes. IL-33 deficiency results in an altered Treg phenotype but may not impact tumor burden because it also negatively affects anti-tumor immunes. For example, ILC2, Th9, and CD8 T cells may also depend on IL-33 signaling for their recruitment and function in tumors. Therefore, IL-33 neutralizing antibodies may be a valuable therapeutic tool in combination with other immune therapies that can compensate for the loss of IL-33 signaling in other cell types. A recent report has also shown that a bifunctional antibody can mediate cell type-specific inhibition of Wnt signaling (Lee et al., 2018). A CTLA-4-ST2 bispecific antibody may thus be able achieve Treg-specific abrogation of ST2 signaling, and/or deletion of CTLA+ST2+ cells, many of which are likely to be Treg cells.

Our study of CD4+ T cell heterogeneity in tumors has highlighted mechanisms of tumor immunosuppression that may be fruitful targets for cancer immunotherapy. The potential challenges of inhibiting IL-33 signaling to disrupt tumor Treg function, however, exemplify the difficulties of targeting pleiotropic cytokines and other immune mediators. The context-specific functions of many immune regulators contribute to the heterogeneity of tumor immune responses. Granzyme B, for example, may be important
for anti-tumor CD8 and NK cell-mediated killing of tumor cells, but may also be used by Tregs to kill effector T cells. Nevertheless, an improved understanding of the diversity of tumor immune responses and the nuances of IL-33 signaling in different cell types may reveal strategies to inhibit specific functions or targets of IL-33.
REFERENCES


APPENDIX 1

PD-1 blockade is ineffective in a genetic mouse model of lung adenocarcinoma

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A.L. and T.J. designed the study; A.L. performed all of the experiments and wrote this appendix.
ABSTRACT

PD-1 blockade therapy is now used as a first and second-line treatment for NSCLC and can stimulate durable anti-tumor immune responses in a subset of patients. It remains unclear which patients will respond to PD-1 inhibition, and how PD-1 inhibition alters tumor immunosuppression over time. In this study, we characterized the response to PD-1 inhibition in a genetic mouse model of lung adenocarcinoma expressing a strong T cell antigen. We demonstrate that PD-1 is ineffective in generating a sustained anti-tumor T cell response in spite of elevated expression of both PD-1 and PD-L1 in the tumor microenvironment. Our data hint that Treg expansion and activity may mitigate any pro-inflammatory effect of PD-1 blockade, suggesting a possible mechanism of resistance to checkpoint inhibition.
INTRODUCTION

Non-small-cell lung cancer (NSCLC), which accounts for about 85% of all lung cancers, is often diagnosed at an advanced stage and carries a poor prognosis (Molina et al., 2008). Recently, therapeutic strategies have aimed to enhance the immune response to tumors in order to complement traditional chemotherapeutic regimens and targeted cancer therapies (Pfirschke et al., 2016; Ramakrishnan et al., 2010; Vanneman and Dranoff, 2012). Lymphocytes, macrophages, and other immune cells have long been observed within or around tumors (Bremnes et al., 2011), suggesting that tumors mount endogenous immune responses. However, in-depth histopathological study has revealed that there is considerable diversity in cancer-associated immune responses (Fridman et al., 2012), and that tumor and stromal cells, as well as infiltrating immune cells, may dampen anti-tumor inflammation and promote immune tolerance for tumor growth (Motz and Coukos, 2013; Rabinovich et al., 2007). Therapies that disrupt immunosuppressive signaling in tumors may thus restore the ability of host immune responses to prevent tumor growth.

The programmed death 1 (PD-1) receptor is a negative costimulatory molecule on T cells that may contribute to immune evasion by cancer cells, especially because many tumors express programmed death ligand 1 (PD-L1), the ligand that binds and activates PD-1 (Dong et al., 2002; Iwai et al., 2002; Keir et al., 2008). Antibody-mediated PD-1 and PD-L1 blockade agents have been approved as first and second line treatment for NSCLC (Peters et al., 2018), and the latest follow-up studies suggest that the overall five-year survival of patients treated with nivolumab, a PD-1 antibody, is 16%, compared to 4% in patients treated with docetaxel (Gettinger et al., 2018). However, it remains
unclear why only a fraction of patients respond to PD-1 and PD-L1 blockade. The correlation between response to therapy and expression of PD-L1 is not well established (Creelan, 2014), and one mouse model of Kras-driven lung adenocarcinoma, a form of NSCLC, showed meager responses to PD-1 blockade in spite of tumor PD-L1 expression (Akbay et al., 2013). Other immunosuppressive mechanisms in the tumor may account for the limited success of anti-PD-1 therapy, including the activity of regulatory T cells (Tregs), an immunosuppressive T cell population. Previous studies have suggested that PD-1 negatively regulates Treg function (Currie et al., 2009; Franceschini et al., 2009; Sage et al., 2013), and we therefore hypothesize that PD-1 blockade and/or PD-1 deficiency may exacerbate Treg-mediated immune tolerance in tumors. We thus sought to determine whether PD-1 blockade or PD-1 deficiency enhances the immunosuppressive activity of Tregs, mitigating the anti-tumor effect of PD-1 blockade.

Antibody-mediated PD-1 blockade was first shown to have preclinical efficacy in transplantable models of cancer, leading us to ask whether PD-1 inhibition would have similar effects in tumor models that involve strong Treg-mediated immunosuppression. Our lab has developed an inducible, autochthonous model of lung adenocarcinoma where activation of an oncogenic allele of Kras and loss of p53 is driven by intratracheal delivery of a lentivirus bearing Cre recombinase (KP: LSL-Kras\(^{G12D}\), p53\(^{fl/fl}\)) (DuPage et al., 2009). These mice allow us to study endogenous immune responses to tumors, and by expressing ovalbumin (Ova), a known T cell antigen, in tumor cells we can also monitor tumor-specific cytotoxic T cell (CTL) responses (DuPage et al., 2011). Consistent with prior studies (Granville et al., 2009), tumor growth in our mouse model
is accompanied by an expansion of regulatory T cells (Tregs) that express high levels of PD-1 (Joshi et al., 2015). One advantage of using the KP model is that mice can be treated with PD-1 blockade at various timepoints during tumor development to determine the effect of tumor stage on efficacy. The anti-tumor T cell response can also be monitored over a longer period of treatment, allowing us to characterize how T cell phenotypes evolve in response to checkpoint inhibition, and perhaps identifying pathways of resistance. In this study we observed slight phenotypic alterations in the Treg population as a result of short-term PD-1 inhibition, but ultimately minimal effect of the therapy on tumor development and associated immune responses.

RESULTS

PD-1 and PD-L1 are highly expressed in the KP tumor microenvironment

We observed a sharp increase in PD-1 expression in CD8+ and Foxp3-negative CD4+ T cell populations during tumor progression in KP mice (Figure 1a). Interestingly, PD-1 expression was high even at an intermediate timepoint in regulatory T cells (Tregs), and remained high in mice with advanced disease. We wondered whether tumor or immune cells would be the predominant source of PD-L1. PD-L1 did not appear to be co-expressed by CD3+ cells. While some Nkx2.1+ tumor cells stained faintly for PD-L1 (Figure 1b), the population expressing the greatest levels of PD-L1 appeared to be dendritic or myeloid cells, which is consistent with the co-expression in some cells of PD-L1 and CD11c (Figure 1c).
Figure 1. PD-1 and PD-L1 are highly expressed in the KP tumor microenvironment
A. Flow cytometric analysis of PD-1 expression on CD8+, Foxp3- CD4+, and Foxp3+ CD4+ Treg T cells isolated from tumor-bearing lungs. Each histogram represents a single mouse.
B. Representative immunofluorescent staining of lung from late-stage (week 20 p.i. with Lenti-LucOS) KP tumors.
C. Same as in B.
A short pulse of PD-1 inhibition has minimal effect on T cell proportions in the KP model.

To assess the short-term response to PD-1 inhibition in KP tumor-bearing mice, we treated late-stage (week 22 p.i.) KP mice with two doses of anti-PD-1 therapy and measured T cell responses (Figure 2a). We were able to confirm binding of the PD-1 antibody, but not isotype control, to T cells in the lung (Figure 2b). Interestingly, in this timeframe we observed no changes in CD3+, CD8+, or CD4+ T cell proportions in tumor-bearing lungs, draining (mediastinal) lymph node (msLN), or spleen (Figure 2c–d). We also observed no change in the percentage of SIINFEKL-tetramer positive CD8+ T cells (data not shown). Of note, there was a slight expansion of the tumor-associated Treg population in treated mice, and their phenotype was slightly altered (Figure 2e–f). The expression of ICOS was elevated, but the expression of PD-1 was lower, in Tregs in anti-PD-1-treated mice.
Figure 2. Short-term dosing of anti-PD-1 has minimal effect on T cell proportions but slightly alters Treg numbers and phenotype
A. Schematic of short-term PD-1 inhibition.
B. Barplot representing flow cytometric analysis of staining for rat IgG2a to indicate PD-1 antibody binding in mice treated with isotype control antibody compared to anti-PD-1.

C. Barplot of proportions of CD3+ T cells of i.v. neg cells in control and anti-PD-1-treated mice in indicated tissues.

D. Barplot of proportions of CD3+ T cells of i.v. neg cells, and CD4+ and CD8+ T cells of CD3+ T cells, in control and anti-PD-1-treated mice in tumor-bearing lung.

E. Barplot of the proportion of Foxp3+ Treg cells of CD4+ T cells in the lung and spleen of control and anti-PD-1-treated mice.

F. Barplot of the expression level of ICOS and PD-1 in Tregs (black) and Foxp3-CD4+ (Tconv, red) from tumor-bearing lungs in control and anti-PD-1-treated mice.
Long-term PD-1 inhibition has no effect on tumor development

Given the subtle effects of short-term PD-1 blockade, we next asked whether starting treatment earlier, and continuing treatment over an extended period of time, would result in greater efficacy of PD-1 inhibition. We treated mice at week 15-16 p.i. with anti-PD-1 for four weeks (Figure 3a). We observed a slight, but statistically insignificant, decrease in the proportion of CD4+ T cells and increase in the proportion of CD8+ T cells in treated mice (Figure 3b). However, the proportion of SIINFEKL tetramer-positive cells was not different between animals treated with control and anti-PD-1 antibody. Furthermore, tumor growth appeared to be unperturbed by anti-PD-1 treatment (Figure 3c-d).
Figure 3. Long-term dosing of anti-PD-1 has no effect on tumor development of associated immune responses
A. Schematic of long-term treatment with anti-PD-1 therapy. CT indicates timepoints where micro-CT imaging was performed.
B. Barplot of proportions of CD4+ and CD8+ T cells of CD3+ T cells in control and anti-PD-1-treated mice.
C. Barplot of SIINFEKL-tetramer-positive CD8+ T cells in control and anti-PD-1-treated mice.
D. Tumor growth curves for mice treated with isotype control (black) and anti-PD-1 antibody (red) generated by micro-CT measurement. Each curve represents a tumor from a mouse; three tumors were measured per mouse.
DISCUSSION

In spite of durable responses to anti-PD-1 therapy observed in some NSCLC patients, we have failed to observe short or long-term tumor responses to PD-1 blockade in the KP model. T cells in late-stage tumors express high levels of PD-1, and a number of cell types in the tumor microenvironment appear to express PD-L1. Nevertheless, PD-1 inhibition produced subtle to no changes in T cell proportions in tumor-bearing mice.

Short-term dosing with anti-PD-1 does appear to expand Tregs, which express lower levels of PD-1, suggesting that Treg function is enhanced shortly after PD-1 inhibition. A swift compensatory increase in Treg effector function may explain the lack of effect of long-term PD-1 inhibition on tumor growth. The role of PD-1 in controlling Treg function remains unclear, although a recent report has suggested that PD-1-positive Tregs secrete IFNg and are dysfunctional in cancer (Lowther et al., 2016). Further studies of the profile of Treg cells in PD-1-treated animals may provide insight on the impact of PD-1 inhibition on Treg activity.

In addition to inducing a compensatory Treg expansion, PD-1 blockade may also be ineffective in the KP model due to inhibitory signaling through other checkpoint pathways. The combination of anti-CTLA-4 therapy with anti-PD-1 therapy has achieved equivalent or greater efficacy than either monotherapy in a number of trials, and appears to have an advantage over monotherapy in patients with low PD-L1 expression in particular (Hellmann et al., 2017; Larkin et al., 2015). CTLA-4, LAG-3, and TIM-3 expression are elevated in T cells in KP mice with advanced disease (DuPage et al., 2011; Joshi et al., 2015), and may represent alternative pathways by which CTL activity
is suppressed in late-stage tumor-bearing mice. Preliminary data suggests that combination PD-1 and CTLA-4 checkpoint blockade in KP mice with advanced disease still has minimal effect, but the addition of anti-CTLA-4 may result in a slight reduction in Treg numbers in treated mice (data not shown).

Response to checkpoint blockade has also been associated with tumor mutational burden (Hellmann et al., 2018). Although the somatic mutation load of tumors from KP mice is considerably lower than that of carcinogen-associated tumors in patients (McFadden et al., 2016; Westcott et al., 2015), our inclusion of the strong T cell antigens Ova and SIY in Lenti-LucOS is thought to model an immune response to a strong neoantigen. Nevertheless, it is possible that the immunogenicity of Ova is unphysiological, resulting in a skewed response to checkpoint blockade. Future studies in mice with antigens of varying affinities and levels of partial tolerance may approximate human tumor immune responses more faithfully. Furthermore, the finding that checkpoint blockade is particularly effective against tumors with microsatellite instability suggests that anti-PD-1 may be effective in KP tumors with additional mutations in DNA repair.

There is also growing evidence that PD-1 therapy is more effective in patients with tumors with significant immune infiltration (Tumeh et al., 2014). Indeed, certain tumor genotypes and/or TGFβ signaling have been shown to result in a non-inflamed tumor phenotype characterized by exclusion of immune cells (Mariathasan et al., 2018; Spranger et al., 2015). Large KP tumors are characterized by reduced T cell infiltration (DuPage et al., 2011), suggesting that the poor efficacy of checkpoint blockade may also be due to the lack of tumor-resident or adjacent immune cells available to respond.
However, recent data from mouse models suggest that KP tumors are more inflamed and recruit more T cells than EGFR-mutant tumors (Busch et al., 2016), which is consistent with early reports of greater efficacy of PD-1 inhibition in KRAS-mutant human tumors as compared to EGFR-mutant tumors (Kim et al., 2017; Lee et al., 2018). The KP model may thus represent a subset of tumors that are inflamed, but nevertheless fail to respond to checkpoint inhibition due to the influence of other strong immunosuppressive mechanisms.
## SUPPLEMENTARY TABLES

### Supplementary Table 1. Antibodies used for flow cytometry.

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MATERIALS AND METHODS

Mice

KP mice have been previously described (DuPage et al., 2011). All studies were performed under an animal protocol approved by the Massachusetts Institute of Technology (MIT) Committee on Animal Care. Mice were assessed for morbidity according to MIT Division of Comparative Medicine guidelines and humanely sacrificed by CO₂ asphyxiation prior to natural expiration.

For in vivo labeling of circulating immune cells, anti-CD45-PE-CF594 (30-F11, BD Biosciences, 1:200) was diluted in PBS and administered by IV injection 2 minutes before harvest (Anderson et al., 2012).

For PD-1 treatment studies, mice were assigned to treatment groups after measurement of tumor burden by microCT in order to ensure that both groups have equivalent average tumor burden. 200ug of PD-1 antibody (29F.1A12, generously provided by Gordon Freeman) or rat IgG2a isotype control (2A3, BioXCell) were administered intraperitoneally (i.p.) every 3 days for both the short and long-term dosing regimens.

MicroCT Measurement of Tumor Burden

In vivo imaging of tumor-bearing mice was performed using an eXplore CT 120 microcomputed tomography (microCT) system (Northridge Tri-Modality Imaging Inc.). Mice were imaged under isoflurane anesthesia in groups of 4 in a custom mouse holder. Scanner settings were as follows: 720 views, 360 degree rotation, 70 kVp, 50 mA, 32 ms integration time with 2x2 detector pixel binning (isotropic nominal resolution of 50 microns). Images were reconstructed using the MicroView software (Parallax
Innovations). Rough tumor burden measurements for assigning mice to treatment
groups was done using OsiriX DICOM Viewer (Pixmeo SARL). Tumor volume
measurements were long-term monitoring were made using ImageJ and Fiji to generate
3D tumor reconstructions.

**Lentiviral Production and Tumor Induction**

The lentiviral backbone Lenti-LucOS has been described previously (DuPage et
al., 2011). Lentiviral plasmids and packaging vectors were prepared using endo-free
maxiprep kits (Qiagen). Lentiviruses were produced by co-transfection of 293FS* cells
with Lenti-LucOS or Lenti-Cre, psPAX2 (gag/pol), and VSV-G vectors at a 4:2:1 ratio
with Mirus TransIT LT1 (Mirus Bio, LLC). Virus-containing supernatant was collected 48
and 72h after transfection and filtered through 0.45um filters before concentration by
ultracentrifugation (25,000 RPM for 2 hours with low decel). Virus was then
resuspended in 1:1 Opti-MEM (Gibco) - HBSS. Aliquots of virus were stored at -80C
and titered using the GreenGo 3TZ cell line (Sánchez-Rivera et al., 2014).

For tumor induction, mice between 8-15 weeks of age received 2.5 x10^4 PFU of
Lenti-LucOS intratracheally as described previously (DuPage et al., 2009).

**Tissue Isolation and Preparation of Single Cell Suspensions**

After sacrifice, lung lobes were allocated for immunofluorescent analysis (usually
lower right lobe) and/ or flow cytometric analyses. Lungs were placed in 2.5mL
collagenase/ DNAse buffer (Joshi et al., 2015) in gentleMACS C tubes (Miltenyi) and
processed using program m_impTumor_01.01. Lungs were then incubated at 37C for
30 minutes with gentle agitation. The tissue suspension was filtered through a 100 um
cell strainer and centrifuged at 1700 RPM for 10 minutes. Red blood cell lysis was
performed by incubation with ACK Lysis Buffer (Life Technologies) for 3 minutes. Samples were filtered and centrifuged again, followed by resuspension in RPMI 1640 (VWR) supplemented with 1% heat-inactivated FBS and 1X penicillin-streptomycin (Gibco), and 1X L-glutamine (Gibco).

Spleens and lymph nodes were dissociated using the frosted ends of microscope slides into RPMI 1640 supplemented with 1% heat-inactivated FBS and 1X penicillin-streptomycin (Gibco), and 1X L-glutamine (Gibco). Spleen cell suspensions were spun down at 1500 RPM for 5", and red blood cell lysis with ACK Lysis Buffer (Gibco) was performed for 5". Cells were filtered through 40 um nylon mesh and, after centrifugation, resuspended in supplemented RPMI 1640. Lymph node suspensions were filtered through a 40 um nylon mesh, spun down at 1500 RPM for 5", and resuspended in supplemented RPMI 1640.

For ex vivo T cell stimulation experiments to detect intracellular cytokines, 0.5 x 10⁵ cells were plated in a 96-well U-bottom plate (BD Biosciences) in RPMI 1640 (VWR) supplemented with 10% heat-inactivated FBS, 1X penicillin-streptomycin (Gibco), 1X L-glutamine (Gibco), 1X HEPES (Gibco), 1X GlutaMAX (Gibco), 1mM sodium pyruvate (Thermo Fisher), 1X MEM non-essential amino acids (Sigma), 50uM beta-mercaptoethanol (Gibco), 1X Cell Stimulation Cocktail (eBioscience), 1X monensin (BioLegend), and 1X brefeldin A (BioLegend). Cells were incubated in a tissue culture incubator at 37C with 5% CO₂ for 4 hours.

**Staining for Flow Cytometric Analysis**

Approximately 0.5-1 x 10⁶ cells were stained for 15-30 minutes at 4C in 96-well U-bottom plates (BD Biosciences) with directly conjugated antibodies. After staining,
cells were fixed with Cytofix/ Cytoperm Buffer (BD). Samples that were destined for Foxp3 or other transcription factor staining were fixed with the Foxp3 Transcription Factor Staining Buffer Kit (eBioscience). Transcription factor staining was performed right before analysis using the Foxp3 Transcription Factor Staining Buffer Kit (eBioscience); staining was performed for 45" at 4C. Analysis was performed on an LSR II (BD) with 405, 488, 561, and 635 lasers. Data analysis was performed using FlowJo software (BD). Directly-conjugated antibodies are listed in Supplementary Table 1. SIINFEKL-Kb tetramer was prepared using streptavidin-APC (Prozyme) and SIINFEKL-Kb monomer from the NIH Tetramer Core.

**Immunofluorescence Staining**

Lung lobes and spleens allocated for IF were perfused with 4% paraformaldehyde in PBS and fixed overnight at 4C. After fixation, lung lobes and spleen were perfused with 30% sucrose in PBS for cryoprotection for 6-8h at 4C. Tissues were then perfused with 30% optimum cutting temperature (O.C.T.) compound (Tissue-Tek) in PBS and frozen in 100% O.C.T in cryomolds on dry ice. 6 um sections were cut using a CryoStar NX70 cryostat (Thermo), and air-dried for 60-90 minutes at room temperature. Sections were incubated in ice-cold acetone (Sigma) for 10 minutes at -20C and then washed 3 x 5 minutes with PBS. Samples were permeabilized with 0.1% Triton-X-100 (Sigma) in PBS followed by blocking with 0.5% PNB in PBS (Perkin Elmer). Primary antibodies were incubated overnight. Primary antibodies used were hamster anti-CD11c (N418, BioLegend), rat anti-PD-L1 (10F.9G2, BioLegend), hamster anti-CD3e (145-C11, BD Biosciences), and rabbit anti-Nkx2.1 (EP1584Y, Abcam). After washing 3 x 5", samples were incubated in species-specific secondary antibodies
conjugated to Alexa Fluor 568, Alexa Fluor 488, or Alexa Fluor 647 at 1:500. Sections were then fixed in 1% PFA and mounted using Vectashield mounting media with DAPI (Vector Laboratories).

Immunofluorescence tissue section images were acquired using a Nikon 80 Eclipse 80i fluorescence microscope using 10x and 20x objectives and an attached Andor camera.

**Statistical Analyses**

Unpaired, two-tailed Student's t tests, Tukey's multiple comparisons tests, and Sidak's multiple comparisons tests were used for all statistical comparisons.
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