Mechanisms of response and resistance to anti-PD-1 immunotherapy

A thesis submitted to fulfil requirements for the degree of Doctor of Philosophy

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Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Jarem Jeff Alan Edwards
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Authorship attribution statement

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As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

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Abstract

Immune checkpoint inhibition with anti-PD-1 and anti-CTLA-4 monoclonal antibodies has revolutionised the treatment of metastatic melanoma. For the first time, durable treatment responses are being observed in a significant subset of patients beyond 10 years. Still, most patients fail to respond or develop acquired resistance, highlighting the need to improve immunotherapies that will benefit the broader patient population. As part of this effort, a greater understanding of the mechanisms of response and resistance to checkpoint-based immunotherapies is required. Key to this is the need to identify the exact immune cell phenotypes responsible for anti-PD-1 and/or anti-CTLA-4 response, including the factors responsible for their recruitment and retention in tumours. Doing so will assist in the development of strategies that can be used to boost these critical populations in patients that lack them. Currently clinical trial investigations are exploring novel strategies for overcoming resistance to anti-PD-1 immunotherapy, specifically through the targeting of alternative checkpoint receptors. Additionally, current treatment practices are being utilized by clinicians to try and rescue anti-PD-1 refractory metastatic melanoma patients through second-line treatment with combination anti-CTLA-4+ anti-PD-1 therapy. While exciting, these approaches are in their early stages of development and implementation. Thus, there is an opportunity to explore and answer such questions that will assist in the advancement of these therapeutic approaches. In this thesis, we investigate the cells underlying response to anti-PD-1 checkpoint immunotherapy and show that CD103+ tumor resident CD8 T cells are strongly associated with patient overall survival in melanoma and expand significantly early during treatment with anti-PD-1. We show that IL-15 expression, but not tumor mutation burden, is associated with a higher density of these cells in patient tumors. Next, we investigate the expression profile of alternative checkpoint markers in primary, regional, and metastatic melanoma disease, providing a foundation for the interpretation of biomarker data to assist clinical decision making in patients receiving novel checkpoint inhibitors after anti-PD-1 failure. Specifically, we show that only a small subset of tumor-infiltrating leukocytes expresses alternative co-stimulatory and co-inhibitory markers at any stage of disease, with PD-1 negative tumors lacking alternative targets compared to PD-1 positive tumors. Lastly, we investigate predictive biomarkers of response to second-line combination ant-PD-1+ anti-CTLA-4 after failure to first-line anti-PD-1 therapy. We characterize these patient tumors and identify the proportion of TCF7+ CD8 T cells and CD4 T cells as immune phenotypes associated with second-line response to combination treatment. Melanoma patient tumors that were unresponsive to anti-PD-1 monotherapy contained T cells and exhibited MHC class 1 expression yet contained low proportions of CD103+ tumor resident CD8 T cells. Collectively, this thesis represents a significant step forward in our understanding of the mechanisms of response and resistance to immunotherapy, which serves to advance the field of immunotherapy and help a higher proportion of cancer patients achieve long-term remission.

Foreword

Melanoma is the deadliest form of skin cancer and remains a significant problem in Australia and in the world. For a long time, metastatic melanoma was considered a lethal disease, however significant treatment advances have revolutionised patient management, reduced treatment burden, and drastically improved patient overall survival. The most durable and efficacious class of treatments to date are checkpoint inhibitors targeting receptors CTLA-4 and PD-1. Melanoma being an immunogenic cancer recruits various T cell and other immune cell phenotypes into tumors, which is believed to be integral to immunotherapy response. Despite these advances however, the majority of patients fail checkpoint inhibition immunotherapy. Part of the problem is our lack of understanding regarding the exact immune targets of these therapies as well as the mechanisms of resistance that prevent optimal responses. Tissue resident memory T cells are a memory T cell phenotype that has critical importance in the control of diseases at epithelial sites, and their role within cancers is beginning to be appreciated. Here we review our current understanding of melanoma, historical and current melanoma treatment practices, T cells and tissue resident memory T cells, as well as our current understanding regarding the immune cell targets of checkpoint immunotherapy and the various mechanisms of resistance.

Introduction

Melanoma

Origins and Epidemiology

Malignant melanoma is a serious skin cancer that begins with the uncontrolled proliferation of melanocytes that predominantly reside in the epidermis and around hair follicles of the skin. While melanoma only accounts for a small 4% of dermatologic cancers, it makes up nearly 80% of all skin cancer related deaths (Miller & Mihim, 2006). Australia is recognised internationally to have one the highest incidence rates of melanoma in the world. The DALY study, a Global Burden of Disease Study for melanoma performed in 2015, found that the incidence and morbidity rates associated with melanoma are increasing worldwide, with Australasia (Australia and New Zealand) leading the way, followed by North America, and Europe (Karimkhani et al., 2017). Within Australia, the statistics are sobering. Taken directly from the Australia Institute of Health and Welfare, it is estimated that for the year 2021 alone, 16, 878 (9,869 males + 7,009 females) new skin diagnoses of melanoma will be made (Australian Institute of Health and Welfare, 2021). This will represent 11.2% of all new cancer diagnoses and 2.7% of all cancer-related deaths for the year 2021. In 2001, melanoma was the fourth most commonly diagnosed cancer in Australia, after colorectal, breast, and prostate cancers (Australian Institute of Health and Welfare, 2021). As of 2021, melanoma is now the third most commonly diagnosed cancer in Australia, clearly showing that despite vigorous and aggressive campaigns to spread awareness, melanoma is only becoming more prevalent in the population. If the incidence of melanoma is stratified by age, it reveals that adults between the age of 20-39 are the driving force for these trends, followed by adults aged 40-59. These are also the two age groups in which melanoma makes the "top ten" for most common causes of death. Some have speculated that Australia's wrestle with melanoma is due to its predominantly fair-skinned population, high ultra-violet (UV) radiation, and cultural emphasis on outdoor recreational activity and tanning (Karimkhani et al., 2017). In any case, it represents a significant social and health burden to Australian society and is often disaffectionately referred to as Australia's "National Cancer".

Pathogenesis

Although certain individuals may be more susceptible to acquiring melanoma due to genetic predispositions and a family history of melanoma, the majority of cases occur because of strong and intermittent UV exposure (Gilchrest, Eller, Geller, & Yaar, 1999). This leads to genetic changes in the skin, over production of growth factors, and the induction of DNA-damaging oxygen species, that together, affect the normal functioning and growth of melanocytes in the skin (Gilchrest et al., 1999; Thompson, Scolyer, & Kefford, 2005). When irreparable damage occurs, melanocytes proliferate uncontrollably, generating self-contained malignancies that are initially restricted to the epidermis and dermis. At the molecular level, upon UV exposure, keratinocytes (a major cellular component of the epidermis) produce the hormone melanocyte stimulating hormone (MSH) (Leonardi et al., 2018). MSH interacts with melanocytes via the melanocortin receptor 1 (MC1R) to promote the upregulation of intra-signalling pathways involved in the production of melanin, a biochrome that absorbs and dissipates UV light to minimise the carcinogenic effects of UV exposure. However, when there is inadequate melanin and an over-exposure of UV light, somatic mutations can occur in the DNA of cells comprising the skin, including melanocytes. The most common somatic mutations leading to malignant

melanoma are in genes involved in cellular growth and proliferation (*RAS, B-RAF, PTEN* - phosphatase and tensin homolog) cell cycle control (*CDKN2A*, cyclin-dependent kinase inhibitor 2A), and cellular replicative capacity (*TERT* - telomerase reverse transcriptase) (Leonardi et al., 2018). It is important to realise that a single mutation in any one of these genes is often insufficient to cause malignancy. This is best demonstrated by the fact that 80% of non-malignant naevi (melanocyte crests, also known as moles) contain mutations in the *BRAF* gene (Poynter et al., 2006), an important gene involved in the MAPK pathway, and which currently is a target of metastatic melanoma systemic therapy. Thus, the transformation of normal melanocytes to malignant melanoma often occurs when cumulative mutations in the DNA occur over time that allows them to escape the surveillance of the immune system (Leonardi et al., 2018).

Ethnicity

Cutaneous melanoma disproportionately affects individuals with light skin. This fact has been born out of studies showing that the highest incidence and mortality rates are within countries predominantly made up of Caucasian populations (ie Australasia, North America, and Europe) (Karimkhani et al., 2017). Caucasian populations have a relatively low melanocyte/melanin density in the skin compared to darker skinned individuals, which allows more carcinogenic UV light to penetrate the epidermis. Indeed, studies have shown that darker skinned individuals are far more effective (up to 50%) at reducing UV-B light (the more carcinogenic form of UV light) than lighter skinned individuals (Kaidbey, Agin, Sayre, & Kligman, 1979). Another reason why lighter skinned individuals are often at greater risk, is due to the fact that these ethnicities are often associated with germ-line polymorphisms in the MC1R gene (gene coding for the MC1R on melanocytes), making melanocytes less responsive to the hormone MCH for melanin production (Miller & Mihim, 2006). However, it would be wrong to call melanoma an exclusively "white-man's" disease. Statistics from the Australia Institute of Health and Welfare clearly show that melanoma also affects Aboriginal and Torres Strait Islander peoples in Australia (Australian Institute of Health and Welfare, 2021). Furthermore, cutaneous melanoma is only one subset of melanoma (albeit the most common). Mucosal and acral melanomas, which arise from melanocytes within the mucosal barriers (ie nose, gut) and palms and soles of feet (acral) are non-UV induced but are known to predominantly affect populations of Asian and darker skinned ethnic groups (Mao, Qi, Zhang, Guo, & Si, 2021). These melanoma subtypes are also less responsive to current systemic treatments, and thus melanoma is a shared social and health burden for all ethnic groups.

Diagnosis and Staging

Melanoma presentation on the skin is usually diagnosed by a dermatologist and can generally be done by eye with proper lighting, magnification and identification of various melanoma features, including asymmetry, border irregularity, colour variation, and a diameter greater than 6 mm – the so-called ABCD system of diagnosis (Thompson et al., 2005). If a lesion is identified or in doubt, it is excised and will undergo histopathological examination by a pathologist to confirm diagnosis and determine the level of invasion into the dermis and related structures (which will later determine primary stage). If a melanocytic lesion is 0.8-1mm in thickness or is <0.8mm but ulcerated (absence of an epidermis structure), a sentinel lymph node biopsy will be recommended to determine involvement of the local draining lymph nodes of the primary site (Thompson et al., 2005). These biopsies will once again be examined by a pathologist to determine macroscopic or microscopic involvement of the lymph nodes. If distant metastasis (distant organ involvement) is suspected or warrants investigation, various

body imaging techniques (ie PET, MRI or CT) may be employed to determine the extent (if any) of metastatic disease.

Melanoma can broadly be divided into four stages of disease progression. These include the following:

Stage I. The tumour is confined to the epidermis /dermis at the primary site.
Stage II. Tumour cells begin to dissociate radially and locally from the primary site, but not metastasized beyond the skin.
Stage III. Tumour cells are dissociated and are present within local draining lymph nodes but have not yet travelled to distant sites (regional-lymph node metastatic melanoma).
Stage IV. Characterized by the presence of metastatic melanoma at distant sites (organs and brain) from the primary and regional (draining lymph node) sites.

Each of these stages can be further divided into sub-stages depending on the extent of progression within the limits defined by that particular stage. The most comprehensive and clinically recognised staging system is the American Joint Committee on Cancer (AJCC) melanoma staging system, which currently is in its eighth edition (Emily & Gershenwald, 2018). According to this edition, Stage I and II melanomas can be further categorised (T categories) based on tumour thickness and ulceration status. Stage III melanomas are subdivided (into N categories) based on the number and extent of lymph node and non-lymph node regional involvement and primary disease (T stage). Lastly, distant metastatic disease can be sub-grouped (by M stage) based on the organ type affected as well as CNS or non-CNS involvement.

Surgical Treatment History- Current

Up to 90% of melanomas are diagnosed at the primary or loco-regional stages of disease, and the primary treatment for such patients is elective surgery (B. Curti & Faries, 2021). Surgical treatment for melanoma disease has evolved drastically over the years. Historically, excision of primary skin lesions involved 5cm wide margins (5cm from last point of detectable melanoma) and prophylactic removal of regional lymph nodes (B. Curti & Faries, 2021). This represented a significant treatment burden to patients in terms of wound healing, cosmetics, and lymphedema, which resulted in a number of successive prospective randomised trials to determine the necessity of such wide margins. Safety margins for thin primary melanomas (<2cm thick) were initially reduced to 2cm, and later reduced to 1cm, based on data showing that no difference in disease recurrence time with wider margins (Khayat et al., 2003; Veronesi & Cascinelli, 1991). For intermediate and thick primary melanomas, clinical trial data now indicate that 2cm margins are safe. However, there are ongoing trials to determine whether 1cm margins are sufficient in these primary melanomas, including the MelMarT-II trial (ClincialTrials.gov number NCT03860883) (the Melanoma Institute of Australia is a participating site). With regards to elective lymph node dissection, historically Herbert L.Snow was the first to recommended routine complete lymph node dissection (CLND), even in patients without clinical evidence of regional metastases (Snow, 1892). This was eventually replaced with the practice of performing sentinel lymph node biopsies, where only the lymph nodes directly involved in the draining from the primary site of disease (determined through a procedure called lymphatic mapping) are removed. Individuals with negative sentinel lymph nodes (primary involvement only) were spared a CLND, whereas those with positive sentinel lymph nodes would then go on to have all lymph nodes within the region removed. The practice of removing all lymph nodes in an affected area was thought to reduce the chances of

melanoma relapse and metastasis. However, two revolutionary clinical trials (one performed by the German Dermatological cooperative group) called DeCOG-SLT and MSLT-II (Multicenter Selective Lymphadenectomy Trial 2) unequivocally demonstrated that there was no melanoma-specific survival benefit nor any clinically meaningful disease-free survival/distant metastasis-free survival benefit in the complete lymph node dissection group compared to the observation groups (Faries et al., 2017; Leiter et al., 2016). These trials changed clinical practice and justified routine ultrasound testing and clinical observation for stage III patients, thereby limiting the significant treatment burden associated with CLND.

Systemic Treatment History - Current

The treatment of metastatic melanoma has come a long way in the last decade resulting in significant advances in patient overall survival (OS) and progression free survival (PFS). Yet, for a very long time (up until 2004), there was no systemic treatment that provided any survival benefit to patients. In fact, before 2011 metastatic melanoma was considered a fatal disease by 18 months from diagnosis, while the median overall survival was just 9 months (Luke, Flaherty, Ribas, & Long, 2017). In the 1970s, Dacarbazine (a chemotherapy agent) was approved by the FDA for the treatment of end stage metastatic melanoma based on data showing a modest improvement in PFS (3-6 months) in a small percentage of patients (10-20%) (Luke & Schwartz, 2013). Dacarbazine had not been shown to provide any survival benefit and the toxicity profile was poor (nausea, bone marrow suppression, vomiting etc). In the early 1990's clinical trials with high dose IL-2 therapy (intravenous IL-2 infusions) were performed in metastatic melanoma patients and demonstrated modest objective response rates (16%) with a small subset of patients showing durable responses (Atkins et al., 1999). While this was the first therapy to utilise the potential of the immune system in stage IV melanoma, the side effect profile was relatively dangerous, meaning that it was only relevant in select patient groups (the fit and healthy). IFN-alpha therapy was approved in 1996 based on the results of the ECOG 1684 trial, which showed modest improvements in relapse-free survival (RFS) and OS in the stage III melanoma adjuvant setting (post-surgery)(Kirkwood et al., 1996). Subsequent trials with IFN-alpha, however, showed no survival benefit despite RFS benefits, and IFN-alpha was never used in end-stage metastatic melanoma. It wasn't until the development and approval of BRAF inhibitors in 2011, that significant survival outcomes were achieved for metastatic melanoma patients. These developments originated from the finding that BRAF mutations in the V599E (later corrected to V600E) region of the *BRAF* gene comprised 59-66% of melanomas, one of the highest mutation rates of any cancer (18% for colorectal cancer, gliomas 11%, lung cancers 3%, ovarian cancers 4% and breast cancers 2%) (Davies et al., 2002). Subsequent studies showed that the mutation rate was closer to 50% (Akbani et al., 2015). In the original study, the authors also demonstrated that an amino acid change from Valine to Glutamic acid at region 600 resulted in increased kinase activity in the BRAF protein (probably because the insertion of a negatively charged residue acted as a phosphorylation event), providing evidence that the BRAF mutation was the most common oncogenic event in melanoma (Davies et al., 2002). At the time, it was clear that the RAS-RAF-MEK-ERK MAP kinase pathway was one of the key cellular signalling pathways responsible for cell growth, proliferation and differentiation, however, later studies confirmed that mutations in BRAF actually increased the activation of the RAF-ERK pathway (Wan et al., 2004). Given the strong scientific rationale for the inhibition of BRAF in melanoma, a number of BRAF inhibitors were developed, tested, and eventually approved for melanoma. The first in-class agent was vemurafenib. In the phase 3 BRIM 3 trial with vemurafenib vs. dacarbazine, the objective response rate (ORR) by RECIST criteria was 48% versus 5%, and the median progression-free

survival (PFS) was 5.3 months versus 1.6 months (Chapman et al., 2011). Dabrafenib, a subsequent BRAF inhibitor showed similar results (ORR 50% vs 6% and a median PFS of 5.1 months verses 2.7 months) in their phase 3 BREAK-3 trial (Hauschild et al., 2012). Adverse events were manageable albeit the non-melanoma skin malignancy rate was high in patients (15-20%) (Chapman et al., 2011; Hauschild et al., 2012). These represented an extraordinary breakthrough in the treatment of metastatic disease, yet resistance to BRAF-inhibitor therapy was common and responses short-lived. One of the key resistance mechanisms was identified to be upregulation of the MEK pathway, primarily due to c-Raf activation (Antony, Emery, Sawyer, & Garraway, 2013; Montagut et al., 2008). MEK inhibitors were soon tested in monotherapy and showed significant efficacy (Flaherty, Robert, et al., 2012). However, it was the combination of BRAF and MEK inhibitors, which demonstrated the highest response and PFS rates. Results from a phase 1/ 2 study of dabrafenib in combination with trametinib (MEK inhibitor) demonstrated an ORR of 76% and a median PFS of 9.4 months, versus an ORR of 54% and 5.8 months PFS, respectively, among those treated with dabrafenib alone (Flaherty, Infante, et al., 2012). While the percentage of patients with a grade 3 or 4 adverse event was higher in the combination therapy, the incidence of keratoacanthomas was decreased, and the tolerability of the combination therapy was better than monotherapy. Phase 3 studies (COMBI-v and COMBI-d) later confirmed these findings, which led to the approval of combination Dabrafenib and Trametinib by the FDA for metastatic melanoma in 2013. In parallel to these developments, a new class of therapies was beginning to be explored that targeted receptors on T cells; receptors that were shown to be important in limiting T cell activation. This class of therapies would later be termed “checkpoint inhibitors”. With the comparative unsuccessfulness of immune targeting therapies until then however, the field of immune oncology was treated lightly by clinicians. Yet the work of scientists provided strong rationale for targeting these immune cell receptors in melanoma. As early as 1995, James Allison, who would later be awarded the Nobel Prize, discovered that the presence of B7-2 (CD86) could partially inhibit T cell activation and proliferation via another receptor on T cells, called cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) (Krummel & Allison, 1995). Indeed, crosslinking of CTLA-4 together with the T cell receptor and a costimulatory molecule, CD28, strongly inhibited T cell proliferation and IL-2 secretion by T cells. To understand the significance of this, it is critical to understand how T cells are activated. It is now widely understood that CD8 naïve T cells require at least two signals to become properly activated. The first is recognition of MHC class 1 presented cognate antigen via the T cell receptor. The second is a costimulatory signal that occurs via CD80 or CD86 (B7-1 or B7-2) on antigen presenting cells (APCs) and CD28 on T cells (Waldman, Fritz, & Lenardo, 2020). Allison and colleagues showed that CTLA-4 acted in opposition to CD28 and could preferentially engage with CD80/CD86 ligands with higher affinity to disrupt the second signal, thereby limiting T cell activation. Later studies would demonstrate that CTLA-4 could in fact remove and degrade CD80 and CD86 via trans-endocytosis, abrogating CD28 costimulation (Qureshi et al., 2011). This discovery led Allison and others to hypothesise that the poor immunogenicity in many tumors might be due to the absence of the second signal in T cells (via CTLA-4). Indeed, in murine models of melanoma, they later demonstrated that antibodies targeting CTLA-4 was sufficient to promote the rejection of tumors, including preestablished tumors (Leach, Matthew, & James, 1996). Ipilimumab and tremelimumab were the first monoclonal CTLA-4 targeting antibodies to be tested in clinical trials. In two phase 3 trials exploring the efficacy of ipilimumab (CA184-002; Ipilimumab + gp100 vaccine vs ipilimumab monotherapy vs gp100 vaccine monotherapy, and CA184-024; Ipilimumab + dacarbazine vs dacarbazine monotherapy) the addition of ipilimumab was found to have superior ORR (11-15%), and overall survival when compared to chemotherapy, albeit with 10-15% of patients treated with ipilimumab exhibiting Grade 3 and 4 adverse events, some of which were life-threatening

(Hodi et al., 2010; Robert et al., 2011). The real excitement for ipilimumab and other checkpoint inhibitors (later on) came from long-term survival data, which suggested durable responses in a subset of melanoma patients. Indeed, in the largest retrospective analysis of pooled OS data from phase 2 and 3 clinical trials with ipilimumab in metastatic melanoma patients, Schadendorf and colleagues showed that the survival curves began to plateau around 3 years (at around a 20% survival rate), with follow-up to 10 years (Schadendorf et al., 2015). Up until this point, no systemic therapy could boast such long-term durable responses. Even combination Dabrafenib and Trametinib, which maintained the highest ORR (76%) for any systemic therapy, was known to have limited durability of response over time. With the relative success of ipilimumab as the first checkpoint inhibitor, clinical trials advanced rapidly for another targeting PD-1. Reports a decade earlier, had shown that disruption of the PD-1 gene caused auto-immune-like conditions in murine models, highlighting PD-1 as another potential coinhibitory receptor on T cells that might act to keep T cell activation in check (Nishimura, Nose, Hiai, Minato, & Honjo, 1999). Soon thereafter, the ligand for PD-1 was identified as PD-L1 (member of B7 family), and studies showed that the engagement of PD-1 (on T cells) and PD-L1 (on APCs) acted as a brake of sorts to limit T cell proliferation, cytokine secretion, and activation (Freeman et al., 2000). Honjo and his lab, which led the efforts on PD-1, were able to show that over-expression of PD-L1 on tumor cells provided an escape pathway from the host immune system, while blockade of the PD-1 and PD-L1 axis made tumors susceptible to the effects of immune system (Iwai et al., 2002). In the clinic, two anti-PD-1 inhibitors were being tested: Nivolumab and Pembrolizumab. Nivolumab (Checkmate 037) and Pembrolizumab (KEYNOTE-002) were initially tested in the second-line treatment setting (after ipilimumab failure) for metastatic melanoma and showed ORR ranging from 20-40% (Ribas et al., 2015; Weber et al., 2015). Phase 3 studies followed (Checkmate-066 and KEYNOTE-006) in the first line setting, clearly showing that anti-PD-1 provided clinical benefit in the first line setting as well. KEYNOTE -006, which tested pembrolizumab vs ipilimumab showed that the estimated 6-month PFS rates were 47.3% for pembrolizumab every 2 weeks, 46.4% for pembrolizumab every 3 weeks, and 26.5% for ipilimumab (Robert, Schachter, et al., 2015), while Checkmate-066, which tested nivolumab vs. dacarbazine, demonstrated median PFS of 5.1 months vs. 2.2 months, and 1-year OS rates of 72.9% vs 42.1% for nivolumab and dacarbazine, respectively (Robert, Long, et al., 2015). These data propelled anti-PD-1 as the undisputed leading checkpoint inhibitor in metastatic melanoma both in terms of efficacy and safety. However, like ipilimumab, more exciting was the durable responses observed with long-term follow-up in patients who had had a complete response to anti-PD-1 immunotherapy (Warner et al., 2020). Much like with BRAF and MEK inhibitors, clinician Thought Leaders began to test the combination of nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4) in metastatic melanoma based on data from murine models suggesting that the efficacy of combination treatment would be twice as either therapy alone (Curran, Montalvo, Yagita, & Allison, 2010). Indeed, reports from the scientific community suggested that there would be synergy between the two agents based on distinct but complimentary mechanisms of action. It was thought that anti-PD-1 most likely acted in the periphery of non-lymphoid tissues and at the later stages of T cell activation, whereas anti-CTLA-4 was thought to act during the priming stage of the immune response within lymphoid organs (Waldman et al., 2020). While the exact mechanisms of action were undefined (and remain poorly understood in many respects today), a pivotal clinical trial by Larki and colleagues clearly demonstrated the superior efficacy of combination anti-PD-1 and anti-CTLA-4 therapy, over either agent as monotherapy. In this phase 3 trial named Checkmate 069, the median PFS for combination treatment was 8.9 months, vs. 6.9 months and 2.9 months for nivolumab and ipilimumab, respectively (Larkin et al., 2015). However, combination treatment also had the worse toxicities, with 55% of patients experiencing grade 3 or 4 adverse

events in the combination treatment arm compared to 16.3% and 27.3% in nivolumab and ipilimumab arms, respectively (Larkin et al., 2015). Despite this, combination therapy has now become the standard of care for many patients with metastatic melanoma. In the last few years, extensive research has been conducted exploring the use of combination immunotherapy in special patient cohorts. The ABC study, led by Prof. Georgina Long, demonstrated for the first time the intracranial activity of nivolumab and ipilimumab immunotherapies in patients with melanoma brain metastases, and showed the superior efficacy of combination anti-PD-1+ anti-CTLA-4 therapy in the brain over nivolumab (G. V. Long et al., 2018). This study changed clinical practice for patients with asymptomatic melanoma brain metastases. In addition to special cohorts, excitement has recently revolved around using immunotherapies earlier in the course of disease and before surgery (neo-adjuvant therapy). NeoTrio and PRADO are two neoadjuvant trials currently being explored in stage III melanoma patients, which explore the use of combination pembrolizumab and dabrafenib + trametinib (NeoTrio) or nivolumab and ipilimumab (PRADO) before surgery. Early data suggests that neo-adjuvant therapy may exhibit higher complete response rates and limit surgical lymph node dissections (Blank et al., 2020). In terms of new treatments on the horizons, a plethora of trials are currently examining alternative co-inhibitory and co-stimulatory checkpoint inhibitors/agonists. Recent data from ASCO 2021 suggests that a monoclonal antibody targeting LAG-3 may be efficacious in combination with nivolumab vs nivolumab alone (Lipson et al., 2021). In summary, the treatment landscape has changed drastically over a decade and has provided clinically meaningful improvements in patient OS for those with metastatic disease. It is important to recognise that while we have come so far, the majority of patients still end up failing (either through primary or acquired forms of resistance) the best systemic treatments, and thus there is work still to be had to meet these unmet needs. Given the success of targeting the immune system, a greater understanding of the exact mechanisms and immune cells involved in tumor clearance and immunotherapy response may help improve current therapies and design others.

The immune system, T cells, and Tissue resident T cells

The immune system and T cells

The human body is exposed to a variety of pathogens and foreign entities within the environment. If left unchecked, these cause disease and mortality in their hosts. For this reason, humans have developed a vast and somewhat complicated system of cells, proteins, messenger molecules, barrier tissues and chemicals, which work collectively to eliminate pathogens from the body in a quick and effective manner. This synchronized network is what we call the immune system. A key event in the activation of the immune system is the process whereby antigen presenting cells (APCs), such as dendritic cells, migrate to draining lymph nodes or other secondary lymphoid organs (SLO) and activate important cells necessary to eliminate the threat. One such key player is the T lymphocyte (T cell). T cells are important in the clearance of a variety of infectious agents including viruses, bacteria, parasites and even cancers. When a naïve T cell encounters its cognate antigen for the first time, an activation phase follows, marked by rapid growth and proliferation coupled with a change in metabolism (Blattman et al., 2002; O'Neill, Kishton, & Rathmell, 2016). This metabolic change, though inefficient, supports rapid growth and effector function, particularly for effector T cell subsets by enhancing IFN-gamma gene expression (Chang et al., 2013). Simultaneously, T cells can also differentiate into other distinct T cell states, each programmed with various degrees of functionality and longevity. Once antigen has been cleared, the vast majority of antigen experienced effector T cells undergo apoptosis and die, in what has been termed the contraction phase (Sabbagh et al., 2004). A small subset of antigen experienced T cells destined to survive

and persist become part of the memory phase. Traditionally, memory T cells have been defined based on their expression of CCR7 and CD45RA, giving rise to the common memory subsets, including central memory (TCM; CCR7+ CD45RA-), effector memory (TEM; CCR7- CD45RA-), and terminally differentiated effector memory (TEMRA; CCR7- CD45RA+) (Sallusto, Lenig, & Förster, 1999); however, stem cell-like memory T cells (TSCM), which exhibit particular self-renewal capabilities and multipotency (Gattinoni et al., 2011), and tissue resident memory T cells (TRM), which reside permanently in peripheral and epithelial/barrier surfaces and do not enter the circulation (Thomas Gebhardt et al., 2009; L. K. Mackay et al., 2012), have recently been added to the T cell memory repertoire. Memory populations offer a number of advantages to the host. For one, memory T cells do not require APC presentation, but instead, are able to recognise and respond to their antigen independently. Memory T cells also have more potent effector functions than naïve T cells, elicit rapid recall during re-infection, show greater proliferative capacity and migration potential, and can persist for years in the absence of antigen (Mueller, Gebhardt, Carbone, & Heath, 2013). For many years, memory T cells were thought to circulate continuously between blood and tissues based on studies showing distinctive circulating patterns for naïve (lymph and blood) and memory T cells (blood and tissues) (Cahill, Poskitt, Frost, & Trnka, 1977; C. Mackay, Wayne, Malcolm, & Ross, 1988; C. Mackay, Wendy, & Lisabeth, 1990). Years later however, a pivotal study by Sallusto and colleagues showed that memory T cells could be divided into functionally distinct subsets based on their expression of CCR7, a chemokine receptor important for homing into lymphoid organs (Sallusto et al., 1999; N. Sharma, Benechet, Lefrançois, & Khanna, 2015). These memory subsets were termed central memory (Tcm) and effector memory (Tem) T cells. As part of their study, Tcm cells were shown to express CCR7, enabling them to traffic from blood into secondary lymphoid organs, while Tem cells didn't express CCR7, and so circulated mainly between blood and non-lymphoid tissues (Sallusto et al., 1999). Despite these important nuances however, it was still widely believed that memory T cells in the blood and tissue were in equilibrium with each other, and thus, for many years, the majority of work in humans was performed on peripheral blood with the results being extrapolated to TEM populations within tissues.

Emergence of Tissue Resident Memory T Cells

Just as evidence was coming out in support of Sallusto's model, other work was beginning to suggest that the model was incomplete in its current state. Klonowski and colleagues performed parabiosis studies in mice, a procedure wherein the blood supply of two animals are joined. During their experiments they discovered that some organs, including the brain and intestines, contained a population of memory T cells that were not in equilibrium with the circulation (Klonowski et al., 2004). This was later confirmed in other parabiosis experiments for the lung, epidermis of skin, and the genital tract (Iijima et al., 2014; Jiang et al., 2012; Teijaro et al., 2011). These results suggested the presence of another population of memory T cells that were independent from the circulating memory pool. Gebhartd and colleagues were the first to clearly demonstrate the resident nature of these cells in mice. In their model, sensory ganglia containing a persistent latent form of HSV and a population of CD8+ T cells specific for the pathogen were transplanted underneath the kidney capsule of recipient mice. When the latent infection was reactivated, they observed that T cells exclusive to the graft were able to undergo secondary stimulation with no apparent involvement of lymph nodes (Thomas Gebhardt et al., 2009). Furthermore, these cells failed to recirculate despite local or systemic infection with HSV, demonstrating that these memory cells were resident within the graft. In a follow-up study years later, Gebhartd and colleagues validated these observations by utilizing an adoptive transfer model, thereby proving, in more models than one, that these memory cells were

resident in tissues and independent from the circulating memory pool in blood and lymph(T Gebhardt et al., 2011) . The authors named these cells “tissue resident memory T cells”, and in line with the already existing memory nomenclature, these cells are now abbreviated as Trm. Following the discovery of tissue resident memory T cells in mice, it wasn’t long until this population was also identified in humans (Clark et al., 2012; Kumar et al., 2017; Purwar et al., 2011; Sathaliyawala et al., 2013; Woon et al., 2016). Perhaps one of the most compelling evidences for the existence of these cells in man came from a clinical study, wherein patients suffering from cutaneous T-cell lymphomas were treated with a drug called alizumambab (Clark et al., 2012), an antibody that inhibits CD52 function on the surface of T cells, and which leads to the depletion of all circulating T cells in blood. Clark and colleagues observed that while all central memory T cells were eliminated from the blood and skin, a population of memory T cells reminiscent of a Tem phenotype persisted in the skin during and after treatment. The authors concluded that Tcm cells were depleted because they transited between blood and tissue, while the resident memory population was spared because they did not enter the circulation (Clark et al., 2012). More recently, multiple studies with HLA-mismatched T cells in organ transplant recipients have provided further evidence for Trm in humans (Bartolomé-Casado et al., 2019; Lian et al., 2014; Snyder et al., 2019; Zuber et al., 2016). In each of these studies, whether they be transplants of the skin, gut, lung or small intestine, donor T cells have not been observed in the host circulation but have been observed to persist within the donor organ for years. It has now been established that Trm do exist in humans and that they have a unique molecular and transcriptional phenotype, mechanisms for retention and maintenance, and function, which sets them apart as a key T cell in the immune system.

The Unique Molecular and Transcriptional Phenotype of Trm Cells

Trm cells are associated with a number of molecular markers, some considered more unique to Trm than others. Initially, Gebhardt and colleagues observed that tissue resident memory T cells in the epidermis of the skin had high levels of CD103, but not circulating cells (T Gebhardt et al., 2011; Thomas Gebhardt et al., 2009). Other studies followed that showed that TGF-beta dependent expression of CD103 on Trm in the epidermis of skin, brain and intestinal tract was necessary for Trm persistence and survival in these tissues (L. Mackay et al., 2013; Wakim, Woodward-Davis, & Bevan, 2010). It is now clear that not all Trm express CD103, and it is mainly associated with CD8+ Trm at epithelial surfaces (Casey et al., 2012; T Gebhardt et al., 2011; Ma, Mishra, Demel, Liu, & Zhang, 2017; L. Mackay et al., 2013; Purwar et al., 2011; Sathaliyawala et al., 2013; Wakim et al., 2010, 2012). A more encompassing Trm marker is CD69, a C-type lectin, that has also been found to be constitutively expressed on Trm (Casey et al., 2012; Thomas Gebhardt et al., 2009; L. Mackay et al., 2013). While CD69 has been traditionally viewed as an early activation marker (Schuurman, van Wichen, & de Weger, 1989), it also has a role in regulating the retention of T cells in tissues in the absence of activation (L. Mackay et al., 2015; Shiow et al., 2006; Woon et al., 2016). A study exploring the human Trm signature of CD8+ and CD4+ T cells in lymphoid and mucosal organs from healthy donors, identified CD69 as a definitive identification marker of human Trm based on the fact that it could correctly identify cells with a Trm signature (a set of 31 genes conserved across tissues and lineages) (Kumar et al., 2017). However, it is important to recognise that Trm-like cells have been observed without CD69 expression (Steinert et al., 2015). A number of other molecular markers have been associated with Trm, including CXCR6, CD101, PD-1 and CD49a (Cheuk et al., 2017; Kumar et al., 2017), which may help to differentiate heterogeneity within the Trm population, as is likely the case (Cheuk et al., 2017; Christo et al., 2021; Kumar et al., 2017; Kurd et al., 2020). Indeed, the molecular marker CD49a has

been shown to distinguish Trm with distinct effector function; CD49a+ Trm excreted IFN-gamma and were critical in the pathogenesis of vitiligo, whereas CD49a- Trm excreted IL-17 and were drivers of psoriasis pathogenesis (Cheuk et al., 2017). In our own lab, the combination of markers CD103 and CD69 (in the absence of activation markers) has enabled us to identify Trm with downregulation of genes associated with tissue egress(Woon et al., 2016).

In addition to these molecular markers, Trm cells also display a unique transcriptional phenotype when compared to other memory cells. Mackay and colleagues were the first to comprehensively show this. They compared the RNA of virus-specific CD103+ CD8+ Trm cells in the skin, lung and gut of mice with virus-specific Tcm and Tem CD8+T cells of the spleen (L. Mackay et al., 2013). Among the genes differentially expressed between Trm and non-Trm T cells were genes involved in tissue retention and egress (*Itgae, S1pr1, Cd69, Itag1, Ccr7*), and inhibitory function (*Cd244, Ctla-4*). This expounded and confirmed the work of Wakim and colleagues exploring Trm cells in the brain of mice (Wakim et al., 2012). More recent work in humans have shown key homologies with the mouse Trm core gene signature, along with additional nuances. Human CD4 and CD8 Trm have downregulation of genes involved in exit cues (*CD62L, S1PR1, KLF2 S1PR5, CX3CR1*), upregulation of genes involved in tissue retention and tissue homing (*CXCR6, CD69, ITGA1, ITAGE* and *CD101* (for CD8 Trm)), genes involved in rapid cytokine responses (*IFN-γ*, *IL-2, IL-17, NOTCH*), metabolism (*HIF-1a, BHLHE40, FABP4/FABP5*), anti-inflammation (*IL-10*), decreased cell turnover (*KI67*), and differentiation and homeostasis (*RUNX3*) (Hombrink et al., 2016; Kumar et al., 2017; C. Li et al., 2019; Milner et al., 2017; Pan et al., 2017; Woon et al., 2016). In mice, elevated levels of the transcriptional regulators *Blimp-1* and *Hobit* are critical for Trm development and maintenance (L. K. Mackay et al., 2016) but appear to be less important in human Trm (Kumar et al., 2017). Altogether, however, these studies demonstrate that Trm cells are distinct from their circulating counterparts.

Molecular Mechanisms of Tissue Retention

One of the key mechanisms in which Trm cells are able to maintain residency in tissues is through down regulation of the sphingosine-1-phosphate (S1P) receptor 1, S1P1 (Skon et al., 2013). S1P1 is a chemokine receptor that is expressed on T lymphocytes. It is also sensitive to S1P, a chemokine that is expressed at high concentrations in the blood/lymph and low concentrations in tissues (Matloubian et al., 2004). The disparity in S1P concentrations enables T cells to exit tissues and enter circulation. In addition to S1P1, expression of the zinc finger transcription factor, Kruppel-like factor (KLF) 2, is also necessary for T cell migration out of tissues. KLF2 positively regulates S1P1, such that down regulation of KLF2 drives the down regulation of S1P1, subsequently leading to T cell retention in tissues (Bai, Hu, Yeung, & Chen, 2007; Carlson et al., 2006; Skon et al., 2013). A number of studies in mice (L. Mackay et al., 2013; Skon et al., 2013; Wakim et al., 2012) and humans (Woon et al., 2016) have shown that Trm populations express lower levels of *KLF2* and *S1PR1* relative to their circulating counterparts, indicating that this is a general requirement for Trm formation. Recently, the role of sphingosine-1-phosphate (S1P) receptor 5 (S1P5) has been delineated and appears to also play a significant role in Trm retention in tissues (Evrard et al., 2022). The expression of the receptors CD69, CD103, and CD49a on Trm have a functional role in the retention of T cells. Shiow and colleagues found that downregulation of S1P1 by interferon α/β stimulation resulted in the coordinated upregulation of CD69 (Shiow et al., 2006). This and other studies showed that CD69 prevented the chemotactic responsiveness of S1P1 to S1P and potentiated the down-modulation of S1P1 (Bankovich, Shiow, & Cyster, 2010; Shiow et al., 2006). Later, Mackay and colleagues, demonstrated that this was a primary mechanism of T cell retention in CD69+

CD103+ Trm cells (L. Mackay et al., 2015). With regards to CD103, it is upregulated upon exposure to TGF-beta and can act as a receptor that binds to E-cadherin on epithelial cells (K. L. Cepek, Parker, Madara, & Brenner, 1993; K. Cepek et al., 1994; El-Asady et al., 2005). Work in our own laboratory has also shown that CD103+ CD69+ Trm cells localize near the epithelium of tissues (typically places where E-cadherin is expressed) and that these had the lowest levels of expression for *S1PR1* and *KLF2* genes relative to CD103- CD69+ Trm cells and CD103- CD69- circulating cells (Woon et al., 2016). TGF-beta induces CD103 expression on Trm and increases their longevity in tissues (L. Mackay et al., 2013). Therefore, CD103 is likely to have an important functional role in the retention of CD8+ T cells, and particularly, in tissues that are epithelial in nature and where E-cadherin is expressed. Lastly, CD49a is known to pair with CD29 to form the heterodimer, VLA-1, which has the capacity to bind with collagen at peripheral sites, thus also being a potential molecule important for Trm retention (Topham & Reilly, 2018).

Generation and Maintenance of Trm Cells

It is unlikely that tissue resident memory T cells require the persistence of cognate antigen for their maintenance within tissues, marking them as a true memory population of cells (Casey et al., 2012; L. K. Mackay et al., 2012; Wakim et al., 2010). Early studies in the skin showed that antigen presentation was necessary for the generation of CD8+ Trm cells but dispensable for their maintenance in mouse skin (L. K. Mackay et al., 2012), brain (Wakim et al., 2010) and intestines (Casey et al., 2012). In humans, the persistence of donor Trm for years in organ transplant recipients suggests that they are able to survive independently of antigen (Bartolomé-Casado et al., 2019; Lian et al., 2014; Snyder et al., 2019; Zuber et al., 2016). However, it is likely that this is not true for Trm cells in all tissue types, particularly in the lung, where Trm have been shown to wane months after infection and require multiple antigen re-exposures to develop persistent, durable populations (Slütter et al., 2017; Van Braeckel-Budimir, Varga, Badovinac, & Harty, 2018). Why this is the case is not known, although it has been suggested that the local tissue environment (high oxygen) may not be conducive to Trm survival (reduced levels of anti-apoptotic molecules, reduced sensitivity to key memory cytokines (IL-7, IL-15) (Zheng & Wakim, 2021), highlighting the importance of the local niche in determining Trm function and durability, as others have recently shown (Christo et al., 2021). Upon antigen re-exposure, secondary Trm are formed from pre-existing Trm in the skin but can be aided by pre-curser populations in the circulation (Simone L. Park et al., 2018). Importantly, these Trm were not found to be displaced with the influx of new Trm with differing antigen specificities (Simone L. Park et al., 2018).

The importance of the local milieu and cytokines for the generation and maintenance of Trm cells is clear. Wakim and colleagues first observed that CD8+ Trm cells failed to survive after isolation from the brain and failed to undergo recall expansion when placed in the blood (Wakim et al., 2010). Given the importance of IL-7 and IL-15 in the maintenance of central memory and effector memory T cells, respectively (J Geginat, Sallusto, & Lanzavecchia, 2001; Jens Geginat et al., 2003), and given evidence that TGF-beta could up regulate CD103 on non-resident CD8+ and Trm-like cells (Casey et al., 2012; K. L. Cepek et al., 1993), it was assumed that many of these *in situ* cues for survival were largely driven by cytokines. In line with this notion, Mackay and colleagues showed that the development of Trm cells in the skin required TGF-beta and IL-15. When ovalbumin specific transgenic CD8+ T cells deficient in the type 2 TGF-beta receptor were activated and injected into the dermis of mice, they failed to express CD103 and maintain residency compared to the wild-type cells (L. Mackay et al., 2013). Furthermore, the generation and survival of these CD103+ CD8+ Trm cells were dependent on

IL-15, as fewer Trm cells were generated/ maintained in the skin of IL-15 -/- mice when compared to wild type at 2 weeks and 4 weeks post-infection (L. Mackay et al., 2013). The requirement of IL-15 and TGF-beta for the development and maintenance of Trm has since been demonstrated in multiple organs (Takamura, 2018; Zheng & Wakim, 2021), and in humans (Woon et al., 2016). A recent study by the Mackay lab showed that much of the variability in Trm cell function, durability and malleability could be attributed to unequal responsiveness to TGF-beta in different tissues (skin and liver) (Christo et al., 2021).

Certain transcription regulators as well as their repression have been associated with Trm cell development and maintenance. Hobit and Blimp are two that are upregulated in Trm cells of mouse skin, gut, liver, and kidney, and were shown to be crucial in controlling a transcriptional program important for tissue retention (L. K. Mackay et al., 2016). Loss of Hobit/Blimp compromised Trm development but not circulating memory T cells. In humans it is less clear whether Hobit/Blimp drive similar programs as Hobit upregulation has not been observed in human Trm and circulating memory T cells can also express Hobit (Kumar et al., 2017; Vieira Braga et al., 2015). Repression of T-bet and Eomes also appear to be important for TGF-beta signalling, with residual T-bet but not Eomes expression being important for IL-15 responsiveness (Hombrink et al., 2016; L. K. Mackay et al., 2015). Another transcription factor, Notch, is critical for Trm maintenance, probably through upregulation of CD103 as has been shown in human lung (Hombrink et al., 2016). More recently, Runx3 and Bhlhe40 were shown to be important in the differentiation and homeostasis of Trm by limiting genes involved in tissue egress and promoting mitochondrial fitness, respectively (C. Li et al., 2019; Milner et al., 2017).

Protective Function of Trm cells in infectious diseases

The functional relevance of tissue resident memory T cells has been thoroughly summarised in a number of recent review articles (Okla, Farber, & Zou, 2021; Sasson, Gordon, Christo, Klenerman, & Mackay, 2020), and points to their significant role in both infectious diseases and within cancer. Initial reports found that Trm in HSV models provided superior protection after secondary infection compared to their circulating counterparts (Thomas Gebhardt et al., 2009; L. K. Mackay et al., 2012). Within humans, despite the depletion of circulating T cells (in alizumambab-treated patients), patients displayed a remarkable ability to withstand infections, suggesting that populations of Trm cells in the skin and other peripheral sites were sufficient alone in providing adequate immune protection in many instances (Clark et al., 2012). Since then, it is now recognised that Trm cells play a vital role, in many cases a superior role, in the protection against various pathogens within different organ sites; lungs (Pizzolla et al., 2017; Teijaro et al., 2011; Turner et al., 2014; T. Wu et al., 2014), liver (Pallett et al., 2017), intestinal gut (Sheridan et al., 2014), reproductive tract (Schenkel, Fraser, Vezys, & Masopust, 2013), nose (O'Hara et al., 2020), secondary lymphoid organs (Thom, Weber, Walton, Torti, & Oxenius, 2015; Woon et al., 2016) and more (Muruganandah, Sathkumara, Navarro, & Kupz, 2018; Sasson et al., 2020). One of the reasons for this is that Trm are often located proximally to the sites of pathogen entry, thereby acting as sentinels where they bypass the delays associated with T cell recruitment from the circulation. Trm are also enhanced with a high degree of polyfunctionality and effector function, including potent expression of cytotoxic cytokines (TNF-alpha, IFN-gamma) and molecules (Granzyme B) during recall responses (Hombrink et al., 2016; Schenkel et al., 2014). Indeed, one study showed that the RNA transcripts for these effector functions were constitutively expressed and deployment ready (Hombrink et al., 2016). Presumably, this would also contribute to rapid and effective Trm protective functions. In addition to de novo Trm properties critical for pathogen control, other

studies have shown that Trm cells may also "sound the alarm", where they act as triggers for alerting and recruiting other immune cells to the site of infection (Ariotti, 2014; Schenkel et al., 2014, 2013). Indeed, IFN-gamma and other inflammatory chemokines by Trm helped sequester circulating memory CD8+ T cells to local sites of infection (Schenkel et al., 2013). It has also been shown that Trm reactivation involved the up regulation of VCAM-1 (an adhesion molecule important for lymphocyte migration) on nearby vascular endothelium, and that this in turn helped recruit B cells, natural killer cells, and even mature dendritic cells (Schenkel et al., 2014). Another study demonstrated that reactivated CD8+ Trm cells in skin were also capable of altering the local microenvironment by up regulating broadly activating anti-microbial agents, such as the antiviral product IITM3 (Ariotti, 2014). Together, these provide evidence to suggest that Trm cells are not only capable of rapid recall towards specific cognate antigen but may also play an important functional role in triggering immune responses to non-specific pathogens.

Protective function of T cells and Trm cells in cancers

Cancers can be recognised by the immune system in many ways, including via self-mutated antigens (tumor antigens), over-expression of proteins (including viral), cancer testis antigens, and others, all of which may result in strong immune responses directed towards tumors (Boon, Coulie, & Van Den Eynde, 1997). Histologically, this phenomenon is observed by the fact that lymphocytes infiltrate, interact, and kill tumour cells directly. "Cancer immunoediting" is a theory that aims to describe the interplay between tumour and immune cells over the course of disease progression. There are three phases - Elimination, Equilibrium, and Escape (Dunn, Old, & Schreiber, 2004). Elimination is the first phase wherein lymphocytes recognise and kill tumor cells resulting in the vast majority of tumour cells being eliminated, albeit some immune-resistant tumor cells remain. During the equilibrium phase, immune-resistant tumor clones are viable but are kept in check through pressures exerted by the immune system. During the escape phase, tumor cells undergo adaptations that allow them to overcome the pressures of the immune system, ultimately leading to their uncontrolled growth and metastasis. One of the strongest prognostic factors associated with the control of tumor growth and metastasis is the presence of tumor infiltrating lymphocytes (TILs), which has been associated with patient overall survival and/or disease relapse free survival in virtually every cancer type (Azimi et al., 2012; Jerome Galon et al., 2006; D. Zhang et al., 2019; L. Zhang et al., 2003). Metanalyses have confirmed these in recent years (Berele, Cai, & Yang, 2021; Fu et al., 2019; G. Gao, Wang, Qu, & Zhang, 2019; Gooden, De Bock, Leffers, Daemen, & Nijman, 2011; Idos et al., 2020). CD8+ T cells, in particular, have been a strong prognostic factor, likely due to their ability to recognize tumour antigens and perform cytolytic killing of tumour cells. Indeed, tumour-infiltrating CD8+ T cells have been shown to correlate with patient survival in breast cancer (Mahmoud et al., 2011), ovarian cancer (L. Zhang et al., 2003), melanoma (Erdag et al., 2012; Kohlhapp et al., 2015; Van Houdt et al., 2008), colorectal cancer (Jerome Galon et al., 2006; Oberg, Samii, Stenling, & Lindmark, 2002; Pagès, 2007), and gastrointestinal stromal tumors (Rusakiewicz et al., 2013). These observations have given impetus to the development of CD8+ T cell immunoscoring systems that aim to provide clinical value to clinicians and their patients (Azimi et al., 2012; Jérôme Galon et al., 2012). Of course, CD8 T cells are themselves a heterogenous population, and recent technological advancements have allowed better profiling of CD8 T cell subsets and their association with patient survival. Trm represent a subset of CD8 memory T cells that have now been extensively studied in the context of cancer within recent years. However, at the beginning of this thesis, our understanding of Trm in cancer, their prognostic effect, as well as their phenotype and function in the tumor micro-environment, was far more limited. Two studies in high-grade serous ovarian cancer and non-

small lung carcinoma reported a subset of CD103+ CD8+ T cells that strongly correlated with patient survival and with better prognostic value than total CD8 T cells (Djenidi et al., 2015; John R. Webb, Milne, Watson, DeLeeuw, & Nelson, 2014). Indeed, in a retrospective cohort of 101 stage 1 non-small lung carcinoma patients, it was shown that for every 50 CD103+ CD8 T cell incremental increase in the tumor, the risk of relapse or death was reduced by 16% and 12%, respectively (Djenidi et al., 2015). In ovarian cancer, tumors predominantly infiltrated by CD103- CD8 T cells showed poor prognosis equivalent to tumors with no CD8 T cell infiltration (John R. Webb et al., 2014). While these cells expressed CD103 (an important tissue residency marker) as well as inhibitory receptors like PD-1 (associated with Trm transcriptomic signature), it was unclear whether this cell subset truly represented the resident phenotype described in early models with infectious diseases. In non-small lung carcinoma, CD103+ CD8+ T cells were negative for CCR7 and CD62L and positive for CD69 and CD45RO, suggesting a Trm memory phenotype, however this had not been demonstrated in ovarian cancer. Nevertheless, these studies suggested that specific CD8 T cell phenotypes, particularly those reminiscent of a Trm phenotype, might be more functionally important for tumor control and patient survival. Given that Trm-like cells in cancers are continuously exposed to antigen (due to the chronic nature of the disease), to avoid any nomenclature mistakes arising through technicalities of whether true memory can exist in the continuous presence of antigen, hereafter Trm within cancers will be termed tumor resident T cells.

Checkpoint Immunotherapy Targets and Resistance Mechanisms

As we have already discussed, anti-PD-1 and anti-CTLA-4 checkpoint inhibitors represent the most advanced class of therapies for the treatment of metastatic melanoma and various other solid malignancies. They target the immune system (mostly T cells) and can provide durable responses (greater than 10 years) in patients with objective responses. Despite this, the majority of patients fail to respond because of primary resistance (innate non-response) or acquired forms of resistance (initial response but then progression) (P. Sharma, Hu-Lieskovan, Wargo, & Ribas, 2017), and therefore there is an unmet need for improving these therapies to broaden responses amongst patients. For a long time (and in many respects still is the case today), the exact mechanisms of response to anti-PD-1 and anti-CTLA-4 checkpoint inhibition, as well as the exact immune cell populations targeted by these therapies, have been poorly understood. However, this is critical for understanding why some patient tumors respond and others fail. In addition to mechanisms of response, a greater understanding of the mechanisms of resistance, both tumor intrinsic and extrinsic, are essential to improving the efficacy of current immunotherapies. In this part of the review, we will examine the developments in our understanding of the immune cell targets of immunotherapy as well as the various mechanisms by which T cell immunity in the tumor microenvironment (TME) may be supressed to reduce immunotherapy efficacy.

Immune cell targets of anti-PD-1 and anti-CTLA-4 immunotherapy

Anti-PD-1 immunotherapy: Whilst many immune phenotypes have been associated with response to anti-PD-1 immunotherapy, including macrophages (Gordon et al., 2017), monocytes (Krieg et al., 2018), and NK cells (H. Lee et al., 2019), it is widely accepted that the predominant target are tumor specific CD8 T cells. An early and pivotal study investigating biomarkers of response and resistance to anti-PD-1 therapy in melanoma showed that high CD8 T cell densities, but not CD4 T cell densities, in pre-treatment biopsies correlated with response (Tumeh et al., 2014). Specifically, proliferation of intratumoral CD8 T cells correlated most significantly with radiographic reduction in tumor size (Tumeh et al., 2014). Others have

described immune gene signatures for CD8 cytolytic activity and upregulation of T cell associated chemokine/cytokines as predictors of response using transcriptomic analysis in patient biopsies (Litchfield et al., 2021; Rooney, Shukla, Wu, Getz, & Hacohen, 2015). Whilst a significant step forward in our understanding of anti-PD-1 targets, CD8 T cells themselves are a heterogenous population, and it is now understood that many CD8 T cell populations infiltrating tumors can be "bystander populations" (specific for epitopes unrelated to cancer) that have no functional relevance on anti-tumor immunity (Simoni et al., 2018). Because CD8 T cells are not all the same, subsequent studies tried to examine the exact CD8 T cell phenotypes linked with durable responses. CXCR5+ CD8+ T cells were first defined as the subset of CD8 T cells providing the proliferative burst after anti-PD-1 immunotherapy in a chronic viral infectious model (Im et al., 2016). CXCR5+ CD8+ T cells were enriched for genes associated with memory, self-renewal, mitochondrial fatty acid β-oxidation, and survival compared to CXCR5- CD8+ T cells (Im et al., 2016). Additionally, these cells expressed higher levels of costimulatory (CD28 and ICOS) but lower coinhibitory (TIM-3, LAG-3) receptors. This is relevant given other studies have shown that CD28 mediated co-stimulation, and not just the T cell receptor (TCR) activation, may be a target of PD-1 mediated T cell suppression (Hui et al., 2017). Later, a study utilizing single cell mass cytometry to dynamically examine immune infiltrates in melanoma specimens demonstrated that the expansion of exhausted-like CD8 T cells was primarily associated with response to anti-PD-1 (Wei et al., 2017). These CD8 T cells expressed inhibitory receptors (LAG-3, TIM-3, PD-1) but retained function. Other studies corroborated these findings and suggested that the presence of Eomes+ memory CD8 T cells in tumors might also be associated with anti-PD-1 response (Gide et al., 2019). Recently, TCF7+ CD8+ T cells were identified from single-cell sequencing in metastatic melanoma patient tumors and were shown to correlate strongly with clinical outcome to anti-PD-1 monotherapy as well as combination anti-PD-1 + anti-CTLA-4 immunotherapy (Sade-Feldman et al., 2018). TCF7+ CD8+ T cells expressed genes linked to memory, activation, and cell survival, while TCF7- CD8+ T cells were enriched for genes associated with an exhausted-like signature (Sade-Feldman et al., 2018). *TCF7* is an important transcription factor involved in the Wnt-signalling pathway and is associated with a self-renewal transcriptional program vital for stem cell-like memory cells (Gattinoni et al., 2011). Current work is ongoing to examine in greater detail the contribution and functional relevance of each of these CD8 immune subsets.
Anti-CTLA-4 immunotherapy: The immune cell targets of anti-CTLA-4 immunotherapy is less clear. While the presence of CD8+ memory T cells have been implicated in response to anti-CTLA-4 (Tietze et al., 2017), it is more likely that CD4 T cell subsets also play a key role. Early studies demonstrated that patients treated with ipilimumab had higher ICOS+ CD4 T cells in on-treatment biopsies of tumor and peripheral blood compared to baseline (Liakou et al., 2008). This led to an increase in the effector T cell to Treg ratio, irrespective of treatment outcome (Liakou et al., 2008). Later studies suggested that these ICOS+ CD4 T cells had a TH-1 phenotype based on gene expression analysis and their expression of IFN-gamma (Binnewies et al., 2019; Ji et al., 2012; Wei et al., 2017). These ICOS+ TH-1like CD4 T cells were observed to expand in response to anti-CTLA-4 treatment in both murine models and melanoma patient samples (Binnewies et al., 2019; Wei et al., 2017), suggesting that they might indeed be a target. However, evidence to support their predictive value in treatment outcome has been far more limited. T-regulatory cells express high levels of CTLA-4, and there is strong evidence in murine models that anti-CTLA-4 depletes T-regs in the tumor microenvironment (Binnewies et al., 2019; Buchan et al., 2018; Simpson et al., 2013). Simpson and colleagues demonstrated that the depletion of Tregs occurred through the recognition of the Fc domain of the CTLA-4 antibody by Fc gamma receptor (CD16) positive macrophages (Simpson et al., 2013). These CD16+ macrophages used this recognition to perform receptor mediated phagocytosis of Treg cells. More recent studies have corroborated these findings (Binnewies et al., 2019; Buchan et

al., 2018), though T-regulatory depletion is yet to be identified as a mechanism of action for anti-CTLA-4 in humans. Despite this, an analysis of human melanoma tumours from patients treated with ipilimumab showed that amongst T cells, B cells and NK cells, CD16 and CD68 were the only markers significantly higher in responding cutaneous metastases compared to non-responders, suggesting that macrophages may play a role in anti-CTLA-4 response (Balatoni et al., 2018).

T cell persistence a hallmark of response to immunotherapy

While there are various CD8 T cell phenotypes that have been implicated as immunotherapy targets, one characteristic that is common to all is their programmed innate capacity to persist in the tumor microenvironment. This is likely an important feature that enables them to adapt to the harsh conditions of the TME and yet retain functional and proliferative potential during immunotherapy response. Indeed, the exhausted-like CD8 T cells described by Wei and colleagues (the only phenotype to expanded in response to anti-PD-1 therapy) as well as the Eomes+ CD8 T cells described by Gide and colleagues, share many features with the tumor resident/tissue resident CD8 T cell population, which (as has been reviewed) as a population, is known to persist in tissues for years and exhibit remarkable longevity and recall functions. Exhausted-like CD8 T cells and Eomes+ CD8 T cells expressed memory markers (CD45RO+, CCR7/CD62L-), upregulation of inhibitory markers (PD-1, LAG-3, TIM-3, TIGIT), and markers involved in tissue retention (CD69 and CD103) (Gide et al., 2019; Wei et al., 2017), all of which are common to the tumor resident/tissue resident core signature. It is known that the effectiveness of checkpoint blockade may be independent of circulating T cells (Spranger et al., 2014) and more recent studies now confirm the potential role of tumor resident CD8+ T cells in immunotherapy response (discussed extensively in chapter 1 of this thesis). Besides their molecular mechanisms of tissue retention, it is not clear what exactly enables tumor resident CD8 T cells to persist in the TME, although they likely rely on alternate energy sources (exogenous lipids vs glucose) that are probably more abundant in the TME and associated with metabolic programming suited for longevity vs short-lived effector cells (O'Neill et al., 2016; Pan et al., 2017). Indeed, there is evidence to suggest that PD-1 blockade reduces lipid uptake by tumor cells whilst simultaneously increasing lipid uptake via Fabp4/Fabp5 on tumor resident CD8 T cells, providing a metabolic edge that may translate to improved function (Lin et al., 2020). One interesting feature of tumor resident T cells is that their generation at one tumor site may not necessarily restrict their protective features and long-term surveillance properties at other sites. Indeed, a recent study showed that resident memory T cells in tumors were associated with the presence of similar tumor resident T cell clones at tumor-distant sites (Christian et al., 2021), providing a rationale for the sustained clinical responses observed with immune checkpoint inhibitors. In addition to the exhausted-like resident phenotype, TCF7+ CD8 T cells and CXCR5+ CD8 T cells also share properties that are conducive to persistence in the TME. For example, both subsets were described as "stem cell-like" CD8 T cell populations with enhanced self-renewal and proliferative capacity (Im et al., 2016; Sade-Feldman et al., 2018). These CD8 T cells would therefore most likely be stem cell memory T cells (TSCM), which are known to harbour one of the most undifferentiated T cell states. Differentiation status, or how far a T cell has departed from its naïve state, is a major factor defining T cell functionality and persistence. TSCM and TCM, being relatively undifferentiated, have strong proliferative and self-renewal capacity as well as multipotency relative to TEM, yet do not show the same capacity as TEM to produce robust quantities of effector cytokines (IFN-gamma) upon antigen rechallenge (Sallusto et al., 1999)(Gattinoni et al., 2012). The mechanism for this increased proliferative and self-renewal capacity may be

due to telomere lengths. Indeed, as T cells progress further along the differentiation pathway, the lengths of their telomeres (important for maintaining DNA integrity during cellular replication) become shorter (Sallusto et al., 1999; Weng, Levine, June, & Hodes, 1995), severely limiting their capacity for long-term self-renewal. Similar to tumor resident CD8 T cells, it appears that TCF7+/ CXCR5+ CD8 T cells mitochondrial fatty acid oxidation rather than glucose metabolism (Im et al., 2016; Sade-Feldman et al., 2018), another characteristic conducive to long-term persistence.

Resistance mechanisms to immunotherapy

Crucially, resistance to checkpoint blockade immunotherapy involves the absence or suppression of T cells in the tumor microenvironment (Tumeh et al., 2014). Indeed, there are various mechanisms, both intrinsic to the tumor (de novo in tumor cells) and extrinsic (tumor microenvironment) that have been associated with the exclusion or suppression of T cells in tumors.

Dysregulation of growth and self-renewal signals

Cytokines represent important growth factors that are essential for immune cell differentiation, proliferation, and persistence. It is becoming clear that the cytokine milieu of the tumor microenvironment, including the dysregulation of normal cytokine homeostasis, can have profound effects on the immune landscape and the persistence of particular immune cell subsets in the TME. ***TGF-beta*** Recently, the aberrant expression of transforming growth factor- beta (TGF-beta) in tumors has been linked to T cell exclusion and resistance to checkpoint blockade-based immunotherapies (Mariathasan et al., 2018; Tauriello et al., 2018). In preclinical models of colorectal cancer, TGF-beta in the stroma was associated with reduced TIL infiltration and higher incidences of tumor metastasis (Tauriello et al., 2018), whilst in urothelial cancer, increased TGF-beta signalling in patient fibroblasts was associated with poorer response to immunotherapy (Mariathasan, 2018). In both studies, blocking TGF-beta signalling enhanced T cell infiltration into tumors, increased tumor susceptibility to PD-1/PD-L1 based immunotherapy, delayed metastasis, and increased survival. In line with this, others have shown that surface-bound TGF-beta on T-reg cells can supress intratumoral effector T cells and that blockade of surface-bound TGF-beta can abrogate these effects (Budhu et al., 2017). Research to identify the exact mechanisms behind TGF-beta mediated exclusion of TILs is still ongoing, however there is evidence to suggest that TGF-beta may promote a pro-tumor environment by either polarising Th1 CD4 helper T cells to a suppressive phenotype, inducing macrophages to an M2 phenotype by dampening aerobic glycolysis and promoting OXPHOS metabolism, or by supporting angiogenesis (Stuelten & Zhang, 2021). Interestingly, and somewhat paradoxically, TGF-beta in tumors also appears to be important for the induction and enrichment of CD103+ CD8+ tumor resident T cells. An early study in the context of graft-versus-host disease (GVHD) showed that CD8 T cells deficient in TGF-beta receptor were unable to express CD103 and were less efficiently retained (El-Asady et al., 2005). A more recent study found that epithelial tumor islets rich in TGF-beta were enriched for CD103+ tumor resident CD8 T cells (Boutet et al., 2016). Thus, while TGF-beta severely impacts the number and effector functions of CD8 T cells in tumors, it may also play an important role in the development of certain CD8 T cell subtypes associated with persistence in the tumor microenvironment. ***IL-15/IL-2*** As previously mentioned, IL-15 and IL-2 represent cytokines indispensable for self-renewal, proliferation and effector functions in memory and effector T cells, respectively. In the context of the tumor microenvironment these cytokines also have an

effect on the magnitude and type of T cell anti-tumor responses. For example, deletions in IL-15 of patient-derived colorectal tumors have been shown to exhibit decreased IL-15 protein expression and lower densities of proliferating lymphocytes, including T cells, translating into higher risk of tumor recurrence and reduced patient survival (Mlecnik et al., 2014). Others have shown that IL15/IL15alpha receptor complexes are able to increase T cell infiltration into tumors, particularly those of a memory phenotype, and work synergistically with anti-PD-1 to rescue exhausted T cell phenotypes (Desbois et al., 2016). To date however, IL-15 targeting anti-cancer monotherapies have shown little clinical efficacy, and others suggest that IL-15 may expand unrelated intratumoral memory CD8 T cell populations with little anti-tumor capacity (A. L. Doedens et al., 2016). IL-15 is also known to promote FAO metabolism in T cells over glycolysis-based metabolism (van der Windt et al., 2012). Given that glycolysis is essential for effector cytokine production in T cells (Chang et al., 2013), it is possible that IL-15 induces T cell fates suited for persistence in the TME at the expense of some functionality. Indeed, inhibition of glycolysis in CD8 T cells promoted a cell fate characteristic of memory T cells with better survival properties and longevity post adoptive transfer yet with decreased expression of effector molecules granzyme B and perforin relative to wild type CD8 T cells (Sukumar et al., 2013). In line with this, IL-2 therapies, which promote T cell glycolysis, have shown greater clinical efficacy, particularly in melanoma patients, and efforts are currently underway to improve them in the context of checkpoint inhibitors. A recent study demonstrated that IL-2 complexes in murine models could work synergistically with anti-PD-1 therapy to increase survival and CD8 infiltration in tumors compared to PD-1 monotherapy alone (Caudana et al., 2019). One of the possible limitations for IL-2 therapy is the fact that IL-2 itself, while promoting T cells with heightened effector functions, may push T cells to a terminally differentiated state not conducive to longevity in the TME (Kishton, Sukumar, & Restifo, 2017; Sukumar et al., 2013). This highlights the need for cytokine therapeutics which promote both longevity of CD8 T cell responses and effector functions. Another limitation to IL-2 therapy is its ability to promote T-reg proliferation and function via the high affinity IL-2 subunit receptor, CD25 (Furtado, De Curotto Lafaille, Kutchukhidze, & Lafaille, 2002). Indeed, regulatory T cells can outcompete effector T cells for IL-2 resulting in higher Treg:CD8 T cell ratios (Vignali, Collison, & Workman, 2008). In line with this, patients treated with bolus high dose IL-2 therapy or IL-2 based adoptive T cell therapy show expansion in T-reg populations with potent suppressive function (Ahmadzadeh & Rosenberg, 2006; Brandenburg et al., 2008; Sim et al., 2014). Future IL-2 based therapies will need to overcome the challenge of off target expansion and persistence of pro-tumor immune cells in order to provide increased efficacy to patients. In line with this, preclinical models have shown that IL-2 complexes in combination with anti-CTLA-4 therapy, but not anti-PD-1 therapy, can increase CD8: Treg ratios in the tumor (Caudana et al., 2019) leading to the enhancement of other anti-tumor immune cells such as NK cells. ***IL-10*** is a pleotropic cytokine that has traditionally been associated with immunosuppression (Moore, O'Garra, Malefyt, Vieira, & Mosmann, 1993), mainly because of its importance in regulatory T cell function and TH2-like responses (Dennis, Blatner, Gounari, & Khazaie, 2013; Vignali et al., 2008). In some cancers, IL-10 expression is correlated with tumor aggressiveness, metastasis, and poor patient clinical outcomes (Sung et al., 2013; R. Wang et al., 2011). Recently it was shown that T-reg derived IL-10 and IL-35 in the TME work together to drive the expression of inhibitory receptors, exhausted-like states, supressed anti-tumor functions and limited memory differentiation of intratumoral T cells (Sawant et al., 2019). Indeed, loss of T-reg derived IL-10 and IL-35 resulted in downregulation of an exhaustion gene signature and upregulation of a memory associated transcriptional profile. Although both IL-10 and IL-35 were important in this context, IL-35 appeared to be more critical for driving T cells away from memory states than IL-10, suggesting that IL-10 alone does not inhibit formation in the TME. Whilst IL-10 has been implicated as a suppressor of

TIL function, there is now a growing body of evidence to suggest that IL-10 may be necessary for proper anti-tumor CD8 T cell responses, and perhaps even important for long-term persistence of tumor specific CD8 T cells. For example, IL-10 KO in tumor bearing mice significantly reduces IFN-gamma -mediated immune surveillance, while IL-10 over-expression in tumors show higher CD8 densities, more IFN-gamma, and higher MHC expression, translating into repressed tumor growth and better survival (Mumm et al., 2011). Others have shown that inhibition of complement-dependent downregulation of CD8 T cell-derived IL-10 results in CD8 TILs with increased IL-10 expression and effector functions (Y. Wang et al., 2016).

Pro tumor immune cells

The presence of myeloid-derived suppressor cells (MDSCs), T-regulatory cells, and tumor associated macrophages (TAMs) in the tumor microenvironment are poor prognostic features in many cancer types and are associated with resistance to anti-PD-1 and anti-CTLA-4 checkpoint immunotherapy (T. Li et al., 2021; Saleh & Elkord, 2019). Indeed, early reports in melanoma showed that non-responders to ipilimumab had higher densities of MDSCs in the blood (Meyer et al., 2014). Later, it was demonstrated that combination checkpoint blockade with epigenetic modulators could rescue mice refractory to anti-PD-1, with the mechanism of action being through the depletion of MDSCs (Kim et al., 2014). Interestingly, in-vivo studies tracking the fate of anti-PD-1 monoclonal antibodies showed that their engagement with PD-1+ TILs was transient due to their capture by TAMs, limiting the therapeutic efficacy (Arlauckas et al., 2017). In terms of T-regs, residual and highly proliferative TIM-3 expressing T-regs were found in orthotropic models of head and neck squamous cell carcinomas refractory to anti-PD-1, but CD25 mediated Treg depletion induced sensitivity to anti-PD-1 and tumor rejection (Oweida et al., 2018). There are various ways in which these immune subsets have been linked to resistance, however all involve the dampening and suppression of effector T cells in the tumor microenvironment. For example, upregulation of checkpoint ligands on MDSCs and TAMs (PD-L1) or their deactivation of tyrosine kinases (LCK) involved in T cell activation have been proposed as possible mechanisms (Ballbach et al., 2017; Feng et al., 2018). MDSCs have also been shown to recruit other immunosuppressive cells, particularly Tregs that directly inhibit T cell function, through the excretion of IL-10 and TGF-beta cytokines (Huang et al., 2006; Pal et al., 2019; Tomić et al., 2019). Another significant pathway by which MDSCs and TAMs regulate T cell activity is through the modulation of metabolites in the TME. For example, tryptophan can be depleted in tumors by the activity of indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO); enzymes that are expressed in a variety of tumor types by tumor cells/ MDSCs/TAMs, and which convert tryptophan into kynurenine via the kynurenine pathway (T. Li et al., 2021; Pilotte et al., 2012; Uyttenhove et al., 2003). T cell activation and T cell cycle progression are known to be restrained in conditions of limited tryptophan, making them susceptible to apoptosis (G. K. Lee et al., 2002; Munn et al., 1999). While it is evident that tryptophan depletion adversely affects anti-tumor T cell functionality, other immune cells, including T-regs and myeloid derived suppressive cells, can be enhanced in similar conditions. For instance, IDO expression has been associated with higher densities of Treg cells in tumors (Yu et al., 2013, 2011), and tryptophan depletion can induce FOXP3 expression in T cells (A. Curti et al., 2007; Yu et al., 2013, 2011). Additionally, IDO expression in MDSC's was found to be required for MDSCs' immunosuppressive activity on T cells (Yu et al., 2013). The build-up of kynurenine from tryptophan catabolism activates the aryl hydrocarbon receptor (AHR), which upon activation has potent immunosuppressive effects on anti-tumor immunity (Opitz et al., 2011), and leads to AHR-dependent Treg generation (Mezrich et al., 2010). In addition to modulating tryptophan metabolism pathway, MDSCs

and TAMs can directly modulate arginine levels in the TME through expression of arginase-1 and the excretion of iNOS (Rodriguez et al., 2004), which is known to supresses T cell activation, proliferation, and survival (Fletcher et al., 2015; Rodriguez, Quiceno, & Ochoa, 2007; Rodriguez et al., 2004). This suppression is thought to occur in part because low arginine inhibits the re-expression of the CD3-zeta chain in stimulated T cells (Rodriguez et al., 2004).

Hypoxia

Malignant cells proliferate at uncontrolled rates and can outgrow their nutrient supplies, often resulting in underdeveloped vasculature and regions of the TME with limited oxygen. Hypoxia is a poor prognosis factor in many cancer types and is also emerging as a mechanism of resistance to cancer therapies, including immune based therapies (Jing et al., 2019). In the TME, hypoxia has been associated with reduced TIL infiltration and impaired effector function (Hatfield et al., 2015; Jayaprakash et al., 2018; Scharping, Menk, Whetstone, Zeng, & Delgoffe, 2017). Indeed, oxygenation of tumors in preclinical models by drug or respiratory hyperoxia increased the CD8: TIL ratio, IFN-gamma/TNF-alpha production and proliferative capacity of CTLs, and sensitivity to checkpoint blockade, culminating in tumor regression and increased survival (Hatfield et al., 2015; Jayaprakash et al., 2018; Scharping et al., 2017). It is not exactly clear how limited oxygen in the TME leads to supressed CTL function, given that hypoxia actually improves effector cell and effector memory cell function in isolation (Xu et al., 2016). It is possible that hypoxic conditions preferentially promote the survival and persistence of immunosuppressive immune cells, which in turn act to dampen cytotoxic cells in the tumor. In line with, hypoxic zones of tumors have been shown to preferentially attract myeloid derived suppressor cells (Corzo et al., 2010), tumor-associated macrophages (Andrew L. Doedens et al., 2010) and regulatory T cells (Clambey et al., 2012), and promote the upregulation of PD-L1 on MDSCs, TAMs and tumor cells (Barsoum, Smallwood, Siemens, & Graham, 2014; Noman et al., 2014). Central to these hypoxic induced changes on immune cells is the transcription factor, hypoxia-inducible factor 1-alpha (HIF1-alpha), a key transcription factor involved in energy metabolism and cell survival (Papandreou, Cairns, Fontana, Lim, & Denko, 2006). Indeed, hypoxia induced HIF1-alpha selectively enhances FoxP3 expression in T cells and promotes Treg abundance (Clambey et al., 2012), indicating that hypoxic environments, though harsh, may permit and even promote the survival of these cells. Recently, greater nuance for the role of hypoxia induced HIF1-alpha in T-regs has been elucidated. Miska and colleagues showed that T-regs with functional HIF1-alpha in the presence of hypoxia utilized glycolysis pathways for better migratory capabilities in tumors at the expense of some suppressive functions (Miska et al., 2019). Conversely, ablation of HIF1-alpha in Tregs in the presence of hypoxia enhanced immunosuppression at the expense of tumor infiltration, leading to better survival in a murine model of glioma. Somewhat paradoxically, stabilization of HIF1-alpha is also essential for driving glycolytic metabolism, infiltration, and effector function of CD8+ T cells in tumors (Andrew L. Doedens et al., 2013; Palazon et al., 2017). Additionally, metabolites regulated by HIF1-alpha dependent mechanisms, have also been implicated in determining CD8 T cell fate, including cells with a memory phenotype, increased capacity to proliferate and persist in vivo, and enhanced anti-tumor functions (Tyrakis et al., 2016). Together, this highlights the complexity of hypoxia induced changes on function and survival capabilities of both anti-tumor and pro-tumor immune cells in the TME.

Acidosis

Acidity is a common feature of many tumors and represents another challenge for the immune system, both in terms of persistence and function. Malignant cells undergoing aerobic glycolysis for their metabolism, convert pyruvate to lactate via lactate dehydrogenase (LDH). The accumulation of lactate and other acidic metabolites results in a lower intracellular pH and is detrimental to the cell if it is not exported to the extracellular environment (Chiche et al., 2009). Tumor cells in particular, are equipped with a number of transporters/ enzymes that assist in the process of removing acidic derivatives to the extracellular space (Chiche et al., 2009), and over time, contribute substantially to a lower physiological pH in the tumor microenvironment. Immune cells are adversely affected by low pH (Angelin et al., 2017; Brand et al., 2016; Colegio et al., 2014; Dietl et al., 2010; Fischer et al., 2007; Husain, Huang, Seth, & Sukhatme, 2013). Indeed, increased lactic acid production by tumor cells has been shown to impair proliferation, cytokine production, and cytolytic activity of CD8 T cells and NK cells (Brand et al., 2016; Fischer et al., 2007; Husain et al., 2013). Reducing lactate levels by silencing LDHA in melanoma tumors improved CD8/NK cell infiltration and effector functions (Brand et al., 2016). In preclinical models, others have shown that acid-neutralizing bicarbonate in combination with checkpoint or adoptive T cell immunotherapy promoted CD8+ T cell infiltration and anti-tumor responses (Pilon-Thomas et al., 2016), suggesting that therapies capable of normalizing the pH of the TME may bolster the efficacy of current immunotherapies. Whilst acidity is detrimental to the function of many immune subsets, there is also evidence to suggest that certain suppressive immune phenotypes may be more apt to function in acidic surroundings (Angelin et al., 2017; Colegio et al., 2014; Husain et al., 2013). In line with this, tumor-derived lactic acid can suppress glycolysis in pro-inflammatory myeloid cells (Dietl et al., 2010) and promote the polarization of TAMs to an immunosuppressive M2 phenotype (Bohn et al., 2018; Colegio et al., 2014; Dietl et al., 2010). Polarized M2 macrophages upregulate ariginase-1 and other suppressive factors in response to lactic acid (Colegio et al., 2014), while reduced acidity in the TME results in fewer myeloid derived suppressor cells compared to tumors with higher acidity (Husain et al., 2013). Furthermore, regulatory T cells have also been shown to have a natural propensity to persist in acidic environments with little compromise on function (Angelin et al., 2017). The mechanistic basis for this has been linked to metabolism states. Angelin and colleagues found that lactic acid impaired effector T cells utilizing aerobic glycolysis through LDH-mediated NAD depletion, while FoxP3 expression in regulatory T cells supressed myc dependent glycolysis and induced mitochondrial oxidative phosphorylation and higher NAD:NADH ratios, together culminating in resistance towards lactate-mediated suppression of T cell function (Angelin et al., 2017). These findings suggest that immune cells heavily reliant on aerobic glycolysis become functionally impaired in conditions of low glucose and high acidity, perhaps in an effort to persist, while immune cells undergoing mitochondrial based metabolism hold a metabolic state that preferentiates their survival and function in similar environments. It remains to be determined whether CD8 memory subsets undergoing FAO also utilize similar mechanisms to regulatory T cells to help them persist and function in the challenging environment of the TME.

Wnt/ β-catenin signalling pathway dysregulation

The dysregulation of β-catenin, an important transcription factor in the WNT signalling pathway, has been associated with early events in carcinogenesis and in cancer progression within various cancer types (Y. Zhang & Wang, 2020). This is likely due to the fact that the

WNT signalling pathway is involved in cellular proliferation, differentiation, and stem cell renewal (Y. Zhang & Wang, 2020). Under normal regulation, β-catenin levels in the cytosol are degraded by proteosomes through phosphorylation and ubiquitination events controlled by the β-catenin tertiary complex (comprised of AXIN, CK1-alpha, and GSK3- β). However, dysregulation of any of these components can lead to stabilization of β-catenin and subsequent constitutive transcription of genes involved in growth and cell cycle progression. In melanoma, molecular analysis of metastatic melanoma patient samples revealed upregulation in the Wnt/ β-catenin signalling pathway, which was associated with T cell exclusion in the tumor microenvironment (Spranger, Bao, & Gajewski, 2015). Interestingly, forced upregulation of this pathway was associated with resistance to PD-L1/anti-CTLA-4 monoclonal antibody therapy in murine models (Spranger et al., 2015). Later reports identified upregulation of the Wnt/ β-catenin signalling pathway in patient melanoma progressing lesions after combination anti-PD-1 + anti-CTLA-4, highlighting it as a mechanism of resistance to immunotherapy (Trujillo et al., 2019). It is likely that this pathway is important in other cancers with respect to resistance given recent findings that Wnt/ β-catenin signalling is enriched in non-T cell inflamed tumors (Luke, Bao, Sweis, Spranger, & Gajewski, 2019), and that intratumoral T cell densities are associated with response to checkpoint blockade.

Upregulation of alternative checkpoint receptors

T cells require at least two signals to become fully activated and primed for effector functions. Firstly, engagement between the T-cell receptor (TCR) and loaded peptide within the major histocompatibility complex (MHC) molecules I or II, and secondly, along with the right cytokine milieu, a costimulatory signal between costimulatory receptors on T cells and their ligands on antigen presenting cells (APCs) (Waldman et al., 2020). One of the first and most important costimulatory signals is provided by CD28 on T cells and B7-1/B7-2 molecules on APCs. However, many other costimulatory molecules exist, including ICOS, OX40, CD137 and GITR, which also play a role in controlling T cell differentiation and fate. Opposing T cell activation and costimulatory signalling, checkpoint interactions between coinhibitory receptors on T cells and their ligands serve to dampen T cell responses to prevent excessive T cell activation, including the potential dangers of autoimmunity. Many checkpoint coinhibitory receptors have been described including PD-1, CTLA-4, LAG3, TIGIT, VISTA, TIM-3, among others. CTLA-4 expression on T cells competes with CD28 to negatively regulate TCR activation (Krummel & Allison, 1995), and has been shown to do so by capturing the shared ligands, CD80 and CD86, in a process of trans-endocytosis (Qureshi et al., 2011). In the TME, PD-1 expression on T cells and their ligation with PD-L1/PD-L2 on tumor cells/APCs is a major axis exploited by tumor cells to dampen anti-tumor immune responses in many solid malignancies, but can be reversed upon anti-PD-1 checkpoint blockade (P. Sharma et al., 2017). Early reports in mice and in patients refractory to anti-PD-1 therapy detailed the upregulation of alternative inhibitory checkpoint receptors (TIM-3, LAG-3, CD137) in progressing lesions compared to baseline (Koyama et al., 2016; Williams et al., 2017). VISTA expression was upregulated in the immune infiltrate of prostate tumors after two doses of anti-CTLA-4 therapy (J. Gao et al., 2017) and in melanoma patient tumors refractory to anti-PD-1 therapy (Kakavand et al., 2017). Recently RNA transcriptomic analysis of melanoma tumor patient biopsies PRE and EDT (early during treatment) in patients refractory to anti-PD-1 and combination anti-PD-1 + anti-CTLA-4 therapy also revealed high expression of alternative checkpoint receptors (Gide et al., 2019). These observations have led to the “alternative checkpoint hypothesis”, whereby within some patient tumors other checkpoint inhibitory receptors serve to dampen T-cell responses and contribute to severe T-cell exhaustion, such that anti-PD-1 alone is not

enough to rescue T-cell functionality (P. Sharma et al., 2017). Indeed, there is both pre-clinical and clinical evidence to support the notion that the upregulation of alternative checkpoint receptors is a significant mechanism of resistance to current immunotherapies. In murine models of mice refractory to anti-PD-1 or anti-CTLA-4 therapy, the additional blockade of alternative checkpoint receptors (LAG-3, TIGIT, TIM-3) has been shown to overcome resistance and lead to tumor rejection in various cancer types (Johnston et al., 2014; Koyama et al., 2016; Sade-Feldman et al., 2018; Sakuishi et al., 2010; Woo et al., 2012). These studies demonstrated that blockade of alternative checkpoints led to an increase in T cell infiltration into the tumor and improved effector T function, and in some cases, this was achieved through the depletion of pro-tumor immune populations like T-regs or myeloid suppressor cells (Buchan et al., 2018; Oweida et al., 2018). In humans, combination anti-CTLA-4 and anti-PD-1 yields the highest efficacy of objective response rates in melanoma and other cancers, and recently the addition of anti-LAG-3 with anti-PD-1 was shown to improve recurrence free survival in a phase 3 melanoma trial (Lipson et al., 2021). Currently there is a plethora of clinical trials testing the combination of various monoclonal antibodies targeting alternative checkpoint receptors and it remains to be seen how significant the clinical benefit is with these approaches. One challenge for the field will be understanding the expression profiles of these receptors as it relates to response, given little is known.

Low tumor mutation burden

One tumor intrinsic property that can result in resistance to anti-PD-1 and anti-CTLA-4 checkpoint blockade is the lack of tumor mutation burden (TMB), which refers to the total number of single nucleotide variants (SNVs) arising from somatic mutations in tumor DNA. Melanoma and lung cancer, which have significant SNVs because of UV and smoking-induced damage respectively, are some of the highest TMB expressing cancers (Alexandrov, Nik-zainal, Wedge, & Aparicio, 2013). Other common tumor types like breast, prostate, and pancreas cancers have relatively low TMB (Alexandrov et al., 2013). A seminal publication in the New England Journal of Medicine eloquently demonstrated that anti-PD-1 clinical objective response rates closely correlated with TMB across 27 cancer types (Yarchoan, Hopkins, & Jaffee, 2017), clearly showing that low TMB cancers were less responsive to checkpoint blockade. Within cancer types themselves, patients with relatively low TMB, even in high TMB tumors like melanoma, are known to be associated with non-response to anti-CTLA-4 and anti-PD-1 immunotherapies (Newell et al., 2021; Van Allen et al., 2015), whereas relatively high TMB has been reported as one of the strongest predictors of response (Litchfield et al., 2021). However, recent reports suggest that TMB is not predictive of response or resistance in all tumor types (McGrail et al., 2021), suggesting that it may not be universally applicable as a biomarker of resistance. It is unclear exactly why low TMB in some cancer types are associated with non-response. The most common belief is that because TMB closely correlates with the number of available neoantigens in the tumor microenvironment, tumors with low TMB (and therefore neoantigens), should theoretically have lower numbers of immune infiltrates, particularly T cells. While high TMB tumor types generally are well known to have relatively high immunogenicity (Alexandrov et al., 2013), evidence supporting this within patient tumors of the same cancer type is limited and remains controversial.

Defects in antigen presentation pathways and IFN-gamma signalling

Tumor specific CD8 T cells require the presentation of tumor peptides in the context of major histocompatibility complex class 1 (MHC-1). Tumor cells, like all cells in the human body (except at immune privileged sites) express MHC-1, and without this, CD8 T cells would not

be able to recognise tumor antigens and perform their cytotoxic functions. The formation of MHC-1 molecules and the loading of peptides onto MHC-1 is a complex pathway that involves many protein subunits (MHC-1 alpha chain and beta 2 microglobulin (β2m), proteasomes, transporters (TAP), and peptide loading proteins (Tapasin and Calreticulin) (Dhatchinamoorthy, Colbert, & Rock, 2021). Any defect in this pathway, including somatic mutations encoding for any of these components, can prevent proper antigen presentation and subsequent antigen recognition by CD8 T cells (D'Urso et al., 1991). In fact, loss of MHC-1 is a relatively common occurrence in various tumor types (one study documents 30% for melanoma patients resistant to ICI (Sade-Feldman et al., 2016)), probably because it is dispensable for proliferation and growth, and is usually associated with reduced T cell infiltration into tumors and poor outcomes (Dhatchinamoorthy et al., 2021). In the context of anti-PD1 and anti-CTLA-4 immunotherapies, MHC-1 loss is an important mechanism of resistance. Early reports exploring resistance pathways in melanoma patients who were refractory to anti-PD-1 found homozygous truncating mutations in B2M (Zaretsky et al., 2016), or loss of B2M expression (Restifo et al., 1996). A more recent study investigating melanoma biopsies at baseline and at time of progression with anti-PD-1 inhibitors, found that MHC-1 downregulation was a hallmark of resistance and that this downregulation was associated with TGF-beta upregulation in the tumor microenvironment (J. H. Lee et al., 2020). Other studies now show that dysregulation of the MHC-1 antigen presentation pathway is a mechanism of resistance to anti-CTLA-4 (Rodig et al., 2018) and combination anti-PD-1 + anti-CTLA-4 immunotherapies (Paulson et al., 2018), highlighting the importance of intact antigen presentation pathways as a necessary pre-requisite for immunotherapy response. Dysregulation of proper IFN-gamma signalling pathways in tumor cells also represents a significant mechanism of resistance. IFN-gamma produced by activated T cells in the tumor microenvironment improves antigen presentation by causing upregulation of MHC-1 on tumor cells but can also directly inhibit tumor growth and proliferation, as well as recruit other immune cells into the tumor (P. Sharma et al., 2017). Indeed, several studies exploring the transcriptomic differences between responding and non-responding tumor biopsies, across melanoma and other tumors, have shown that upregulation of IFN-gamma and IFN-gamma response genes in tumor cells is associated with immunotherapy response by amplifying the anti-tumor immune response (Ayers et al., 2017; Grasso et al., 2020). Because IFN-gamma signalling not only requires IFN-gamma receptors (IFNG1 and IFNG2) but also intracellular kinases (JAK1/2) and STAT proteins (signal transducer and activators of transcription) for transduction of cytokine signals, somatic mutations in any of these genes can cause impairment of the IFNG signalling pathway. Humans and mice with non-functional IFN-gamma receptor 1 have impaired rejection of tumors upon anti-CTLA-4 blockade (J. Gao et al., 2016). In patients resistant to anti-PD1 based immunotherapy, homozygous mutations in *JAK1* or *JAK2* were identified and resulted in lack of sensitivity to IFN-g but not type 1 interferons (Zaretsky et al., 2016). In another study, *JAK1* and *JAK2* mutations resulted in lack of PD-L1 expression on tumor cells upon IFN-g stimulation despite having a high tumor mutation burden (Shin et al., 2017), suggesting that anti-PD-1 would no longer have a therapeutic benefit by disrupting PD-1/PD-L1 ligand interactions. Recently, other regulators of the IFN-gamma pathway have been identified as resulting in melanoma insensitivity to IFN-g. High levels of LNK, a negative regulator of JAK-STAT was associated with enhanced tumor cell survival, growth, and insensitivity to IFN-g (Ding et al., 2019). Loss of function of ADAR1, an enzyme involved in the A-to-I editing of IFN-g RNA, was found to improve sensitivity of tumor cells to IFN-g through the enhanced recognition of double stranded RNA from ligands PKR and MDA5 (Ishizuka et al., 2019). In the context of anti-PD-1 immunotherapy, loss of ADAR1 was found to overcome resistance to anti-PD-1 by impaired antigen presentation of tumor cells (Ishizuka et al., 2019). It is important to recognise that both dysregulation of IFN-g signalling or MHC-

1 antigen presentation pathways can occur before or even during immunotherapy treatment, and thus early during treatment or progression biopsies may be needed to evaluate resistance to immunotherapy by these mechanisms.

Conclusion

To conclude this review, we have thoroughly discussed melanoma and the treatment landscape in the metastatic setting, highlighting the importance of checkpoint inhibition immunotherapies in transforming patient management and improving patient survival. We have discussed the importance of the immune system, particularly T cells, as it relates to the natural control of tumors as well as their central role in response to immunotherapy. We highlighted tissue resident T cells as a crucial T cell phenotype responsible for the control of diseases at epithelial sites and their emerging role in cancer and as targets to checkpoint blockade. Lastly, we reviewed our current understanding of the immune cell targets of immunotherapy as well as potential mechanisms of resistance contributing to dampened T cell immunity in the tumor microenvironment. A better understanding of these last two points is crucial in our efforts to improve immunotherapy such that a greater proportion of patients achieve long-term durable responses and cancer remission.

Chapter 1

$CD103^+$ Tumor-Resident $CD8^+$ T Cells Are Associated with Improved Survival in Immunotherapy-Naïve Melanoma Patients and Expand Significantly During Anti-PD-1 Treatment.

Chapter 1 - Introduction

The advent of anti-PD-1 and anti-CTLA-4 immunotherapy has indeed revolutionized the treatment of many solid malignancies, most notably metastatic melanoma. While this is an exciting time for patients, still, the majority of patients either fail to respond or succumb to acquired forms of resistance, highlighting the continued unmet need in this area. One of the challenges to improving the efficacy of current immunotherapies, is the fact that very little is known about how the exact mechanisms of response. While our understanding has certainly developed over the years, at the time that this next body of work was written, the mechanism of action for anti-PD-1 immunotherapy and the exact immune cell targets of this therapy were largely unknown. This is especially important because anti-PD-1 checkpoint blockade still remains the most efficacious single agent immunotherapy (Larkin et al., 2015). Early studies investigating human metastatic melanoma biopsies before and after anti-PD-1 treatment, suggested that cytotoxic CD8 T cells (but not CD4 T cells) were the primary target. This was based on the observation that higher CD8 T cell (but not CD4 T cells) densities were associated with response to anti-PD-1 therapy and increased in number early during treatment (Tumeh et al., 2014). While a significant step forward, CD8 tumor infiltrating lymphocytes (TILs) are a heterogenous population and are known to display different phenotypes. Thus, it was hypothesised that only a subset of CD8 TILs were actually responsible for the proliferative burst during immunotherapy treatment. Chronic virus infection models suggested that CD8 T cells expressing PD-1 as well as an "exhausted-like signature" (marked by less responsiveness to antigen), were the likely targets of anti-PD-1 therapy (Jin et al., 2010). Yet still, the exact phenotype of these CD8 T cells remained unknown. Additionally, there was debate about whether anti-PD-1 checkpoint blockade could truly rescue terminally differentiated CD8 T cells. In parallel to this work, investigations were beginning to take place into a new subset of memory T cells in various cancers. Tissue resident memory CD8 T cells, as we have described, were known to be critical in the setting of chronic viral infections and particularly important at controlling infectious diseases of epithelial origin (Woon et al., 2016). In non-small lung carcinomas and high-grade ovarian cancers, emerging evidence suggested that these resident CD8 T cells (termed tumor resident CD8 T cells) existed in tumors and that their presence was strongly associated with patient survival (Djenidi et al., 2015; John R. Webb et al., 2014). Still, whether or not this was true in other cancers was unknown. Furthermore, the factors involved in resident T cell formation in tumors, and how they differed phenotypically from their circulating counterparts in the tumor microenvironment, were also unknown. Yet, these were important concepts in understanding whether the efficacy of anti-PD-1 immunotherapy was dependent on a new influx of CD8 T cells from the circulation into the tumor, or whether anti-PD-1 acted through resident de novo CD8 T cell populations residing in the tumor. We hypothesized that melanoma, being a skin cancer of epithelial origin, would contain tumor resident CD8 T cells in the tumor microenvironment, and therefore would be an appropriate model in which to explore the significance of this CD8 T cell population in the treatment-naïve setting and within the context of anti-PD-1 checkpoint blockade.

The aims and objectives of the current study were as follows:

1. Do tumour resident CD8 T cells exist in melanoma?
2. If so, what is the phenotype of these cells in melanoma and how are they distinguished from their circulating counterparts?
3. Are tumour resident CD8 T cells associated with patient survival in melanoma?
4. Do tumour resident CD8 T cells expand in response to anti-PD-1 and do they present phenotypic markers that would provide a rationale for their being a target of anti-PD-1 immunotherapy?
5. What factors are involved in the recruitment/generation of resident CD8 T cells in melanoma tumours?

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Clinical Trials: Immunotherapy

Clinical Cancer Research

CD103$^+$ Tumor-Resident CD8$^+$ T Cells Are Associated with Improved Survival in Immunotherapy-Naïve Melanoma Patients and Expand Significantly During Anti–PD-1 Treatment

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Abstract

Purpose: Therapeutic blockade of immune checkpoints has revolutionized cancer treatment. Durable responses, however, occur in less than half of those treated, and efforts to improve treatment efficacy are confounded by a lack of understanding of the characteristics of the cells that initiate antitumor immune response.

Patients and Methods: We performed multiparameter flow cytometry and quantitative multiplex immunofluorescence staining on tumor specimens from immunotherapy naïve melanoma patients and longitudinal biopsy specimen obtained from patients undergoing anti PD 1 therapy.

Results: Increased numbers of CD69$^+$CD103$^+$ tumor resident CD8$^+$ T cells were associated with improved melanoma specific survival in immunotherapy naïve melanoma patients. Local IL15 expression levels strongly correlated with these tumor resident T cell numbers. The expression of several immune checkpoints including PD 1 and LAG3 was highly enriched in this subset, and these cells significantly expanded early during anti PD 1 immunotherapy.

Conclusions: Tumor resident CD8$^+$ T cell numbers are more prognostic than total CD8$^+$ T cells in metastatic melanoma. In addition, they are likely to initiate response to anti PD 1 and anti LAG 3 treatments. We propose that the immune profile of these cells prior to treatment could inform strategies for immune checkpoint blockade. *Clin Cancer Res; 24(13); 3036 45. ©2018 AACR.*

Introduction

Immune checkpoints have become a focus of intense research due to their ability to suppress T cell mediated immune responses (1, 2). In the context of cancer, these receptors facilitate escape from antitumor immune responses by tumor cells. In recent years, therapies that block these immune checkpoints, thereby enhancing antitumoral immune responses, have revolutionized the treatment of many solid malignancies. For example, treatment with anti programmed cell death 1 receptor (PD 1) antibodies in patients with advanced stage metastatic melanoma leads to objective and durable responses in approximately 40% of the patients (3 6). Nevertheless, it remains largely unknown why a large proportion of patients fail to respond. Although the mechanism of durable response is believed to be through reinvigoration of quiescent T cells (7), only a small subset of tumor infiltrating T cells (TILs) express these inhibitory receptors (8), and only a proportion of tumor cells express the ligands (9). The characteristics of the tumor infiltrating CD8$^+$ T cells that express immune checkpoints, how important they are for tumor control, and whether they respond to immunotherapy are unclear. Efforts to improve the efficacy of these treatments will inevitably require a greater understanding of this population of immune cells and the factors that regulate their trafficking and function.

Although the critical role of CD8$^+$ T cells in tumor control, particularly in melanoma, has long been recognized (8, 10), mechanistically, effective responses were viewed in the context of continuous recruitment of effector lymphocyte populations from the circulation. Recent evidence from animal models, however, clearly shows that a subset of antigen experienced CD8$^+$ T

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

It remains unknown whether all tumor infiltrating CD8$^+$ T cells are critical for initiating response to immunotherapy. Using rare human tumor biopsy specimens, we demonstrate that tumor resident cytotoxic T cells are not only associated with protective immunity against metastatic melanoma in treatment naïve patients, but also likely to be critical for response to anti PD 1 therapy. We show that the expression of several checkpoint receptors is enriched in these tumor resident CD8$^+$ T cells, and local IL15 could be a factor that regulates their numbers. Understanding the characteristics of antitumor CD8$^+$ T cell subsets, the factors that regulate their retention within human tumors, and mechanisms to reactivate them against tumors will enable the development and utili zation of better systemic treatments to improve outcomes for patients with cancer.

cells become resident within tissue environments, facilitated by local cytokines (11, 12). These resident T cells are not only distinct from their circulating counterparts, but have also been implicated in protective immune responses against many pathogens in both animal models (13 16) and humans (17 20). We therefore hypothesized that these resident T cells could be of great impor tance in cancers. Indeed, there is emerging evidence from different cancers, both in animal models and humans, that there are resident CD8$^+$ T cells within the tumor microenvironment (21 23). There is also some evidence that these resident cells could have a prognostic value in some human cancers (21, 22). However, the exact role of these resident T cells in tumor control and whether they respond to immunotherapy are unknown.

Tissue resident T cells are characterized by their constitutive surface expression of CD69 and CD103. CD69 has long been viewed as an early T cell activation marker; however, it is now clear that tissue resident T cells can constitutively express this receptor in the absence of T cell receptor stimulation. Whether all CD69$^+$ T cells in tissues are long term residents remains controversial. We recently showed in humans that CD103$^+$CD69$^+$ T cells were more reminiscent of tissue resident T cells described in mouse models than CD103 CD69$^+$ T cells as the former had the highest level of downregulation of the tissue exit signals (20). It is however possible that CD103 CD69$^+$ T cells are also retained in tissues for a limited period of time as an intermediate population. The development and persistence of tissue resident T cells depend on local cytokines such as IL15. We have shown that IL15 can not only induce CD69 expression, but also downmodulate tissue exit signals on circulating human CD8$^+$ T cells (20).

In this study, we have examined the prognostic value of tumor resident CD8$^+$ T cells in metastatic melanoma patients prior to immunotherapy and in patients undergoing anti PD 1 immu notherapy. Using multiparameter flow cytometry and multiplex immunofluorescence staining on patient samples selected from the Cancer Genome Atlas (TCGA) study, we have accurately quantified the numbers of tumor resident CD8$^+$ T cells and also determined their phenotype. In addition, we have also examined the local factors that regulate these tumor resident CD8$^+$ T cell numbers. Here, we show that tumor resident CD8$^+$ T cells are stronger predictors of melanoma specific patient survival than total CD8$^+$ T cells in patients who are immunotherapy naive. Our data also show that their numbers may be dependent on local IL15 expression levels. Importantly, tumor resident CD8$^+$ T cells were enriched in many immune checkpoints, and these cells significantly expanded early during anti PD 1 treatment. We therefore propose that immune profiling of these cells prior to immunotherapy could predict outcomes and help determine targets for treatments.

Patients and Methods

Melanoma samples

Archival formalin fixed and paraffin embedded tumor samples from patients treated at Melanoma Institute Australia who were also analyzed in TCGA project were used for immunofluorescence analysis. These samples were obtained as described previously (24). Briefly, patients diagnosed with either primary or metastatic cutaneous melanoma or metastatic melanoma of unknown pri mary treated at Melanoma Institute Australia were recruited to the TCGA study. These patients did not receive any prior systemic treatments, and the site from which the biopsy specimens were obtained had not been previously treated at any time with radiotherapy. Biopsy specimens from resected primary and/or metastatic melanomas were obtained from patients with appro priate informed consent and Institutional Review Board or ethics board approval. Biopsy specimens used for flow cytometry anal ysis were obtained from patients with stage III regional lymph node metastatic melanoma. Tumor dissociates were prepared by digesting with liberase (Sigma) at 37° for 1 hour. Thirteen biopsy specimens, including those from five good responders and eight poor responders, were obtained from patients who were treated with anti PD 1 inhibitor alone (pembrolizumab). Patient selec tion was dependent on the availability of multiple tumor biop sies, before treatment (PRE biopsy) and within 14 days of com mencing treatment (early during treatment, EDT biopsy) for 10 patients. Biopsies from three patients were obtained during treatment (Supplementary Table S1). Patients were classified as "responders" if they had durable stable disease (SD, greater than 6 months), partial response (PR), or complete response (CR) to anti PD 1 antibodies as their best response by RECIST or immune related response criteria (irRC) assessed at 6 to 12 weekly intervals. The study was conducted in accordance with the Nation al Health and Medical Research Council of Australia's National Statement on Ethical Conduct in Human Research. The study was undertaken with institutional Human Ethics Review Committee approval and patient's informed consent. Samples were acquired from the Melanoma Biospecimen Tissue Bank, which included patients from Royal Prince Alfred Hospital, Westmead Hospital, and Melanoma Institute Australia (Protocol No. X15 0454 and HREC/11/RPAH/444).

Quantitative multiplex immunofluorescence assays

All immunofluorescence staining was carried out on 4 μm thick sections using an Autostainer Plus (Dako, Agilent Technol ogies) with appropriate positive and negative controls. Opal Multiplex IHC Assay kit (PerkinElmer) was used as per the manufacturer's protocol. Briefly, paraffin embedded tissue sec tions were first deparaffinized, rehydrated, and treated with anti gen retrieval buffer. Antigen retrieval was performed by boiling in either basic (10 mmol/L Tris base, 1 mmol/L EDTA, 0.05% Tween 20, pH 9.0) or acidic (10 mmol/L sodium citrate, 0.05% Tween

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20, pH 6.0) antigen retrieval buffers. Sections were then incubated with 3% hydrogen peroxide for 10 minutes at room temperature in order to block endogenous peroxide activity. Following this, sections were washed in 1 XTBST and then incubated with a single primary antibody (made up in Da Vinci Green Diluent solution; Biocare Medical) for 35 minutes. Sections were then washed and incubated with a probe antibody (Rabbit or Mouse MACH3 Probe; Biocare Medical) specific to the species of the primary antibody for 10 minutes, which were then washed and incubated for a further 10 minutes with a horseradish peroxidase (HRP) conjugated antibody (Rabbit or Mouse MACH3 HRP; Biocare Medical) specific to the probe. Following this, sections were washed and then incubated with opal fluorophores at a 1:50 dilution made up in tyramide signal amplification reagent (pro vided in the opal kit). For every additional marker, the process was repeated by treating the slides with an antigen retrieval step followed directly by primary antibody staining and then down stream steps. Following this, sections were stained with DAPI for 3 minutes and then mounted using Vectashield. The following primary antibodies were used to identify CD8 expression (Ab4055 clone; Abcam), CD103 expression (Ab129202 clone; Abcam), SOX10 expression (BC34 clone from Biocare Medical), and PD 1 expression (NAT205 clone from Cell Marque). For imaging, a standard fluorescent microscope fitted with an auto mated quantitative pathology imaging system (Vectra) was used in conjunction with Vectra 3.3 software. Images were unmixed and annotated in Phenochart 1.0.4 and inForm 2.2.0.

Flow cytometry analysis

Lymphocytes from freshly isolated or cryopreserved tumor samples were stained with fluorochrome conjugated mAbs for 20 minutes at 4°C. T cell subsets were identified using the fol lowing mAbs: anti CD3, CD8, CD69, and CD103 (all from BioLegend) to identify $CD8^+$ T cells and resident subsets, whereas anti CCR7 (R&D Systems) and anti CD45RA (BD Biosciences) were used for characterizing naïve and memory T cells. Activation and differentiation of T cells were determined using mAbs against CD25, CD137, HLA DR (all from BD Biosciences), and KLRG1 (BioLegend), whereas expression of inhibitory receptors was determined using mAbs against PD 1, LAG3, GITR, CD244, TIM 3 (all from BioLegend), and CTLA 4 (BD Biosciences). $CD8^+$ T cells specific for the melanoma antigen Melan A were identified using an HLA A*0201 restricted MHC class 1 tetramer (ELAGI GILTV; from IMMUDEX). Cells were labeled with Zombie Aqua or Zombie UV to exclude dead cells from analysis. Stained cells were then washed and fixed with 2% paraformaldehyde before analysis on an LSRFortessa flow cytometer (BD biosciences). The data were then processed using FlowJo software (Treestar).

Detection of gramzyme B, perforin, and eomes by intracellular staining

Following extracellular staining, cells were fixed and permea bilized using a Transcription Factor Buffer Set (BD Pharmingen) and then intracellularly stained with mAbs to granzyme B and Eomes (eBioscience), and Perforin (BioLegend) for 20 minutes at 4°C before analysis on a flow cytometer.

Statistical analysis

Graphical and statistical analyses were performed using either Prism version 6.0f (GraphPad Software), TIBCO Spotfire v6.5.2, or CutoffFinder version 2.0. The mean of fluorescent intensity was calculated using the geometric mean within FlowJo v9.7.6. *P* values were performed either by a nonparametric Kruskal Wallis matched pairs test, nonparametric log rank test, or Spearman rho test, where appropriate. *P* values less than 0.05 were considered significant. All variability in the data is shown as SEM. Correla tions between Trm/CD8 IHC score with clinical features were performed using the Spearman ρ method. Melanoma specific survival (MSS) was calculated from the date of surgical resection of stage III melanoma specimen to date of last follow up or death from melanoma as performed in previous studies (24). Kaplan Meier curves were generated using cutoff values that where deter mined using ROC curve analysis that maximized the sensitivity and specificity of the analysis (Cutoff Finder, http://molpath. charite.de/cutoff/; ref. 25). The significance for the survival curves was determined using the log rank test.

Results

$CD103^+$ tumor resident cytotoxic T cell numbers are strongly associated with increased MSS

We began by analyzing TCGA melanoma database (26) to determine whether altered expression of the core signature tissue resident T cell genes provided a prognostic advantage in immu notherapy naïve patients with melanoma. This showed that increased expression of one or more of the following genes *CD69*, *CD103*, *TNFRSF1*8, *CD8a*, or *2B4* (Fig. 1A; Supplementary Fig. S1) indeed was associated with improved survival for patients. In order to separate the role of tumor resident $CD8^+$ T cells from nonresident $CD8^+$ T cells, we then performed multiplex quanti tative immunofluorescence staining on a subset (n 44) of stage III metastatic melanoma samples used for the same TCGA anal ysis. This showed a high degree of heterogeneity for the number of $CD103^+$ tumor resident $CD8^+$ T cell numbers between patients, with some having no resident cells and others having significant numbers of them (Fig. 1B). Although it is known that $CD8^+$ TILs are important for survival in melanoma, we reasoned that it is the $CD103^+$ $CD8^+$ T cells that are of primary importance and there fore determined the effect of these cell numbers on patient survival. Although the number of infiltrating $CD8^+$ T cells was not significantly correlated with survival in this cohort of patients [HR 0.7 (0.3 1.63); P 0.41], there was a trend toward better survival in patients who had a ratio of tumor resident T cells to overall $CD8^+$ T cells of >0.25 [HR 0.36 (0.1 1.21); P 0.085]. The enumeration of $CD103^+$ tumor resident T cells yielded the strongest association with survival [HR 0.39 (0.16 0.95); P 0.032; Fig. 1C and D), with a 5 year MSS of 50% in those with higher counts compared with MSS of 20% in those with lower counts.

Expression of immune checkpoints is highly enriched in $CD103^+$ tumor resident cytotoxic T cells

Having established the importance of tumor resident $CD8^+$ T cells, we next determined the phenotypic characteristics of these cells in order to gain insights into their potential role in tumor control. We isolated TILs from freshly resected metastatic mela noma specimens (n 10) and determined the phenotype of $CD8^+$ T cells by multiparameter flow cytometry. As previously reported (27), this showed that most of the $CD8^+$ TILs were effector memory T cells (TEM, CCR7 CD45RA or TEMRA CCR7 $CD45RA^+$; Supplementary Fig. S2a). Interestingly, on aver age 30% of the $CD8^+$ T cells were $CD69^+CD103^+$ tumor resident

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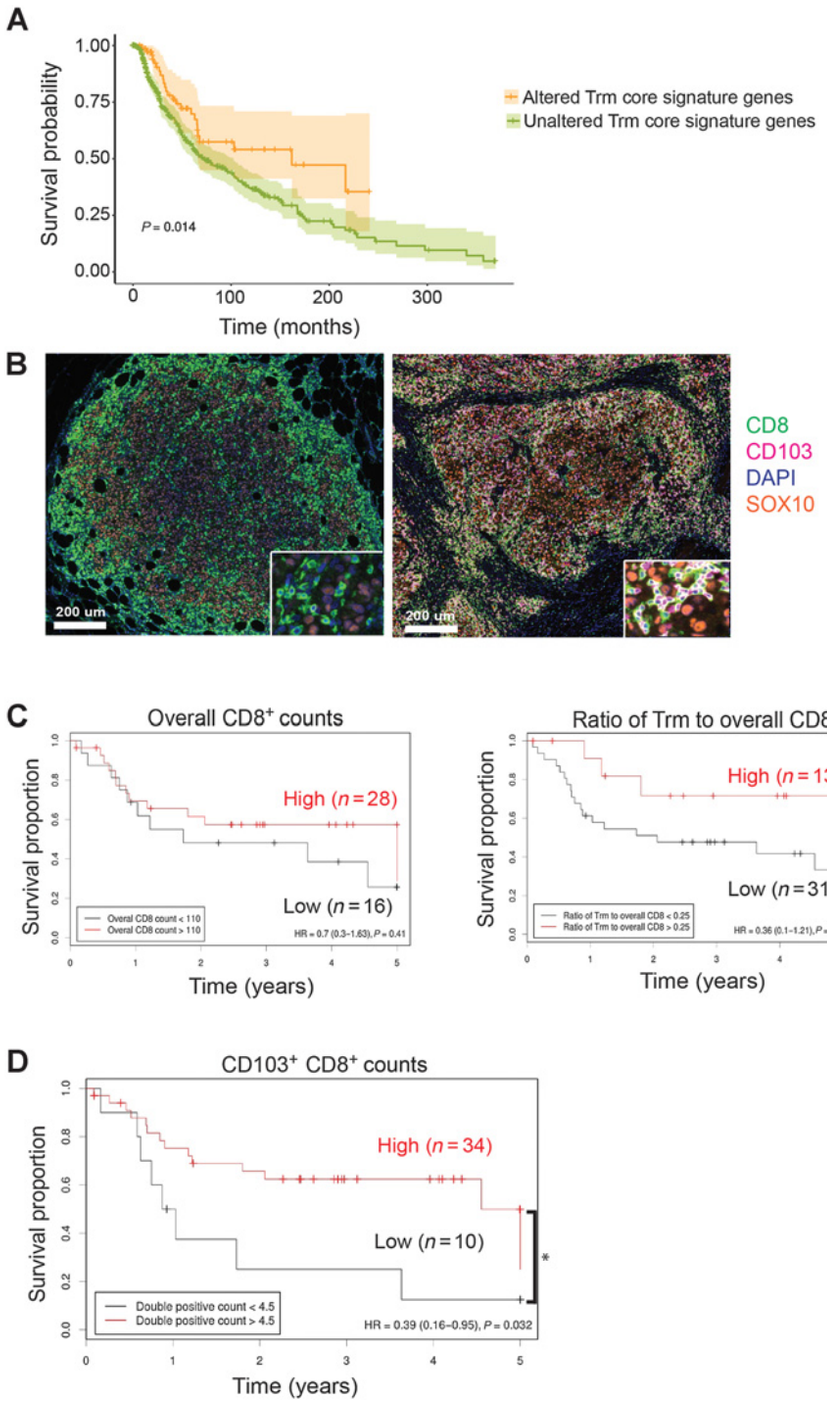

Figure 1.

Description of the protective effect of tumor resident $CD8^+$ T cells. **A,** Kaplan Meier survival curves of melanoma patients from the TCGA database. Patients with improved MSS show altered expression of one or more of the following genes, which are known to be expressed on tumor resident T cells: CD69, CD103, TNFRSF18, CD8A, or 2B4. The confidence intervals (shaded area) and the *P* value for the log rank test are shown on the plot. The cutoff used was mRNA expression z scores (RNA Seq V2 RSEM) $\pm$ 1.5. **B,** Using multiplex tissue immunofluorescent staining on formalin fixed paraffin embedded sections of tumors from a subset of the matched metastatic melanoma specimens utilized in the TCGA analysis, $CD103^+CD8^+$ T cell numbers were enumerated. Figure shows the examples of a patient with a few $CD103^+CD8^+$ T cells (left plot) and a patient with high $CD103^+CD8^+$ T cell numbers (right). Tissues were stained with antibodies for $CD8^+$ T cells (green), CD103 (magenta), the melanoma marker SOX10 (orange), and DAPI (blue). Colocalization of CD8 and CD103 appears as a light pink/white staining. **C,** Kaplan Meier MSS curves for patients with high (red) counts of $CD8^+$ T cells against those with low (black) counts (cutoff 110; left plot) and patients with high (red) ratios of $CD103^+CD8^+$ T cells to $CD8^+$ T cells against those with low (black) ratios (cutoff 0.25; right plot). Statistical differences were calculated using a nonparametric log rank test and displayed as * for $P \leq 0.05$. **D,** Kaplan Meier MSS curves for patients with high (red) counts of $CD103^+CD8^+$ T cells and those with low (black) counts (cutoff 4.5). A tissue microarray slide (TMA) that contained 44 individual regional lymph node melanoma metastases from high TIL areas of the tumor were analyzed. The number of $CD103^+CD8^+$ and $CD8^+$ T cells in each tumor core was counted and analyzed in order to determine the prognostic effect of each.

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T cells (Supplementary Fig. S2b). In line with what has been reported in mice for tissue resident memory T cells (12), these tumor resident CD8$^+$ T cells were KLRG1^{low} TEM like phenotype (Fig. 2A; Supplementary Fig. S2c), suggesting that they were not recently activated effector populations. Also, consistent with this, we found a moderate increase in the expression of some activation markers (CD137 and HLA DR; Fig. 2A), but not others (CD25; Supplementary Fig. S2d), and a moderate increase in the expression of the lytic granules granzyme B in the absence of perforin or granzyme K (Supplementary Fig. S2d), all of which are highly expressed on recently activated effector CD8$^+$ T cell populations.

Studies in mice and our recent work in humans have also shown that tissue resident memory T cells are enriched for the expression of several inhibitory receptors, including PD 1, CTLA 4, and 2B4 (12, 20). We therefore hypothesized that tumor resident CD8$^+$ T cells could be a critical population that expresses immune checkpoints. Indeed, the expression of PD 1 was highest on CD69$^+$CD103$^+$ CD8$^+$ T cells, followed by CD69$^+$CD103 CD8$^+$ T cells (Fig. 2B). The proportion of PD 1$^+$CD103$^+$CD69$^+$ cells was significantly higher than PD 1$^+$CD103 CD69$^+$ or PD 1$^+$CD103 CD69 CD8$^+$ T cells. Similarly, the expression levels of other inhibitory checkpoints LAG 3, 2B4, and TIM 3 were highest in CD69$^+$CD103$^+$CD8$^+$ T cells (Fig. 2B; Supplementary Fig. S2e), with LAG 3$^+$ population significantly higher in CD103$^+$CD69$^+$ subset when compared with the rest. There was, however, no difference in the expression of GITR between the three populations (Fig. 2B), and the surface expression of CTLA 4 was minimal (Supplementary Fig. S2e). When we examined for the expression of two T box transcription factors that regulate T cell responses, we found a trend toward a higher expression of Eomesodermin (Eomes) and Tbet in the CD103$^+$CD69$^+$ subset (Supplementary Fig. S2f) when compared with the other two subsets. In order to verify that these phenotypes were indeed represented in tumor specific CD8$^+$ T cells, we stained TILs with soluble peptide MHC complex to identify Melan A specific CD8$^+$ T cells. This showed that tumor specific resident CD8$^+$ T cells were also enriched for PD 1 and 2B4 (Fig. 2C).

CD103$^+$ tumor resident T cells significantly expand early during anti PD 1 treatment

Previous studies examining the T cell populations responding to anti PD 1 treatment found an expansion of CD8$^+$ TEM cells (28) or PD 1$^+$CD8$^+$ T cells (7) early during treatment. Based on our data, we predicted that the target population for such immunotherapy could be the tumor resident CD8$^+$ T cell population. To test this hypothesis, we analyzed tumor biopsy specimens from advanced stage metastatic melanoma patients being treated with anti PD 1 monotherapy (nivolumab, pembrolizumab), procured before and early during treatment. We grouped patients who had CR, PR, and SD for greater than 6 months as determined by RECIST response criteria as "responders" and patients who had progressive disease and SD for less than 6 months as "non responders" (Supplementary Table S1). As described before, total CD8$^+$ T cell numbers expanded significantly during anti PD 1 treatment (Fig. 3A and B), and there was a significant difference in their numbers at EDT (P 0.039) between those who responded to the treatment against those who did not respond. Quantification of the tumor resident CD8$^+$ T cell numbers showed that, when compared with the baseline, there was significant expansion of CD103$^+$ tumor resident CD8$^+$ T cells early during treatment in the majority of the patients (Fig. 3A and C). We then examined whether there were differences in the number of tumor resident CD8$^+$ T cells between responders to anti PD 1 therapy compared with nonresponders. Despite the small number of responders (n 5) analyzed with early during treatment biopsies, there was a trend toward a greater magnitude of expansion of these cells in responding patients (P 0.07) compared with nonresponders (n 8). This suggests that a threshold of tumor resident CD8$^+$ T cell numbers might be necessary for the effectiveness of anti PD 1 treatment.

Local IL15 expression levels are associated with CD103$^+$ tumor resident T cell numbers

Finally, we examined the local factors that could influence the number of resident T cells within metastatic tumors. In mouse models, cytokines such as IL15, TGFβ, IL7, and IL33 have been implicated in the development of tissue resident memory T cells. We recently showed in humans that IL15 could induce the expression of CD69 and downmodulate the exit signals to potentiate the retention of T cells within tissues (20). Consistent with this, we found a strong correlation between the expression of IL15 and CD8 mRNA in the TCGA database (P 8.3×10^{-7}; Fig. 4A) and more importantly that enhanced IL15 expression was also associated with better patient survival (P 0.0012; Fig. 4B). We therefore determined whether local IL15 levels influenced the CD103$^+$ tumor resident CD8$^+$ T cell numbers within the tumors. Quantitative analysis of samples that either had high or low expression of IL15 RNA in the TCGA study suggests that local IL15 levels may influence CD103$^+$ tumor resident CD8$^+$ T cell numbers within metastatic melanoma (Fig. 4C and D). This suggests that the heterogeneity in these cell numbers between patients could be largely due to local factors that are important for T cell retention.

Discussion

Although therapeutic blockade of immune checkpoints remains a promising treatment strategy for many types of cancers, there is an urgent need to improve its efficacy, reduce the associated toxicity, and develop accurate biomarkers to predict response. Critical to this is a greater understanding of the immune cells that initiate the response and the factors that regulate their numbers. Our collective data demonstrate that CD103$^+$ tumor resident CD8$^+$ T cells are not only associated with tumor control in untreated metastatic melanoma patients, but may also be important for determining responses to anti PD 1 immunotherapy. Here, we show that the expression of PD 1 and LAG 3 is highly enriched to this subset of CD8$^+$ T cells in vast majority of patients, suggesting that these cells are likely to be the initial target of anti PD 1 treatment. In addition, we also found these cells significantly expanded early during anti PD 1 treatment in melanoma specimens taken from patients with metastatic melanoma, further supporting the fact that these tumor resident CD8$^+$ T cells could be initiating the response. In an attempt to identify the factors that regulate these cells, we found local IL15 expression levels to strongly correlate with tumor resident CD8$^+$ T cell numbers.

We have found that tumor resident CD8$^+$ T cell numbers were more prognostic than the total CD8$^+$ T cell counts in untreated

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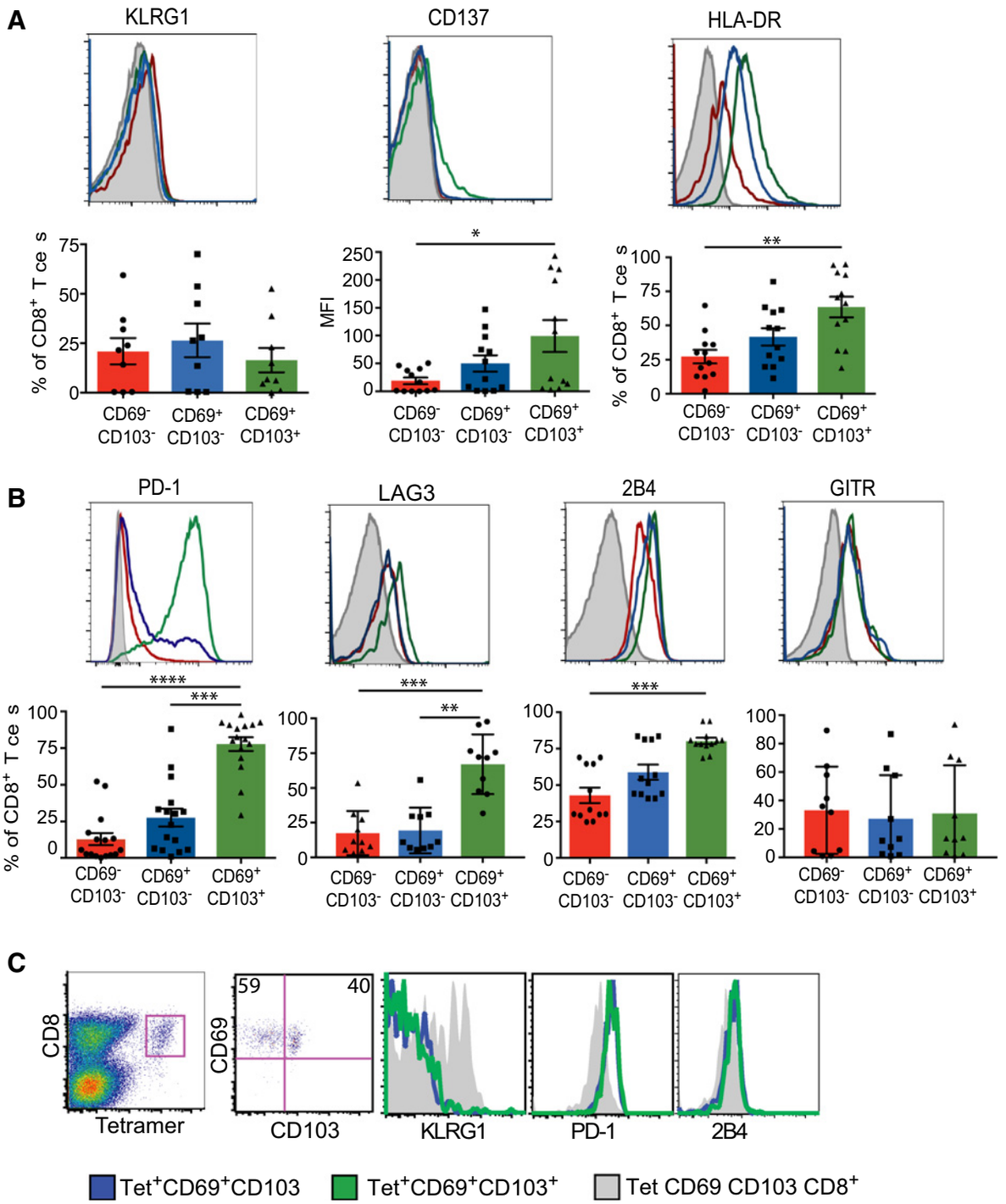

Figure 2.

Phenotype of tumor resident CD8$^+$ T cells. Freshly isolated tumor infiltrating lymphocytes from melanoma patients were stained with antibodies and analyzed by flow cytometry. Antibodies to CD3, CD8, CD103, and CD69 were used to identify tumor resident CD8$^+$ T cells. **A,** The expression levels of KLRG1, CD137, and HLA DR were compared between CD103$^+$CD69$^+$ (green), CD103$^-$CD69$^+$ (blue), and CD103$^-$CD69$^-$ (red) CD8$^+$ T cell populations. Top plot shows the representative histogram plots with FMO controls (gray), and the bottom plot shows the plots representing a minimum of 9 different patient samples with ± SEM. **B,** Similarly, the expression levels of PD 1, LAG3, 2B4, and GITR were compared between CD103$^+$CD69$^+$ (green), CD103$^-$CD69$^+$ (blue), and CD103$^-$CD69$^-$ (red) CD8$^+$ T cell populations. Top plot shows the representative histogram overlay plots with FMO controls (gray), and the bottom plot shows the plots representing a minimum of 10 different patient metastatic melanoma specimens with ± SEM. Statistical differences were calculated using a nonparametric Kruskal Wallis test and displayed as * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, and **** for $P \leq 0.0001$. **C,** Soluble peptide MHC complexes (tetramers) were used to identify CD8$^+$ T cells specific for Melanin A antigen. Plots show the tetramer stain, the expression of CD69 and CD103 on tetramer positive cells, and representative histogram overlay plots of tetramer$^+$CD103$^+$CD69$^+$ (green) and tetramer$^+$CD103$^+$CD69$^-$ (blue) CD8$^+$ T cells for indicated receptors. Tumor specific T cell plots are representative of two different patient samples.

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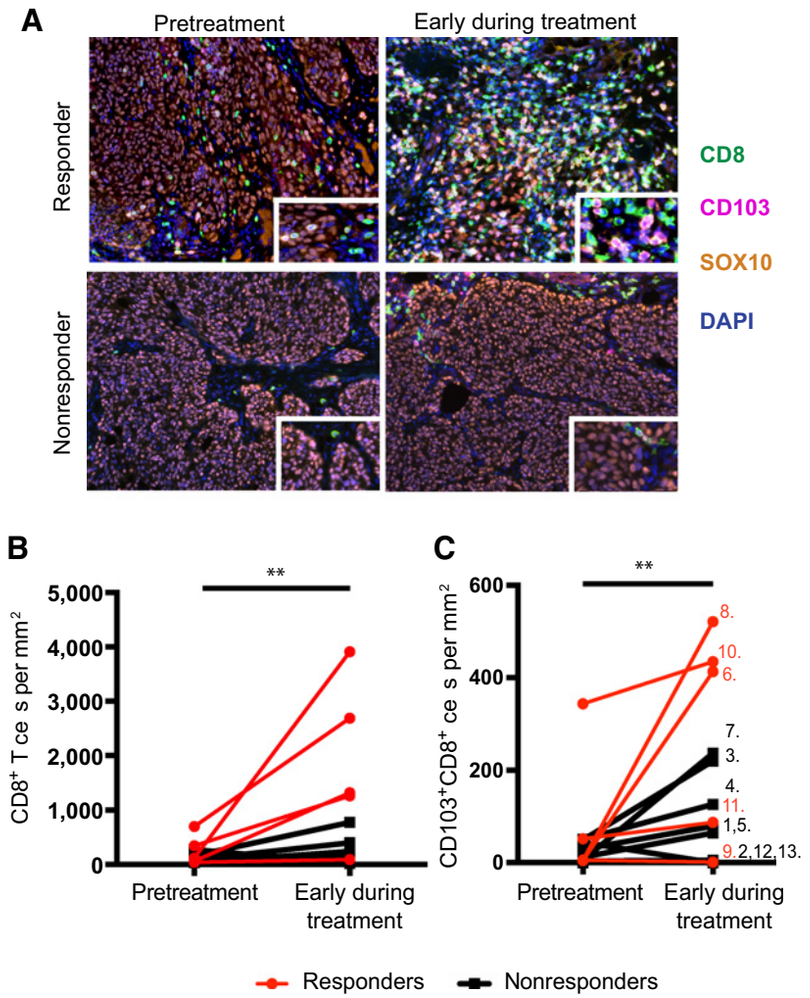

Figure 3.

Tumor resident CD8$^+$ T cells respond to immunotherapy. Tumor biopsies were obtained prior to (pretreatment) and early during treatment from metastatic melanoma patients undergoing anti PD 1 therapy. Paraffin embedded sections were then stained for CD8$^+$ T cells (green), CD103 (magenta), the melanoma marker SOX10 (orange), and DAPI (blue) and CD103$^+$CD8$^+$ T cell numbers. **A,** Example sections from a patient who responded to anti PD 1 treatment (top plots) and a patient who failed to respond (bottom plots) are shown. Quantification of total CD8$^+$ T cells (**B**) and CD103$^+$CD8$^+$ T cell numbers (**C**) in responders ($n = 5$) and nonresponders ($n = 8$) shows a significant expansion of these cells early during treatment. Statistical differences were calculated using nonparametric Wilcoxon matched pairs signed rank test, and ** indicates a P value of < 0.01. Graph points are indexed and matched to patient clinical data in Supplementary Table S1.

melanoma patients. Further studies are required to evaluate the predictive value of these cells. Although total CD8$^+$ T cell counts have previously been associated with patient survival, in the absence of information on subsets, it is difficult to determine whether the protective response was associated with any partic ular subset. Recruitment of tumor specific effector CD8$^+$ T cells to the site of tumors is essential for tumor rejection. It has recently become evident that some of these CD8$^+$ T cells that infiltrate tissues become resident (29, 30). Here, we show that human tumors also contain populations of tumor resident CD8$^+$ T cells, and their numbers are highly variable between patients. Impor tantly, our data clearly demonstrate that the increased presence of tumor resident CD8$^+$ T cells is strongly associated with better MSS in untreated patients. This suggests that tumor resident CD8$^+$ T cells could be critical for immune control of metastatic tumors. Tissue resident CD8$^+$ T cells are characterized by the constitutive expression of CD69 and CD103 (31). In humans, early reports described two populations of resident T cells, CD69$^+$CD103$^+$CD8$^+$ and CD69$^+$CD103 CD8$^+$ T cells (32, 33). CD69 expression, however, can also be induced on T cells upon T cell receptor activation, therefore relying on CD69 expres sion alone to identify resident memory T cells and differentiate them from recently activated effector cells may not be accurate. We recently showed that CD69$^+$CD103$^+$CD8$^+$ T cells in human tissues were more characteristic of the tissue resident memory T cells described in mouse models than CD69$^+$CD103 CD8$^+$ T cells (20). Among the other features that characterize resident cells are a set of core signature genes that are either enhanced (*RGS1*, *TNFRSF18*, *CD244*, *ICOS*) or suppressed (*KLF2*, *S1PR1*, *KLRG1*) in resident cells when compared with their circulating counter parts (12).

Further insight into the critical role of these tumor resident CD8$^+$ T cells became evident when we examined their phenotype. Similar to what has been reported for tissue resident memory T cells, the tumor resident CD8$^+$ T cells were also enriched for several immune inhibitory receptors. It was recently reported that CD69$^+$CD8$^+$ T cells in metastatic melanoma were highly enriched for immune checkpoint receptors (34). However, our data suggest that mainly the CD103$^+$CD69$^+$ subset expressed the highest levels of PD 1 and LAG 3. Together with the fact that these CD103$^+$ T cells expanded significantly during anti PD 1 treat ment, it could be inferred that these cells initiate the response to anti PD 1. Although it has been shown that preexisting CD8$^+$ T cells at the tumor invasive margin was predictive of response to anti PD 1 treatment (7), our data strongly suggest that tumor resident subset of CD8$^+$ T cells is critical. Further supporting

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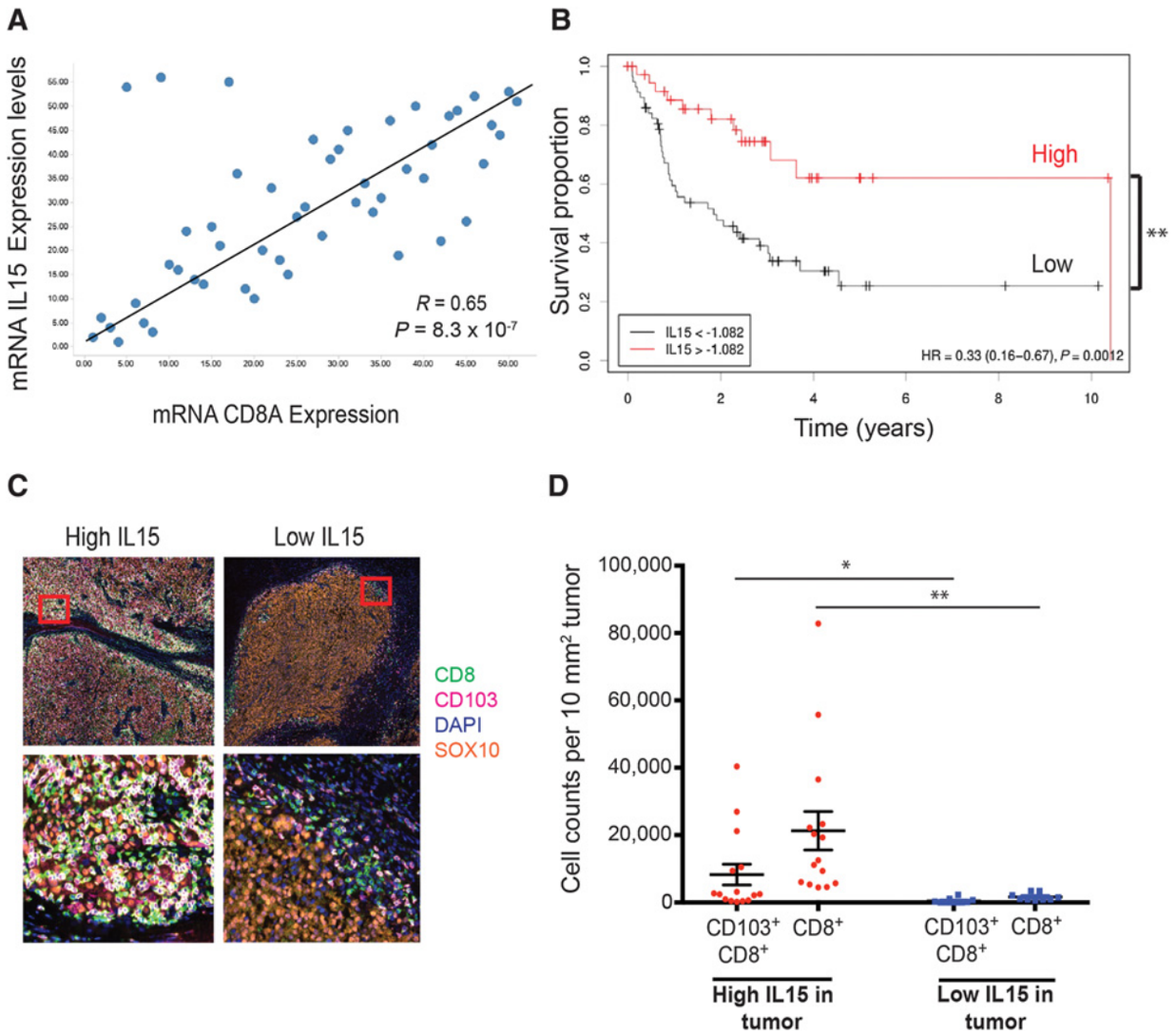

Figure 4.

The impact of IL15 expression levels on tumor resident CD8$^+$ T cells. The expression of IL15 in the tumors of patients with metastatic melanoma was analyzed with respect to CD8 expression levels and patient survival. **A,** Correlation of IL15 and CD8A mRNA expression levels in the tumors of melanoma patients as found in TCGA melanoma data ($P = 8.3 \times 10^{-7}$, Spearman rank correlation coefficient, $r = 0.65$). **B,** Kaplan Meier MSS curve for patients with high (red) IL15 expression levels and low (black) IL15 expression levels from TCGA melanoma cohort. The two groups were compared by analyzing the proportion of patients who survived as a function of time (years). Data were binarized using a threshold of 1.082 for IL15 expression. Statistical differences were calculated using a nonparametric log rank test and displayed as ** for $P \leq 0.01$. **C,** Representative images of patients with either high (left) or low (right) mRNA levels of IL15 in tumors. Top images are low objective views, and bottom images are regions from highlighted (red) area of interest at high power magnification. Paraffin embedded sections from high IL15 mRNA expression ($n = 14$) and low ($n = 11$) mRNA expressing metastatic melanoma tissues from the TCGA analysis were stained for CD8$^+$ T cells (green), CD103 (magenta), SOX10 (orange), and DAPI (blue) and CD103$^+$CD8$^+$ T cell numbers enumerated. Colocalization of CD8 and CD103 is represented by light pink/white staining. **D,** Quantitative analysis of CD8$^+$ and CD103$^+$CD8$^+$ T cell numbers in high (red) or low (blue) IL15 mRNA expressing groups per 10 mm squared area of tumor. Each point represents an individual patient. Statistical significance was calculated using nonparametric Kruskal Wallis test and displayed as * for $P \leq 0.05$ and ** for $P \leq 0.01$.

our work is another very recent study that showed the pheno type of expanding T cell clones during immunotherapy. Wei and colleagues showed that T cell clones that expanded during anti PD 1 treatment expressed high levels of CD69, PD 1, LAG 3, and CD45RO (35), an identical phenotype to the tumor resident CD8$^+$ T cell population we have described. There is also growing evidence from mouse models that tumor resident T cells may play a critical role in checkpoint inhibitor efficacy. For example, recently it was shown that the effectiveness of checkpoint blockade and the expansion of effector populations during treatment may be independent of circulating T cells (36, 37), suggesting that the preexisting tumor resident T cells were sufficient to mediate response. In addition, the failure of circulating T cell numbers to predict response to checkpoint

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blockade could also be attributed to the fact that the critical population is noncirculating and is resident within the tumor environment.

There was significant expansion of tumor resident $CD8^+$ T cells in the vast majority of melanoma patients early during treatment with anti PD 1 drug therapy compared with baseline tissue samples. The magnitude of expansion, however, was higher in responders than nonresponders. It is worth noting that there are some limitations to the interpretation of the data: (1) the tumor biopsies were obtained from different sites between pre and EDT samples, (2) time to EDT samples varied among patients, (3) the responding clones could have changed phenotype and therefore we underestimated their numbers, (4) we were unable to stain for CD69, and (5) there were limited number of biopsies available from patients undergoing immunotherapy. Nevertheless, our data strongly suggest that the numbers of tumor resident $CD8^+$ T cells could be critical in determining the effectiveness of checkpoint blockade. In addition, it has become evident that combination immunotherapies that target multiple immune checkpoints can improve the success rate when compared with monotherapies. In line with this, we have found that tumor resident $CD8^+$ T cells express multiple immune checkpoints and therefore are likely to be regulated by more than one inhibitory receptor. These data need to be confirmed in larger patient cohorts; however, the immune profile of tumor resident $CD8^+$ T cells may provide a rational approach to the selection of therapies for patients who fail monotherapies and provide a basis for the design of clinical trials in this patient population.

Boosting tumor resident $CD8^+$ T cell numbers may be a strategy to improve antitumoral immune responses. Understanding the factors that regulate the retention of tumor resident $CD8^+$ T cells is therefore critical. We have recently shown that IL15 not only downmodulates tissue exit signals on human T cells, but also induces the expression of CD69 and hence may facilitate their retention. IL15 has also been implicated in the development of tissue resident memory T cells in mouse models (12). Our current study shows that the expression levels of local IL15 was highly variable between patients, and importantly, the levels correlated strongly with tumor resident T cell numbers. In the absence of antibodies that can stain CD69 on formalin fixed tissues, we were unable to determine the proportion of $CD69^+CD8^+$ T cells. This also corroborates with the recent finding that majority of $CD8^+$ T cells in TILs express CD69 (34). Therefore, it could be inferred that the local IL15 could be a critical factor in retaining T cells within the tumor environment. Further studies are, however, required to determine the exact role of this cytokine. IL15 could have multiple effects on tumor infiltrating T cells. It can also enhance the survival of memory T cells through the upregulation of prosurvival genes, and it can induce proliferation of T cells in the absence of T cell receptor mediated signals. Although the role of IL15 in antitumoral T cell responses has long been recognized, its ability to retain cells within the tumor environment has not been appreciated. Interestingly, a recent study in human colorectal cancers suggests that IL15 gene deletion by tumor cells could be an immune escape mechanism (38). Another factor that could contribute to tissue retention of T cells is TGFβ, which not only synergizes with IL15 to downmodulate tissue exit signals, but is also necessary for the expression of CD103 (20). How these cytokines are regulated within the tumor environment is unclear; however, tumor infiltrating dendritic cells (DCs) and/or stromal cells can be a source of IL15.

Persistence of tumor resident $CD8^+$ T cells could also be critical for long term tumor control. One possibility is that they are better suited to survive in metabolically challenging tumor environments. A recent study has shown that tissue resident T cells are uniquely dependent on the uptake of free fatty acids and their metabolisms for long term survival (39). The restricted access to glucose within the tumor environment (40) could favor the survival of these resident T cells. Nevertheless, our findings will support the notion that the effectiveness of T cell mediated control of metastatic cancers relies not only on the recruitment of effector T cells, but also on retaining them within the tumor environment.

In conclusion, our work demonstrates that tumor resident $CD8^+$ T cells could be critical for tumor control and are likely to be the population that initiates response to checkpoint blockade. A greater understanding of the characteristics of this population and the crucial factors that regulate their numbers will open new opportunities for novel therapies.

Disclosure of Potential Conflicts of Interest

J.F. Thompson reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, and Provectus. A.M. Menzies is a consultant/advisory board member for Bristol-Myers Squibb, MSD, Novartis, Pierre-Fabre, and Roche. G.V. Long is a consultant/advisory board member for Amgen, Array, Bristol-Myers Squibb, Merck, Novartis, Pierre-Fabre, and Roche. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Edwards, J.S. Wilmott, J. Madore, C. Quek, A. Tasker, A. Ferguson, T. Gebhardt, W. Weninger, W.J. Britton, G.V. Long, R.A. Scolyer, U. Palendira

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.S. Wilmott, A. Ferguson, J. Chen

Study supervision: R.A. Scolyer, U. Palendira

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References

1. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008;26:677–704.
2. Boussiotis VA. Molecular and biochemical aspects of the PD-1 checkpoint pathway. N Engl J Med 2016;375:1767–78.
3. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. N Engl J Med 2015;373:23–34.
4. Robert C, Schachter J, Long G V, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. N Engl J Med 2015;372:2521–32.
5. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, et al. Pembrolizumab versus chemotherapy for PD-L1–positive non–small-cell lung cancer. N Engl J Med 2016;375:1823–33.
6. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. N Engl J Med 2012;109–22.
7. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJM, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 2014;515:568–71.
8. Kakavand H, Vilain RE, Wilmott JS, Burke H, Yearley JH, Thompson JF, et al. Tumor PD-L1 expression, immune cell correlates and PD-1+ lymphocytes in sentinel lymph node melanoma metastases. Mod Pathol 2015;28:1535–44.
9. Herbst RS, Soria J-C, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 2014;515:563–7.
10. Azimi F, Scolyer RA, Rumcheva P, Moncrieff M, Murali R, McCarthy SW, et al. Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. J Clin Oncol 2012;30:2678–83.
11. Adachi T, Kobayashi T, Sugihara E, Yamada T, Ikuta K, Pittaluga S, et al. Hair follicle–derived IL-7 and IL-15 mediate skin-resident memory T cell homeostasis and lymphoma. Nat Med 2015;21:1272–9.
12. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon M-L, et al. The developmental pathway for CD103(+)CD8(+) tissue-resident memory T cells of skin. Nat Immunol 2013;14:1294–301.
13. Jiang X, Clark Ra, Liu L, Wagers AJ, Fuhlbrigge RC, Kupper TS. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. Nature 2012;483:227–31.
14. Mackay LK, Stock AT, Ma JZ, Jones CM, Kent SJ, Mueller SN, et al. Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. Proc Natl Acad Sci 2012;109:7037–42.
15. Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. Nature 2012;491:463–7.
16. Teijaro JR, Turner D, Pham Q, Wherry EJ, Lefrançois L, Farber DL. Cutting edge: tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. J Immunol 2011;187:5510–4.
17. Zhu J, Peng T, Johnston C, Phasouk K, Kask AS, Klock A, et al. Immune surveillance by CD8αα+ skin-resident T cells in human herpes virus infection. Nature 2013;497:494–7.
18. Clark Ra, Watanabe R, Teague JE, Schlapbach C, Tawa MC, Adams N, et al. Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients. Sci Transl Med 2012;4:117ra7–117ra7.
19. Jozwik A, Habibi MS, Paras A, Zhu J, Guvenel A, Dhariwal J, et al. RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. Nat Commun 2015;6:10224.
20. Woon HG, Braun A, Li J, Smith C, Edwards J, Sierro F, et al. Compartmentalization of total and virus-specific tissue-resident memory CD8+ T cells in human lymphoid organs. PLoS Pathog 2016;12:1–19.
21. Djenidi F, Adam J, Goubar a, Durgeau a, Meurice G, de Montpreville V, et al. CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. J Immunol 2015;194:3475–86.
22. Webb JR, Milne K, Watson P, DeLeeuw RJ, Nelson BH. Tumor-infiltrating lymphocytes expressing the tissue resident memory marker cd103 are associated with increased survival in high-grade serous ovarian cancer. Clin Cancer Res 2014;20:434–44.
23. Murray T, Fuertes Marraco SA, Baumgaertner P, Bordry N, Cagnon L, Donda A, et al. Very late antigen-1 marks functional tumor-resident CD8 T cells and correlates with survival of melanoma patients. Front Immunol 2016;7:1–12.
24. Madore J, Strbenac D, Vilain R, Menzies AM, Yang JYH, Thompson JF, et al. PD-L1 negative status is associated with lower mutation burden, differential expression of immune-related genes, and worse survival in stage III melanoma. Clin Cancer Res 2016;22:3915–23.
25. Morello S, Capone M, Sorrentino C, Giannarelli D, Madonna G, Mallardo D, et al. Soluble CD73 as biomarker in patients with metastatic melanoma patients treated with nivolumab. J Transl Med 2017;15:1–9.
26. Akbani R, Akdemir KC, Aksoy BA, Albert M, Ally A, Amin SB, et al. Genomic classification of cutaneous melanoma. Cell 2015;161:1681–96.
27. Baitsch L, Baumgaertner P, Devêvre E, Raghav SK, Legat A, Barba L, et al. Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients. J Clin Invest 2011;121:2350–60.
28. Ribas A, Shin DS, Zaretsky J, Frederiksen J, Cornish A, Avramis E, et al. PD-1 blockade expands intratumoral memory T cells. Cancer Immunol Res 2016;4:194–203.
29. Schenkel JM, Masopust D. Tissue-resident memory T cells. Immunity 2014;41:886–97.
30. Carbone FR, Mackay LK, Heath WR, Gebhardt T. Distinct resident and recirculating memory T cell subsets in non-lymphoid tissues. Curr Opin Immunol 2013;25:329–33.
31. Carbone FR. Tissue-resident memory T cells and fixed immune surveillance in nonlymphoid organs. J Immunol 2015;195:17–22.
32. Thome JJC, Yudanin N, Ohmura Y, Kubota M, Grinshpun B, Sathaliyawala T, et al. Spatial map of human T cell compartmentalization and maintenance over decades of life. Cell 2014;159:814–28.
33. Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJC, et al. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. Immunity 2013;1–11.
34. Boddupalli CS, Bar N, Kadaveru K, Krauthammer M, Pornputtapong N, Mai Z, et al. Interlesional diversity of T cell receptors in melanoma with immune checkpoints enriched in tissue-resident memory T cells. JCI Insight 2016;1.
35. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAAS, Andrews MC, et al. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. Cell 2017;170:1120–1125.e17.
36. Spranger S, Koblish HK, Horton B, Scherle Pa, Newton R, Gajewski TF. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8(+) T cells directly within the tumor microenvironment. J Immunother Cancer 2014;2:3.
37. Williams JB, Horton BL, Zheng Y, Duan Y, Powell JD, Gajewski TF. The EGR2 targets LAG-3 and 4-1BB describe and regulate dysfunctional antigen-specific CD8 + T cells in the tumor microenvironment. J Exp Med 2017 Feb;214:381–400.
38. Mlecnik B, Bindea G, Angell HK, Sasso MS, Obenauf AC, Fredriksen T, et al. Functional network pipeline reveals genetic determinants associated with in situ lymphocyte proliferation and survival of cancer patients. Sci Transl Med 2014;6:228ra37–228ra37.
39. Pan Y, Tian T, Park CO, Lofftus SY, Mei S, Liu X, et al. Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. Nature 2017;543:252–6.
40. Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic competition in the tumor microenvironment is a driver of cancer progression. Cell 2015;162:1229–41.

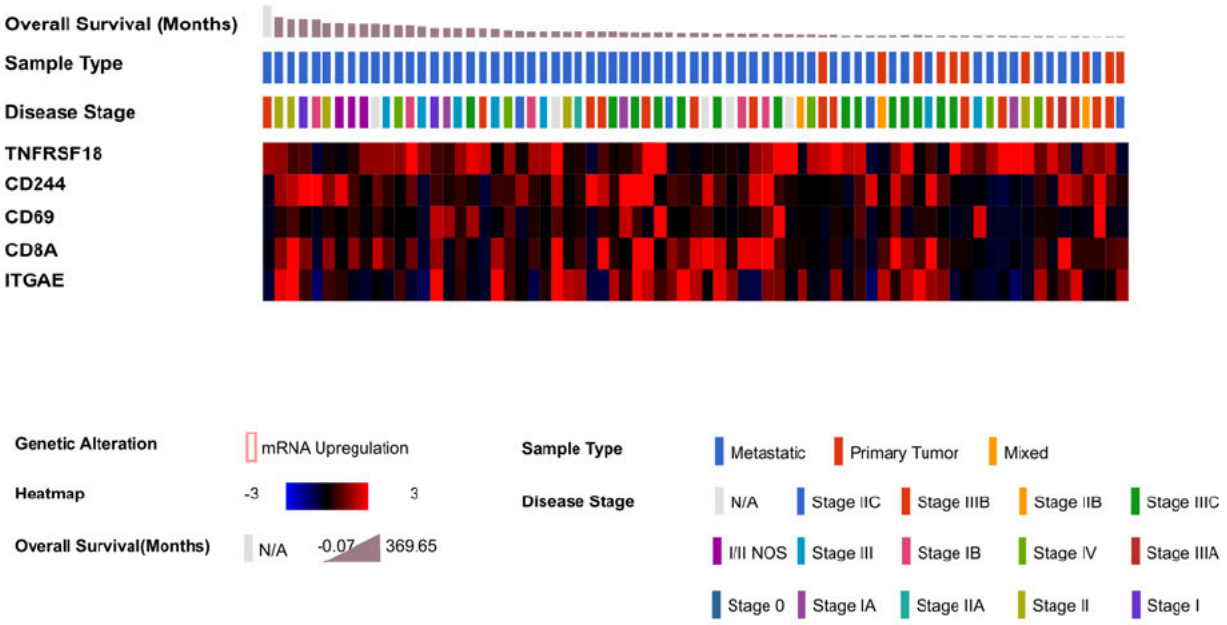
Chapter 1 Supplementary Tables and Figures

Supplementary Table 1. Clinical data of patients used in early during treatment analysis

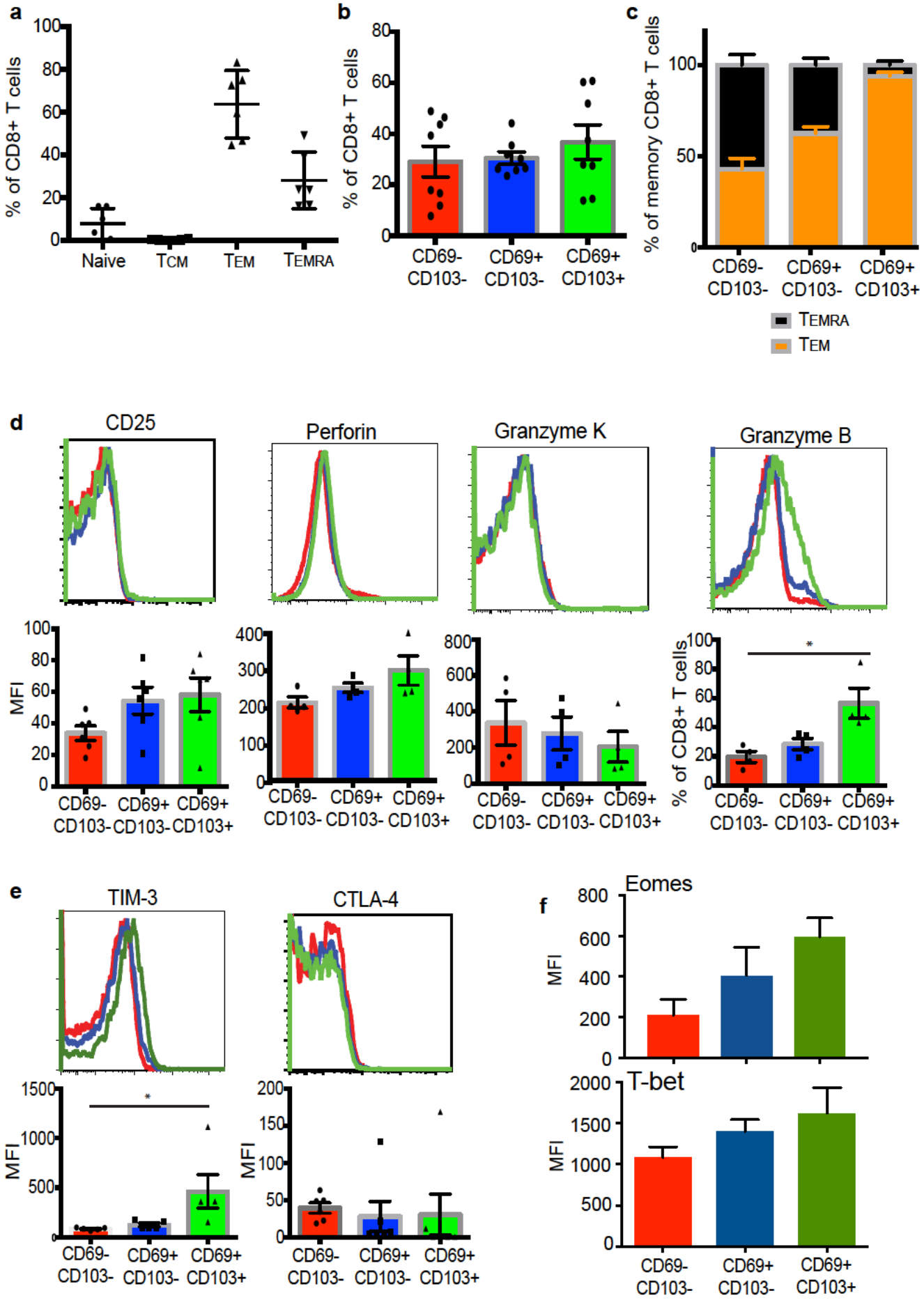
Patient No	Treatment	Best RECIST response	Progression free survival (days)	Overall Survival (days)	Site of PRE biopsy	Days to EDT biopsy	Site of EDT biopsy
1	Nivolumab	PD	9	147	LN	7	Skin
2	Nivolumab	PD	58	58	SQ	6	SQ
3	Pembrolizumab	PD	96	258	SQ	1	Spine
4	Pembrolizumab	PD	120	206	Skin	65	SQ
5	Nivolumab	SD	191	191	SQ	10	SQ
6	Pembrolizumab	non-CR/PD	324	324	SQ	44	SQ
7	Pembrolizumab	SD	335	825	LN	7	SQ
8	Pembrolizumab	CR	460	460	Brain	14	SQ
9	Pembrolizumab	PR	1174	1174	SQ	7	SQ
10	Pembrolizumab	PR	1189	1189	SQ	3	LN
11	Nivolumab	CR	1328	1328	SQ	2	SQ
12	Pembrolizumab	PD	85	279	Skin	11	Skin
13	Pembrolizumab /Placebo + CombiDT	PD	164	333	SQ	56	SQ

PD = partial response, SD = stable disease, CR = complete response
LN = lymph node, SQ = Subcutaneous

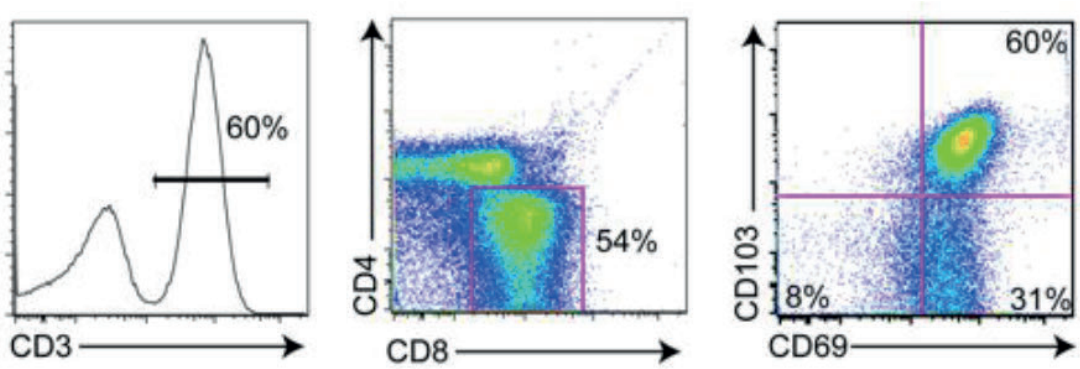
Supplementary Figure 1. Data on genomics



Supplementary Figure 2. Data on flow cytometry analysis



Supplementary Figure 3. Gating strategy used in study



Chapter 1 - Concluding discussion

To conclude this chapter, our study has contributed to our understanding of protective immunity in cancers and has also increased our knowledge of the specific CD8 T cell phenotypes likely targeted by anti-PD-1 immunotherapy. Our findings clearly demonstrate that not all CD8 T cell TILs in melanoma tumors are the same, nor are they equal in their anti-tumor and protective functionalities. Indeed, we have shown that CD69+ CD103+ tumor resident CD8 T cells exist in human melanoma tumors (and make up on average 30% of the total TIL phenotype) and that their presence is associated with patient overall-survival in the treatment-naïve setting. More importantly, we showed that these cells confer greater protection than their non-resident, circulating counterparts, and were specific for melanoma antigens. These findings have important implications because, 1. the presence of resident CD8 T cells could potentially serve as a biomarker for patient survival (at least in stage 3 metastatic disease), and 2. these results suggest that T cells that are adapted to reside and persist in the tumor microenvironment confer greater protection than those transiently entering the tumor. In line with this, we showed that among the important phenotypic differences between resident and non-resident CD8 T cells in the TME, resident CD8 T cells displayed more of an effector memory phenotype rather than a terminally differentiated effector phenotype. Effector memory T cells are known to have greater proliferative capacity and functionality than terminally differentiated cells and therefore would represent a population that could be "reinvigorated" during checkpoint blockade.

One of the main aims of this chapter was to understand the relevance of tumor resident CD8 T cells in anti-PD1 checkpoint blockade. Understanding the targets of anti-PD1 therapy is a critical step forward in improving the efficacy of this therapy, particularly for those unresponsive to current immunotherapy regimes. In this study, we have provided compelling evidence to show that resident CD8 T cells are a likely target of anti-PD-1 checkpoint blockade. Indeed, we showed that tumor resident CD8 T cells expressed the highest levels of the target receptor, PD-1, and expressed high levels of other inhibitory checkpoint receptors, including LAG-3. This is relevant given other studies have demonstrated the importance of PD-1+ CD8 TILs in anti-PD1 immunotherapy (Gide et al., 2019; Tumeh et al., 2014). Additionally, it suggests that tumor resident CD8 T cells may also play a role in the efficacy of other immunotherapies that are emerging, for example, relatlimab, an anti-LAG-3 antibody, which has recently shown efficacy in phase 3 human clinical trials in metastatic melanoma patients (Lipson et al., 2021). We also demonstrated that tumor resident CD8 T cells expanded significantly early during treatment with anti-PD-1 therapy, and that this increase, while not statistically significant, trended higher in responding patients compared to non-responding patients, overall providing further support for this population being a target. Since the completion of this study, other investigations have been made into resident CD8 T cells and anti-PD-1 immunotherapy in melanoma and various other cancers (Duhen et al., 2018; Egelston, Yuan, & Lee, 2019; S.L. Park et al., 2019; Savas et al., 2018) and confirm that this is a population of interest for current and emerging immunotherapies. In future, it will be important to understand the tumor resident CD8 T cell phenotype better, as this may guide the rationale for future therapies directed to individuals that fail anti-PD-1 immunotherapy.

One of the final objectives for this chapter was to determine any factors that might be responsible in the generation/maintenance of tumor resident CD8 T cells in melanoma tumors. This is a relevant question given we have provided strong evidence that these cells play a critical role in protective anti-tumor immunity and that non-responders to anti-PD-1 immunotherapy are likely to lack this important population. We have shown that IL-15 in the

TME has a direct effect on the numbers of CD8 T cells, including the numbers of resident CD8 T cells. Furthermore, we showed that the presence of IL-15 itself is a prognostic feature in melanoma tumors. These results are important but unsurprising, given that early studies investigating resident CD8 T cells in chronic infectious models demonstrated a role for IL-15 in the generation and maintenance of resident CD8 T cells (L. Mackay et al., 2013). Indeed, IL-15 more broadly, is an important cytokine in the maintenance of memory T cells. Where this IL-15 is derived from in the TME is a relevant question that remains to be determined. It will also be important to investigate further factors that may play a role in the generation and maintenance of resident T cells.

Overall, however, this chapter is a significant step forward in our collective understanding of the important CD8 T cell phenotypes involved in the clearance and control of tumors, as well as the specific CD8 T cell phenotypes required for anti-PD-1 immunotherapy response.

Chapter 2

Tumor mutation burden and structural chromosomal aberrations are not associated with T cell density or patient survival in acral, mucosal, and cutaneous melanomas

Chapter 2 - Introduction

In the previous chapter we showed that tumor resident CD8 T cells are a critical anti-tumor T cell population. We also showed that they are a likely target of anti-PD-1 immunotherapy, such that patient tumors lacking resident T cells are unlikely to benefit from current immunotherapies. Of course, more broadly, this is also true for patient tumors lacking cytotoxic CD8 T cells. A critical question then, is what are the factors responsible for the recruitment, maintenance, and retention of CD8 T cell populations in patient tumors? Understanding these factors will enable therapies that can boost CD8 T cell/resident CD8 T cell numbers in the tumors of patients who are unresponsive to current checkpoint blockade. In the previous chapter we showed that IL-15 is likely to be one of the factors responsible for their recruitment and retention. However, at the time that this next body of work was written, an important dogma was becoming more widely accepted within the scientific community, but which had drastic implications for patients lacking effective anti-tumoral immune responses. At the heart of this dogma was the notion that anti-tumoral immune responses were largely predetermined by the number of available neoantigens in the tumor microenvironment. This implied that efforts to boost critical immune populations could only go so far on a backdrop of poor neoantigen load. Neoantigens are non-self-antigens that are produced by tumor cells and not previously recognised by the immune system (Boon et al., 1997). They are thought to arise from mutations in the DNA of tumor cells caused by either ultraviolet light or carcinogenic substance use, such as smoking. Thus, the overall tumor mutation burden (TMB) should correlate with neoantigen load and is expected to correlate with anti-tumor protective immunity (Szeto et al., 2020). While there is evidence that cancers of lower TMB have lower densities of immune infiltrates compared to cancers of higher TMB (Alexandrov et al., 2013), still, no causal relationship between TMB and anti-tumor protective immunity within cancer types had been established. Furthermore, the role of tumor mutation burden on anti-tumor protective immunity in melanoma tumors and its exact effect on resident CD8 T cells densities in particular, were unknown. If TMB truly was the dominant factor in generating tumor specific CD8 T cells and retaining them in the tumor, this would suggest that future efforts needed to be focused on improving neoantigen load and less focused on alternative immune pathways. It would also suggest that non-responding melanoma tumors to anti-PD-1 immunotherapy would unlikely benefit from other immune-based therapies until the neoantigen load in these patient tumors could be improved. While it was known that TMB in cutaneous melanoma was largely comprised of many single nucleotide variants (SNV), it had recently been established that TMB in other melanoma subtypes, including acral and mucosal melanomas (arising in areas of non-UV exposure), comprised primarily of chromosomal structural aberrations (Hayward et al., 2017). Whether or not these mutation types were immunogenic or were associated with higher immune cell densities, including those of a resident phenotype, in acral and mucosal melanomas was also unknown. We hypothesized that tumor mutation burden would be associated with protective anti-tumor immunity in melanoma tumors, and that the densities of resident CD8 T cells would correlate more strongly than other immune cell phenotypes, given their strong protective function and their specificity for tumor specific antigens. We also hypothesized that structural chromosomal aberrations would be associated with higher immune cell densities in acral and mucosal melanoma subtypes.

The aims and objectives of the current study were as follows:

1. What tumor genomic factors are responsible for the recruitment and retention of T cells in patient melanoma tumors?
2. Is TMB in cutaneous melanoma tumors an important factor and does it correlate with the densities of tumor resident CD8 T cells and other immune phenotypes?
3. Is protective immunity in melanoma tumors associated with higher tumor mutation burden?
4. Do structural chromosomal aberration mutations in acral and mucosal melanomas correlate with resident CD8 T cells and other immune phenotypes?

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Tumor Mutation Burden and Structural Chromosomal Aberrations Are Not Associated with T-cell Density or Patient Survival in Acral, Mucosal, and Cutaneous Melanomas

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ABSTRACT

Tumor mutation burden (TMB) has been proposed as a key determinant of immunogenicity in several cancers, including melanoma. The evidence presented thus far, however, is often contradictory and based mostly on RNA sequencing data for the quantification of immune cell phenotypes. Few studies have investigated TMB across acral, mucosal, and cutaneous melanoma subtypes, which are known to have different TMB. It is also unknown whether chromosomal structural mutations [structural variant (SV) mutations] contribute to the immunogenicity in acral and mucosal melanomas where such aberrations are common. We stained 151 cutaneous and 35 acral and mucosal melanoma patient samples using quantitative IHC and correlated immune infiltrate phenotypes with TMB and other genomic profiles. TMB and SVs did not correlate with the densities of $CD8^+$ lymphocytes, $CD103^+$ tumor resident T cells (Trm), $CD45RO^+$ cells, and other innate and adaptive immune cell subsets in cutaneous and acral/mucosal melanoma tumors, respectively, including in analyses restricted to the site of disease and in a validation cohort. In 43 patients with stage III treatment naïve cutaneous melanoma, we found that the density of immune cells, particularly Trm, was significantly associated with patient survival, but not with TMB. Overall, TMB and chromosomal structural aberrations are not associated with protective antitumor immunity in treatment naïve melanoma.

Introduction

Tumor infiltrating lymphocytes (TIL), particularly cytotoxic $CD8^+$ T cells, have long been recognized as a prognostic factor for patient overall survival in multiple cancers types (1) and are also associated with response to anti PD 1 and anti CTLA 4 immunotherapies (2 4). Patient tumors with a higher tumor mutation burden (TMB), the result of increased single nucleotide variants (SNV), are thought to create a proportionally higher number of neoantigens. These neoantigens are recognized as foreign by the immune system, such that an antitumor response can be generated; hence, the greater the number of neoantigens, the greater the immune infiltrate within the tumor (5, 6). This theory is based on (i) the observation that tumor types displaying a high TMB are generally more immunogenic (able to generate an immune response and have greater TILs) than low TMB tumor types (7), (ii) tumors with higher TMB are generally more responsive to anti PD 1 checkpoint inhibitor immunotherapy (5, 8, 9), and (iii) neoantigen specific T cells exist in tumors and are capable of causing tumor regression (10, 11). Despite these observations, data demonstrating a direct relationship between TMB and immune infiltration within specific cancer types are limited, weak, and often contradictory. First, most, if not all, of the studies exploring this relationship have relied on genomic/RNA sequencing data, in which immune gene expression has been used as a surrogate for measuring immune infiltration (12 16). Second, even within genomic studies, there are mixed results in the literature regarding the relationship between TMB and immune cell presence. Some studies have reported increased immune gene expression in high TMB patients within certain cancer types (13, 17), whereas other studies found no correlation in the majority of cancers (15), including melanoma (14). Yet, some studies reported weak correlations ($r < 0.16$) to describe the relationship between TMB and TIL densities (12). Some studies have also reported higher immune gene expression within low TMB patients across multiple cancer types (16). Although it is unclear what is the exact contribution TMB and neoantigen numbers have toward the magnitude and type of an antitumor immune response, it is widely assumed

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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that higher TMB is positively correlated with a higher immune infiltrate in tumors of the same cancer type.

In addition to mutations arising from SNV changes, several cancer subtypes harbor other types of mutations. In melanoma, for example, it has been shown that acral and mucosal melanomas contain more chromosomal structural rearrangement mutations, also termed as structural variant (SV) mutations, than cutaneous melanoma (18). Whether or not these types of mutations are immunogenic or correlate with an increased immune infiltrate in acral and mucosal melanoma tumors is unknown. In this study, we explored the relationship between TMB, SVs, and tumor infiltrating immune cell phenotypes, including tumor resident $CD8^+$ T cells, in 151 cutaneous and 35 acral and mucosal melanoma tumors using quantitative, multiplex immunofluorescence assays. We also validated our findings in a separate cohort of 67 patients with melanoma. We found that TMB and SVs were not associated with the presence of a broad range of immune cell phenotypes in melanoma tumors, including tumor resident $CD8^+$ T cells. Although the presence of $CD8^+$ T cells and tumor resident $CD8^+$ T cells was positively associated with patient overall survival, this was independent of TMB. Our data suggest that TMB and SVs are not determining factors for the presence or absence of immune infiltrates within melanoma tumors.

Materials and Methods

Patients

All patient biopsies were treatment naïve at the time of collection. Fresh frozen tumor tissues from 186 patients and 67 patients were used in the primary and validation cohorts, respectively, for the generation of genomic data, as described previously (18). Patients with corresponding formalin fixed, paraffin embedded (FFPE) tumor tissue were included in the study, and FFPE tumor tissue for each of the patient specimens was stored at room temperature until use for the generation of multiplex IHC data. Patients included in survival analyses did not receive any systemic immune checkpoint or targeted therapy for the duration of their clinical follow up. The study was conducted in accordance with the National Health and Medical Research Council of Australia's National Statement on Ethical Conduct in Human Research. The study was undertaken with institutional Human Ethics Review Committee approval and patient's written informed consent.

Genomic data

Genomic data were generated previously by Hayward and colleagues (18). SNVs and SVs were defined and analyzed from whole genome sequencing data as described previously (18). All SNVs represented protein coding mutations using methods described previously (18). All somatic variants for this study have been deposited in the International Cancer Genome Consortium Data Coordination Centre and are publicly available at https://dcc.icgc.org, under project ID MELA AU. The BAM files have been deposited in the European Genome phenome Archive (https://www.ebi.ac.uk/ega/) with accession number EGAS00001001552. Where appropriate, neoantigen load was calculated using the pVAC Seq (v4.0.10) pipeline (19), whereas the NetMHCpan algorithm (20) was used to estimate binding affinity to HLA genotypes.

Multiplexed immunofluorescence staining

Immunofluorescence staining was carried out on 4 μm thick sections using an Autostainer Plus (Dako Agilent Technologies) and Opal Multiplex IHC Assay Kit (Akoya Biosciences) with appropriate positive and negative controls, as reported previously (21). Briefly, FFPE tumor specimens were deparaffinized and rehydrated by xylene and an Ethanol gradient (100%, 95%, and 70%; Sigma Aldrich). Heat induced antigen retrieval (AR) was performed at 95°C for 20 minutes in pH 9 AR Buffer (Akoya Biosciences). Sections were then cooled and incubated with 3% Hydrogen Peroxide (Sigma Aldrich) for 10 minutes at room temperature, followed by incubation with a single primary antibody against CD8 (1:500; SP16, Cell Marque), CD103 (1:800; EPR4166, ref. 2, Abcam), PD L1 (1:2,000; E13LN, Cell Signaling Therapeutics), CD45RO (1:1,000; UCHL 1, Dako), CD11c (1:1,000; EP1347Y, Abcam), CD68 (1:500; Kp 1, Cell Marque), CD4 (1:500; 4B12, Biocare Medical), CD20 (1:250; L26, Biocare Medical), or SOX10 (1:800; BC34, Biocare Medical) for 30 minutes at room temperature. Following this, samples were either incubated with Opal Polymer HRP (Akoya Biosciences) for 30 minutes (CD103) or incubated with the MACH3 Probe/HRP Polymer Kit (Biocare Medical) for 10 minutes (CD8, PD L1, CD45RO, and SOX10). Finally, sections were incubated with opal fluorophores at 1:100 dilution made up in Tyramide Signal Amplification Reagent (Opal 7 Color IHC, Akoya Biosciences). The AR step was repeated for subsequent stains on the same slide. On the last staining run, DAPI was added to the sample for 5 minutes. All samples were cover slipped using Vectashield (H 1400) and left overnight to dry at 4°C.

IHC staining

Melanoma paraffin embedded tissue sections were cut and prepared for staining as described above, with appropriate positive and negative controls. Sections were incubated with a single primary antibody against CD8 (1:200; SP16, Cell Marque) or PD L1 (1:200; E13LN, Cell Signaling Therapeutics) for 45 minutes at room temperature. Sections were then incubated with a probe antibody (MACH3 Probe, Biocare Medical) specific to the species of the primary antibody for 20 minutes, washed, and then incubated further for 20 minutes with a horseradish peroxidase (HRP) conjugated antibody (MACH3 HRP, Biocare Medical) specific to the probe. Melanoma sections were then stained with 3,3′ diaminobenzidine (Biocare Medical) for 5 minutes and counterstained with hematoxylin for 5 minutes. All samples were cover slipped with xylene and left to air dry at room temperature.

Imaging and staining quantification

The Vectra 3 multispectral slide scanner was used in conjunction with Vectra 3.3 and Phenochart 1.0.4 Software (Akoya Biosciences) to image immunofluorescence staining. Images were then unmixed and analyzed using inForm 2.3.0 Software (Akoya Biosciences). Quantitative analysis was then conducted using the TIBCO Spotfire 3.3.1 from TIBCO. Lymphocyte/leukocyte densities and PD L1 expression was assessed within tumor only (defined by the presence of SOX10 positive staining). This was performed by a trained pathologist (P.M. Ferguson). In nodal metastases, any residual lymph node tissue, associated structures, and/or cells were annotated out and excluded from downstream analyses. Tumor resident $CD8^+$ T cells were quantified using the colocalization of CD103 on $CD8^+$ cells. $CD4^+$ T cells were quantified as $CD68^-$ $CD11c^-$ $CD4^+$ cells. For IHC staining quantification, a trained pathologist (P.M. Ferguson) reviewed each individual case using a conventional upright brightfield microscope and assigned a score to each based on the density of intratumoral CD8 and PD L1 staining. CD8 staining was quantified using a score from 0 to 3, where 0, absent; 1, sparse (<25%); 2, moderate (25% 75%); and 3, dense

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(>75%). PD L1 positivity was determined as the percentage of total cells within the tumor microenvironment (tumor and immune cells, predominantly macrophages) positive for PD L1 (≥1%) in both immunofluorescence and conventional IHC staining using a continuum scale from 0% to 100%.

Statistical analyses

Statistical analyses were performed using Prism version 6.0f (GraphPad Software) or TIBCO Spotfire 3.3.1. Patient characteristics were summarized using frequencies and percentages. *P* values were determined using a nonparametric Dunn multiple comparisons test for comparisons between melanoma subtypes, nonparametric log rank test for survival data, or Spearman rho test for correlations, where appropriate. *P* values less than 0.05 were considered significant. Variability in genomic and immune cell density data was expressed in terms of ± 1 SEM. Correlation plots between genomic (including TMB and SVs) and IHC staining data were performed in Prism using a Spearman correlation test. Clinical outcomes analyzed included melanoma specific survival (MSS). MSS time was calculated as the time from the date of surgical resection of stage III melanoma specimen to date of last follow up or the date of death from melanoma. Survival curves were generated using Kaplan Meier method and stratified by expression of a marker using the median staining intensity value (below and above the median as low and high groups, respectively).

Results

Cutaneous melanoma has higher TMB and immune cell infiltration relative to other subtypes

A total of 186 melanoma samples from 186 patients (Supplementary Table S1), including cutaneous (n 151), acral (n 30), and mucosal (n 5) melanomas, of which 51% were nodal metastases, were stained for CD8, CD103, CD45RO, CD4, CD20, CD68, CD11c, and PD L1 by multiplex immunofluorescence IHC (mIHC). The number of $CD8^+$ lymphocytes per mm^2 within the intratumoral region was significantly higher in cutaneous melanoma (395 ± 50/mm^2) compared with acral melanoma (164 ± 61/mm^2; $P < 0.01$) and also showed trends for being higher relative to mucosal melanoma (137 ± 43/mm^2), although this difference was not statistically significant (**Fig. 1A** and **B**). The densities of tumor resident $CD8^+$ T cells, a subset of $CD8^+$ T cells expressing the E cadherin ligand, CD103, and are thought to be enriched for T cell clones specific to tumor antigens, were also significantly higher in cutaneous melanomas (92 ± 17/mm^2) compared with acral melanomas (23 ± 9/mm^2; $P < 0.01$; **Fig. 1C**). The proportion of tumor resident cells formed a small subset of the total $CD8^+$ T cell populations across all melanoma subtypes, cutaneous (26% ± 2%), acral (13% ± 3%), and mucosal (20% ± 5%; Supplementary Fig. S1A). Acral melanoma, but not mucosal melanoma, tended to harbor a smaller proportion of resident $CD8^+$ T cells compared with cutaneous melanoma, which approached significance (P 0.054; Supplementary Fig. S1A). CD45RO, another marker associated with memory T cells, and PD L1, the ligand that binds to PD 1 on T cells and is upregulated by IFNγ, were expressed highest in cutaneous melanomas (**Fig. 1D**; Supplementary Fig. S1B), along with CD68 (Supplementary Fig. S1E). CD4, CD20, and CD11c displayed modest trends for higher expression in cutaneous melanoma (Supplementary Fig. S1C, S1D, and S1F), together indicating that acral and mucosal melanomas are less immunogenic than cutaneous melanomas and contain fewer immune populations associated with antitumor immunity.

No correlation between immune cells and SNV counts in cutaneous or acral/mucosal melanomas

TMB in cutaneous melanomas had higher protein coding SNV counts (1,281 ± 136) compared with noncutaneous melanomas (acral, 11 ± 74 and mucosal, 59 ± 12; **Fig. 1E**). However, when we analyzed the effect of TMB on TIL densities in cutaneous melanomas only, no correlative relationship between SNV counts and $CD8^+$ T cell densities (Spearman r 0.10; **Fig. 2A**) or $CD103^+CD8^+$ tumor resident T cells (Spearman r 0.04) was observed (**Fig. 2B**), despite increasing evidence that $CD103^+CD8^+$ tumor resident T cells (Trm) represent a subset of T cells specific for tumor antigens (21, 22). We also found no correlation between PD $L1^+$, $CD45RO^+$, $CD4^+$, $CD20^+$, $CD68^+$, or $CD11c^+$ cells and SNV counts (Supplementary Fig. S1G S1L). Correlative relationships between immune infiltrates and TMB did not improve when we focused our analysis on the site of disease (Supplementary Table S2). To avoid any bias on representative regions of tissue microarrays, we also stained a subset of cutaneous (n 17) samples for CD8 and PD L1 on whole slide sections and quantified $CD8^+$ T cell densities and PD L1 expression in each tumor. Consistent with our main findings, no correlation was observed between $CD8^+$ T cell density and SNV counts or PD L1 expression and SNV counts (Supplementary Fig. S2A). Cutaneous tumors with relatively high SNV counts could also simultaneously lack immune infiltration, whereas cutaneous tumors with relatively low SNV counts could show immune infiltrate presence (Supplementary Fig. S2B). We also investigated the effect of TMB and immune infiltrates in acral/mucosal melanomas (n 32). However, we found no correlative relationship between SNV counts and $CD8^+$ T cell densities (Spearman r 0.15), $CD103^+CD8^+$ TILs (Spearman r 0.05), PD L1 expression (Spearman r 0.03), CD45RO positive cells [Spearman r 0.08, not significant (ns)], or other immune cells (Supplementary Fig. S3). Finally, we sought to determine whether any of the known mutation melanoma subtypes (i.e., *BRAF, NF1*, and *NRAS*) were associated with an immune infiltrate presence in cutaneous or acral/mucosal melanomas. No significant trends were found, despite *NF1* positive cutaneous tumors showing a higher average SNV count compared with other known melanoma subtypes (Supplementary Fig. S4).

SV counts in acral and mucosal melanomas do not correlate with $CD8^+$ T-cell densities

In our cohort of patient samples, and similar to what has been published previously by our group (18), acral and mucosal melanomas displayed significantly higher SV mutations (acral, 160 ± 3; $P < 0.0001$ and mucosal, 154 ± 5; $P < 0.01$) compared with cutaneous melanomas (75 ± 4; **Fig. 2C**), despite having lower TMB. We, therefore, sought to investigate whether higher SV counts within acral and mucosal melanoma might be correlated with an immune presence. We found no significant correlations between SV counts and densities of $CD8^+$ T lymphocytes (Spearman r 0.34; **Fig. 2D**) or $CD103^+CD8^+$ tumor resident T cells (Spearman r 0.28; **Fig. 2E**). We also did not found a significant correlation for PD L1 expression (Spearman r 0.22), $CD4^+$ T cells (Spearman r 0.3), $CD68^+$ cells (Spearman r 0.3), and SV counts (Supplementary Fig. S5A, S5C, and S5E). $CD45RO^+$, $CD11c^+$, and $CD20^+$ immune cells were inversely correlated with SV counts (Supplementary Fig. S5B, S5D, and S5F). We investigated this further for $CD8^+$ T cell density and PD L1 expression on whole slide sections in acral (n 21) and mucosal (n 8) melanoma samples containing a higher range of SV counts (52 1,148). Consistent with our findings, no positive correlation was observed between $CD8^+$ T cell density and SV counts or PD L1 expression and SV counts (Supplementary Fig. S6A and S6B), indicating that SV mutations are not a driver of immunity in acral/mucosal melanomas.

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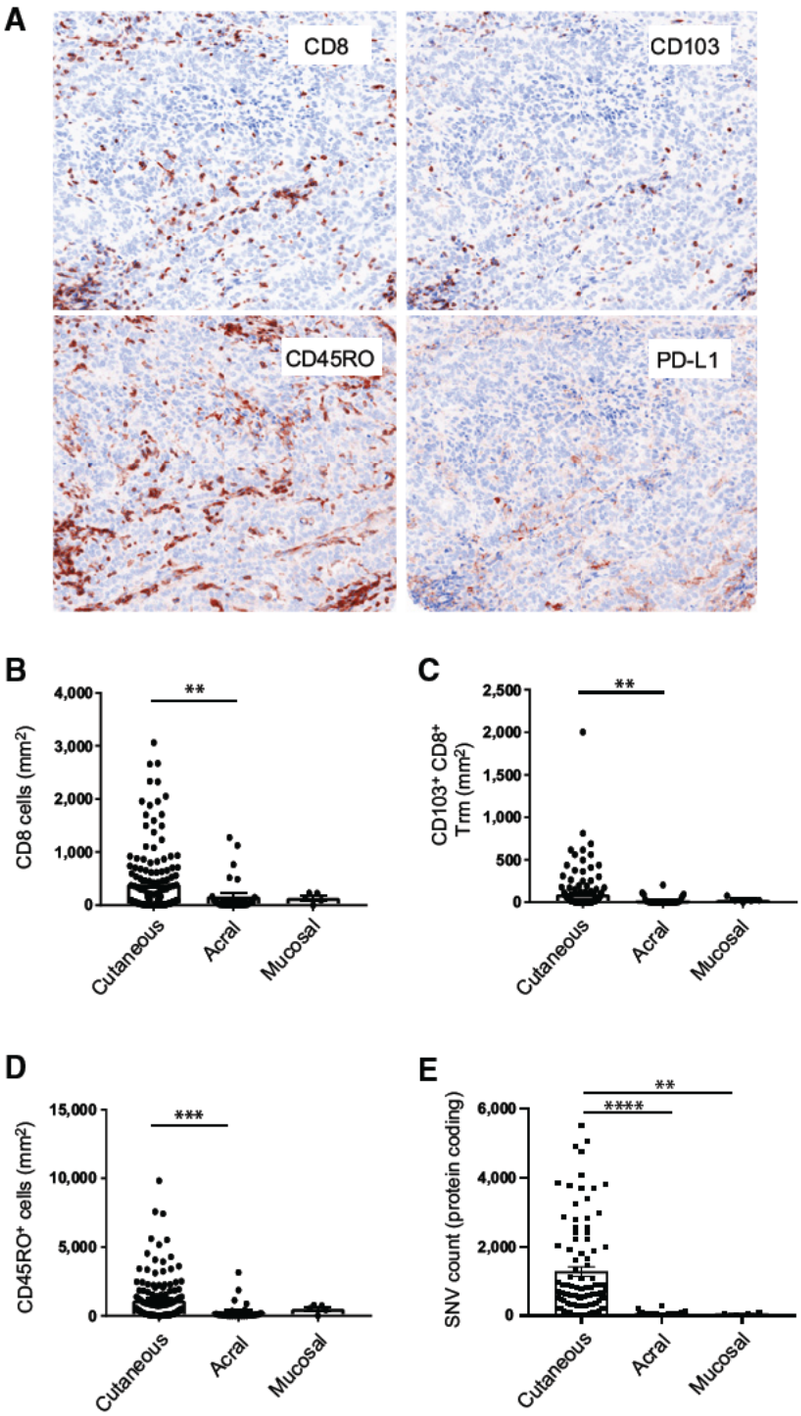

Figure 1.

Density of immune cell infiltration in cutaneous, acral, and mucosal melanoma tumors. **A,** Representative single color images of mIHC staining for CD8, CD103, PD L1, and CD45RO immune markers in melanoma tumors (images taken at 20× magnification). **B,** The mean number of $CD8^+$ T cells per mm^2 of tumor in cutaneous ($n = 151$), acral ($n = 30$), and mucosal ($n = 5$) melanomas. **C,** The mean number of $CD103^+CD8^+$ tumor resident T cells (Trm) per mm^2 in cutaneous ($n = 151$), acral ($n = 30$), and mucosal ($n = 5$) melano mas. **D,** The mean number of $CD45RO^+$ cells per mm^2 in cutaneous ($n = 151$), acral ($n = 30$), and mucosal ($n = 5$) melanomas. **E,** The mean number of SNVs in cutaneous ($n = 97$), acral ($n = 28$), and mucosal ($n = 4$) melano mas. Error bars are ± 1 SEM. *P* values were determined using a nonparametric Dunn multiple comparisons test for comparisons between melanoma subtypes (**, $P \le 0.01$; ***, $P \le 0.001$; ****, $P \le 0.0001$).

$CD8^+$ T-cell densities are independent of TMB in a validation cohort

To validate our findings, we stained whole slide sections for CD8 and PD L1 using conventional IHC in an independent cohort of patient samples (*n* 67; Supplementary Table S3; *n* 56, cutaneous melanoma and *n* 11, acral/mucosal melanoma) and correlated staining with genomic factors of mutation burden. When we compared average SNV counts by density of CD8 staining (0 3) in cutaneous melanoma samples, we found no significant changes in SNV count between all four levels of CD8 density (**Fig. 3A**). Cutaneous melanoma tumors with the least (0) and highest (3) $CD8^+$ T cell infiltration had almost identical average SNV counts (1,647 ± 692 and 1,579 ± 405, respectively). We also explored the predicted neoantigen count from tumor burden in each sample with CD8 density, but found no significant trends (**Fig. 3B**). PD L1 expression was weakly cor related with SNV counts (Spearman *r* 0.27; *P* 0.04; Sup plementary Fig. S7A) and with predicted neoantigens (Spearman

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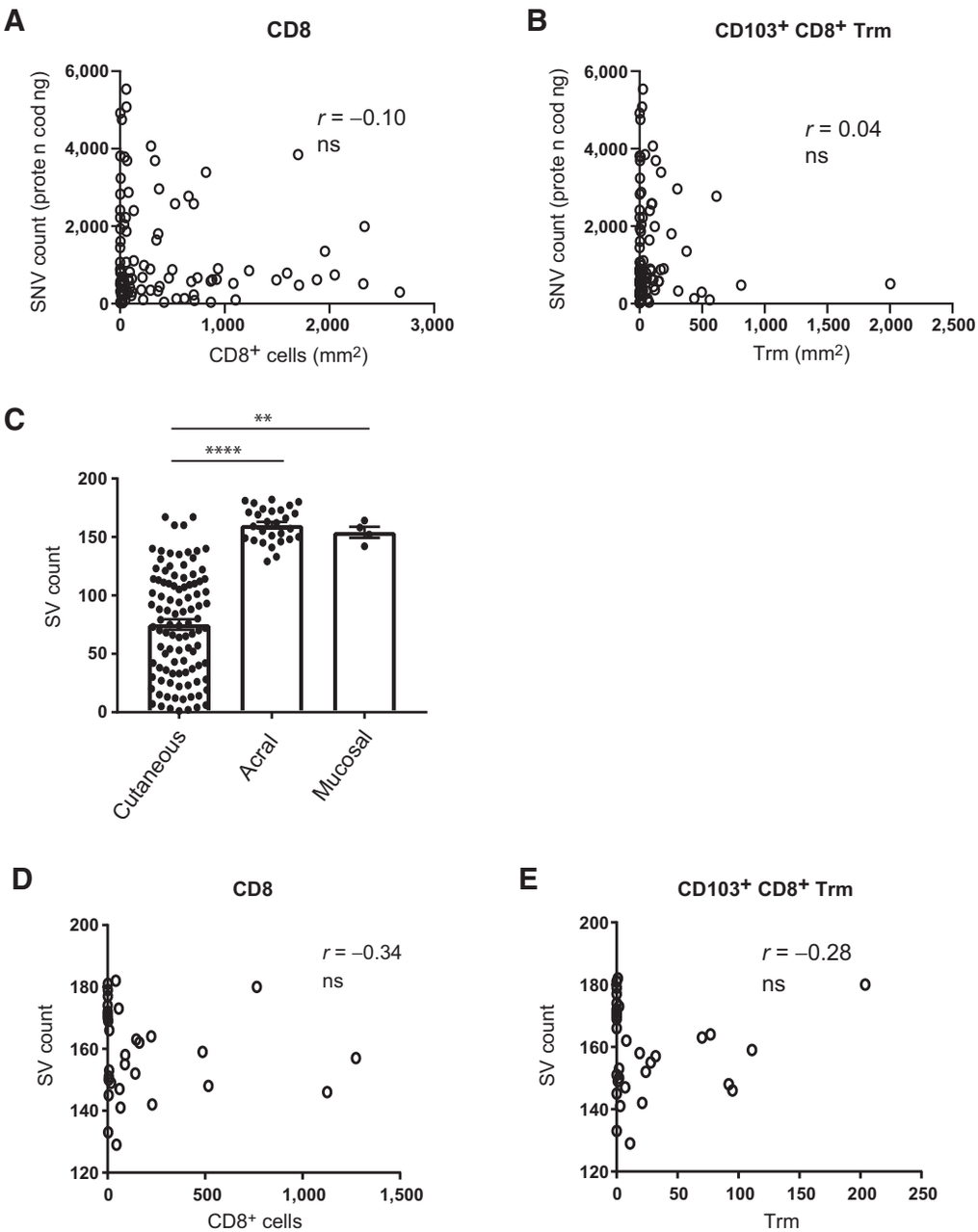

Figure 2.

Correlation between $CD8^+$ T cells, $CD103^+CD8^+$ T cells, and TMB and SV counts in cutaneous and acral/mucosal melanomas. **A,** Spearman correlation (*r*) between SNV counts and number of $CD8^+$ T lymphocytes per mm^2 of tumor in ($n = 97$) cutaneous melanomas. **B,** Spearman correlation (*r*) between SNV counts and number of $CD103^+CD8^+$ Trm per mm^2 of tumor in ($n = 97$) cutaneous melanomas. **C,** The mean number of SVs in cutaneous ($n = 97$), acral ($n = 28$), and mucosal ($n = 4$) melanomas. **D,** Spearman correlation (*r*) between SV counts and $CD8^+$ T lymphocytes per mm^2 of tumor in acral and mucosal melanoma tumors ($n = 32$). **E,** Spearman correlation (*r*) between SV counts and $CD103^+CD8^+$ Trm per mm^2 of tumor in acral and mucosal melanoma tumors ($n = 32$). *P* values were determined using a nonparametric Spearman test [**, $P \le 0.01$; ****, $P \le 0.0001$; ns, not significant ($P > 0.05$)].

r 0.27; *P* 0.04; Supplementary Fig. S7B). Although the number of acral/mucosal samples in this cohort was small (*n* 11), we found no significant trends between CD8 density and average SV counts (**Fig. 3C**) or PD L1 expression and SV counts (Supplementary Fig. S7C), consistent with our previous cohort findings.

MSS for patients with stage III cutaneous melanoma is independent of TMB

We next explored the relationship between TMB and immune infiltrates in a subset of 43 patients with treatment naïve stage III cutaneous melanoma to dissect the role of TMB in patient survival. The median follow up time in this cohort was 24 months

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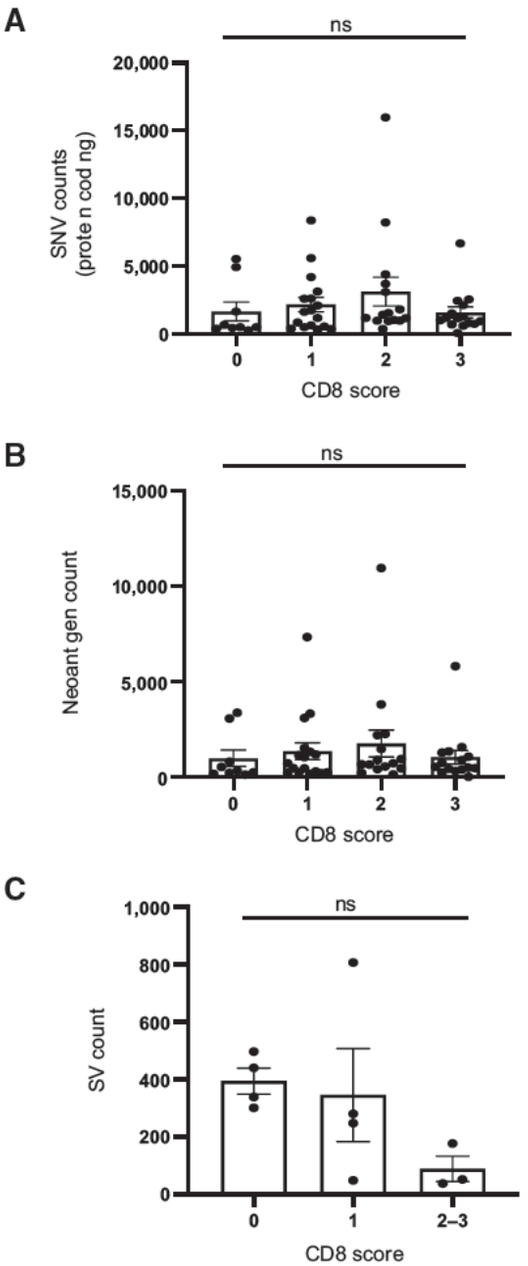

Figure 3.

Validation cohort. **A,** Mean SNVs in cutaneous melanoma tumors ($n = 56$) with varying degrees of intratumoral $CD8^+$ T lymphocyte infiltration (0 3), where 0, absent CD8 infiltration; 1, sparse CD8 infiltration; 2, moderate CD8 infiltration; and 3, dense CD8 infiltration. **B,** Mean neoantigen counts in cutaneous melanoma tumors ($n = 56$) with varying degrees of $CD8^+$ lymphocyte infiltration (0 3). **C,** Mean SV count in samples with varying CD8 scores (0 2) in acral ($n = 8$) and mucosal ($n = 3$) melanomas. Error bars are ± 1 SEM. *P* values were determined using a Dunn multiple comparisons test (ns, $P > 0.05$ considered not significant).

(range, 1 156 months). In each case, the median of each variable was used to separate patients into high and low groups for each variable. Survival analysis found that patients with high TMB (average SNV count, 1,494 and median SNV count, 979) had no significant survival advantage compared with patients with low TMB (average SNV count, 282 and median SNV count, 296; *P* 0.34; **Fig. 4A**). Despite this, patients with high $CD8^+$ T cell densities had significantly longer median MSS (131 months) compared with patients with low $CD8^+$ T cell densities [21 months; HR, 2.5 (1.12 5.8); *P* 0.024; **Fig. 4B**). Tumor resident $CD8^+$ T cells most associated with survival of all the prognostic markers evaluated [HR, 4.1 (1.6 10.0); *P* 0.001; **Fig. 4C**]. High PD L1 expression (>1% cells) also associated with better survival [HR, 2.4 (0.4 1.0); *P* 0.035] compared with patients with low PD L1 expression (Supplementary Fig. S8A). We next investigated whether patient groups with high CD8, $CD103^+CD8^+$ Trm, and PD L1 might have an average increase in TMB compared with CD8, $CD103^+CD8^+$ Trm, and PD L1 low patient groups. We found no differences in the SNV counts between high and low groups (Supplementary Table S4), indicating that the survival advantage associated with high immune cell densities was independent of TMB.

Discussion

We showed that TMB does not correlate with immune cell infil tration in cutaneous and acral/mucosal melanoma tumors, including for $CD103^+CD8^+$ tumor resident memory T cell numbers. These findings remained consistent even when we focused on the site of disease. In melanoma subtypes, where a bias exists for chromosomal structural aberration mutations, we also showed a lack of correlation between these mutation types and the presence of immune cells, likely indicating that these mutations are also not robust elicitors of an antitumor immune response in acral and mucosal melanoma tumors. We also demonstrated that the prognostic effects of immune cell densities, particularly $CD103^+$ tumor resident $CD8^+$ T cells, are not necessarily associated with TMB in cutaneous melanoma. Although it is possible that increased TMB results in enhanced T cell receptor diversity and, therefore, improved quality of the immune response, the fact that TMB did not have an impact on melanoma specific patient survival suggests that it is unlikely to be a key determinant of the protective immunity. Together, our results suggest that immune infiltration in melanomas is independent of TMB, highlighting the importance of other driving factors. Limitations to this study include the moderate sample size of the two patient cohorts, especially the number of acral and mucosal samples with genomic data, and, therefore, it is possible that the study is not powered to detect a significant association between TMB/SVs and immune infiltrates, particularly if those associations are very weak. In addition, no functional characterization of antitumor immunity between high and low TMB tumors was performed. However, despite moderate sample size, known prognostic immune cells/markers, including $CD8^+$ T cells, Trm, and PD L1, were prognostic in this study, whereas TMB was not. A strength to the study was the use of mIHC, which allowed for an accurate assessment of intratumoral lymphocytes and the character ization of complex phenotypes ($CD103^+CD8^+$ Trm). Overall, our data are in line with the results of a number of other studies (14, 15), including a pan cancer analysis utilizing The Cancer Genome Atlas database, where no significant correlations were identified between somatic mutation numbers and $CD8^+$ T cell infiltration scores in the majority of cancers (15).

There are many reasons why TMB may not directly correlate with the magnitude of an antitumor immune response. (i) It is possible that not all the predicted neoantigens from sequencing data are recogniz able to the immune system. For example, tumors that have down regulated MHC class I expression may limit the recognition of neoantigens. (ii) It is probable that neoantigens are not equally

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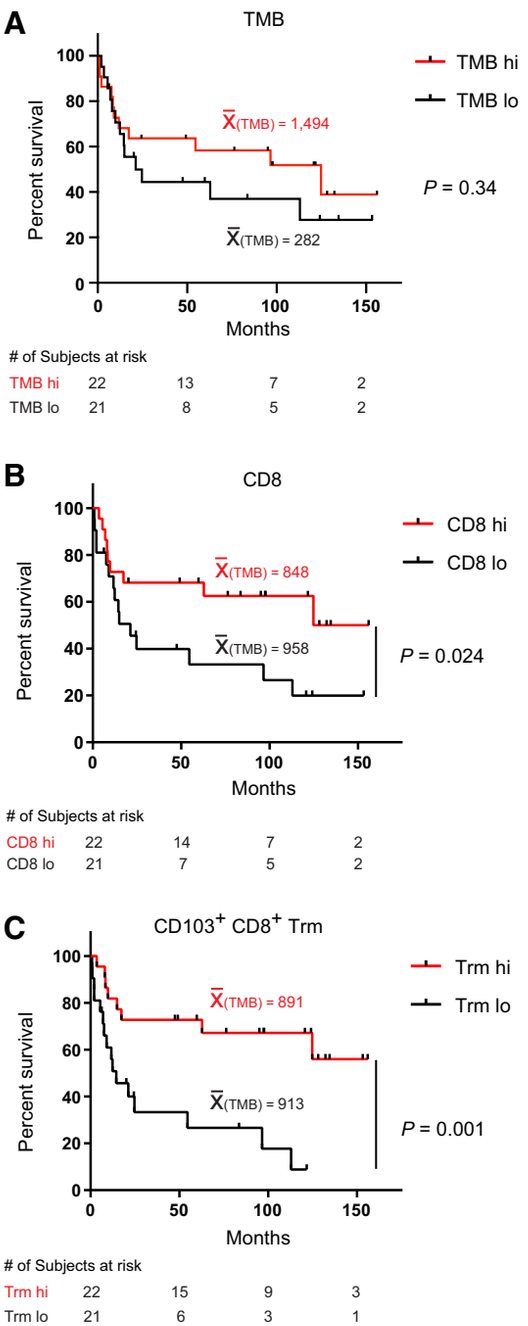

Figure 4.

Prognostic effect of TMB relative to $CD8^+$ T cells and Trm in treatment naïve melanoma patients. **A,** Kaplan Meier curve for 43 patients with stage III cutaneous melanoma with high (hi) TMB [greater than the median (76,310); red] and low (lo) TMB (lower than median; black). **B,** Kaplan Meier curve for 43 patients with stage III cutaneous melanoma with high numbers of $CD8^+$ T cells (greater than the median = 175 cells/mm^2; red) and low numbers of $CD8^+$ T cells (lower than median; black). **C,** Kaplan Meier curve for 43 patients with stage III cutaneous melanoma with high numbers of $CD103^+CD8^+$ Trm (greater than the median = 8 cells/mm^2; red) and low numbers of $CD103^+CD8^+$ Trm (lower than median; black). For each curve, the average TMB is given for high and low groups. *P* values were determined using a nonparametric log rank test and are indicated in panels.

immunogenic and, therefore, may elicit stronger or weaker immune responses depending on the quality of the neoantigen. Indeed, there is evidence to show that clonal neoantigens, rather than subclonal neoantigens, are more associated with immunity in tumors (11). However, this is unlikely to be a strong confounding variable in melanoma, where the overwhelming majority of neoantigens is classed as clonal (23). (iii) Nonmutated antigens, such as cancer testis antigens, which can be expressed in melanomas, may also contribute significantly to antitumor immune responses. (iv) It is possible that TMB is only one of many more dominant factors responsible for recruiting and retaining immune cells in tumors, including the presence of $Batf3^+$ dendritic cells, upregulation of the Wnt signaling pathway (24, 25), and the prevalence of myeloid suppressor cells (26), among others. In conclusion, our data indicate that TMB, SV rearrangements, and known melanoma mutation subtypes are not determining factors for the presence or absence of immune infiltrates within melanoma tumors.

Disclosure of Potential Conflicts of Interest

S.N. Lo reports grants from National Health and Medical Research Council of Australia (program grant) during the conduct of the study. R.P.M. Saw reports personal fees and other from Novartis (advisory board), Bristol-Myers Squibb (speaking honoraria), MSD (advisory board), and QBiotics (advisory board) outside the submitted work. J.F. Thompson reports grants from National Health and Medical Research Council of Australia (program grant) during the conduct of the study, as well as personal fees from GlaxoSmithKline (honoraria and travel support), BMS Australia (honoraria for advisory board participation), MSD Australia (honoraria for advisory board participation), and Provectus Inc. (honoraria and travel support) outside the submitted work. A.M. Menzies reports personal fees from Bristol-Myers Squibb (advisory board), MSD (advisory board), Novartis (advisory board), Roche (advisory board), and Pierre Fabre (advisory board) outside the submitted work. G.V. Long reports personal fees from Aduro (consultant adviser), Amgen (consultant adviser), Bristol-Myers Squibb (consultant adviser), Highlight Therapeutics S.L (consultant adviser), Mass-Array (consultant adviser), Merck (consultant adviser), MSD (consultant adviser), Novartis (consultant adviser), OncoSec Medical (consultant adviser), Pierre Fabre (consultant adviser), Roche (consultant adviser), QBiotics (consultant adviser), Skyline DX (consultant adviser), and Sandoz (consultant adviser) outside the submitted work. J.V. Pearson reports other from genomiQa PTY LTD (genome analysis company; cofounder, equity holder) outside the submitted work. N. Waddell reports grants from National Health and Medical Research Council of Australia (fellowship) during the conduct of the study and other from genomiQa PTY LTD (cofounder, board member, and equity holder) outside the submitted work. N.K. Hayward reports grants from National Health and Medical Research Council (partial funding of work) during the conduct of the study. R.A. Scolyer reports grants from National Health and Medical Research Council of Australia (program grant and fellowship grant) and nonfinancial support from Melanoma Institute Australia during the conduct of the study, as well as personal fees from Royal Prince Alfred Hospital (salary), Qbiotics, Novartis, MSD Sharp & Dohme, NeraCare, Amgen, Bristol-Myers Squibb, Myriad Genetics, and GlaxoSmithKline outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

J. Edwards: Conceptualization, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **P.M. Ferguson:** Formal analysis, investigation, methodology. **S.N. Lo:** Formal analysis. **I. Pires da Silva:** Visualization, methodology, writing–review and editing. **A.J. Colebatch:** Investigation, methodology, writing–review and editing. **H. Lee:** Methodology. **R.P.M. Saw:** Resources, writing–review and editing. **J.F. Thompson:** Resources, writing–review and editing. **A.M. Menzies:** Resources, writing–review and editing. **G.V. Long:** Conceptualization, resources, supervision, funding acquisition, investigation, visualization, methodology, project administration, writing–review and editing. **F. Newell:** Resources, data curation, writing–review and editing. **J.V. Pearson:** Data curation, writing–review and editing. **N. Waddell:** Data curation, writing–review and editing. **N.K. Hayward:** Resources, data curation. **P.A. Johansson:** Resources, data curation, writing–review and editing. **G.J. Mann:** Resources, data curation. **R.A. Scolyer:** Conceptualization, resources, supervision, funding acquisition, investigation, visualization, methodology, project

administration, writing–review and editing. **U. Palendira:** Conceptualization, resources, supervision, investigation, visualization, project administration, writing–review and editing. **J.S. Wilmott:** Conceptualization, resources, software, supervision, funding acquisition, investigation, visualization, methodology, project administration, writing–review and editing.

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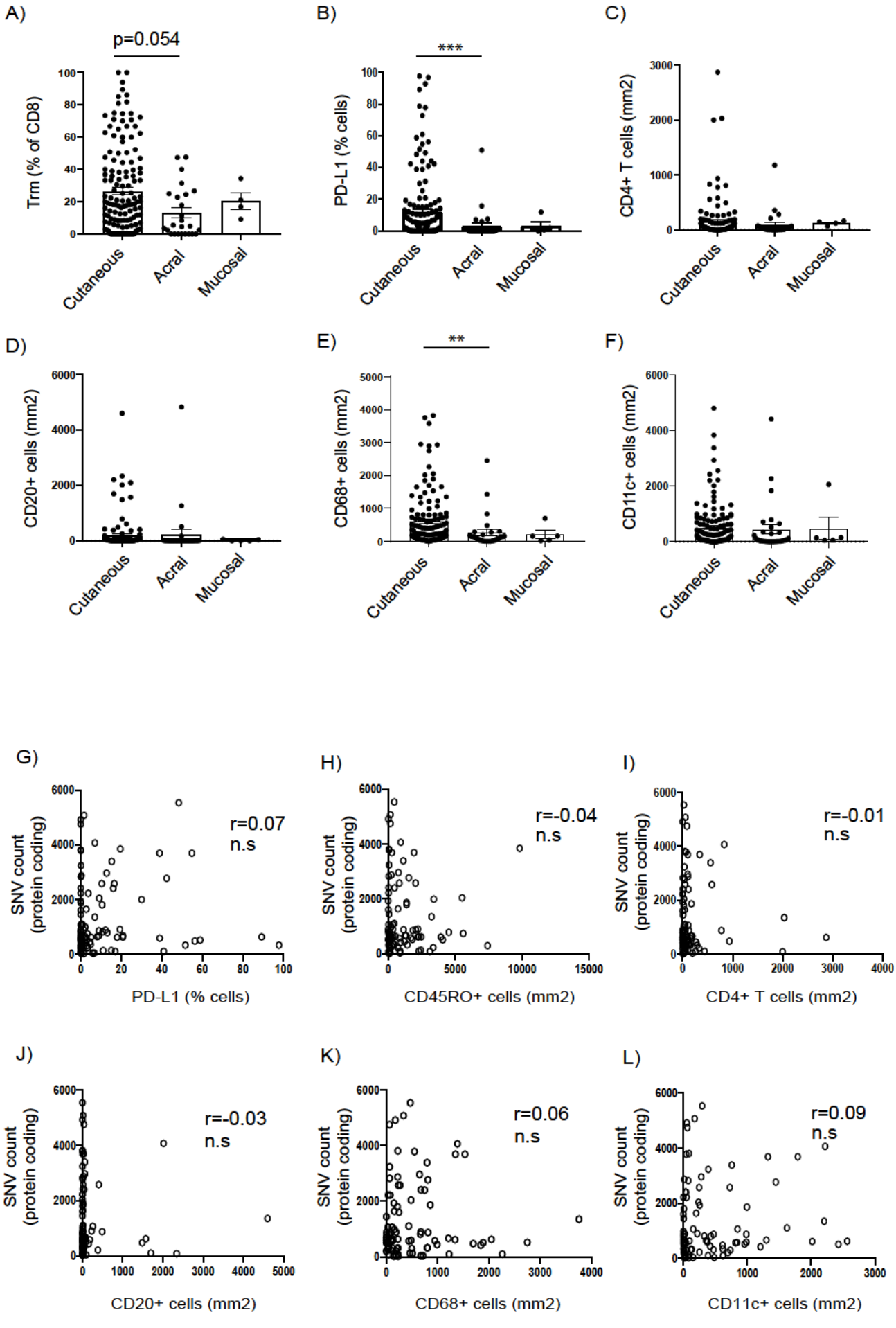
References

1. Barnes TA, Amir E. HYPE or HOPE: the prognostic value of infiltrating immune cells in cancer. Br J Cancer 2017;117:451–60.
2. Gide TN, Quek C, Menzies AM, Tasker AT, Shang P, Holst J, et al. Distinct immune cell populations define response to anti-PD-1 monotherapy and anti-PD-1/anti-CTLA-4 combined therapy. Cancer Cell 2019;35:238–55.
3. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 2014;515:568–71.
4. Vilain RE, Menzies AM, Wilmott JS, Kakavand H, Madore J, Guminski A, et al. Dynamic changes in PD-L1 expression and immune infiltrates early during treatment predict response to PD-1 blockade in melanoma. Clin Cancer Res 2017;23:5024–33.
5. Maleki Vareki S. High and low mutational burden tumors versus immunologically hot and cold tumors and response to immune checkpoint inhibitors. J Immunother Cancer 2018;6:157.
6. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science 2015;348:69–74.
7. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature 2013;500:415–21.
8. Yarchoan M, Hopkins A, Jaffee EM. Tumor mutational burden and response rate to PD-1 inhibition. N Engl J Med 2017;377:2500–1.
9. Champiat S, Ferte C, Lebel-Binay S, Eggermont A, Soria JC. Exomics and immunogenics: bridging mutational load and immune checkpoints efficacy. Oncoimmunology 2014;3:e27817.
10. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Garraway genomic correlates of response to CTLA-4 blockade in metastatic melanoma. Science 2015;350:207–11.
11. McGranahan N, Furness AJ, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 2016;351:1463–9.
12. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell 2015;160:48–61.
13. Safonov A, Jiang T, Bianchini G, Gyorffy B, Karn T, Hatzis C, et al. Immune gene expression is associated with genomic aberrations in breast cancer. Cancer Res 2017;77:3317–24.
14. Spranger S, Luke JJ, Bao R, Zha Y, Hernandez KM, Li Y, et al. Density of immunogenic antigens does not explain the presence or absence of the T-cell-inflamed tumor microenvironment in melanoma. Proc Natl Acad Sci U S A 2016;113:E7759–E68.
15. Varn FS, Wang Y, Mullins DW, Fiering S, Cheng C. Systematic pan-cancer analysis reveals immune cell interactions in the tumor microenvironment. Cancer Res 2017;77:1271–82.
16. Wang X, Li M. Correlate tumor mutation burden with immune signatures in human cancers. BMC Immunol 2019;20:4.
17. Giannakis M, Mu XJ, Shukla SA, Qian ZR, Cohen O, Nishihara R, et al. Genomic correlates of immune-cell infiltrates in colorectal carcinoma. Cell Rep 2016;15:857–65.
18. Hayward NK, Wilmott JS, Waddell N, Johansson PA, Field MA, Nones K, et al. Whole-genome landscapes of major melanoma subtypes. Nature 2017;545:175–80.
19. Hundal J, Carreno BM, Petti AA, Carreno BM, Petti AA, Linette GP, et al. pVAC-seq: a genome-guided in silico approach to identifying tumor neoantigens. Genome Med 2016;8:11.
20. Hoof I, Peters B, Sidney J, Pedersen LE, Sette A, Lund O, et al. NetMHCpan, a method for MHC class I binding prediction beyond humans. Immunogenetics 2009;61:1–13.
21. Edwards J, Wilmott JS, Madore J, Gide TN, Quek C, Tasker A, et al. CD103(+) tumor-resident CD8(+) T cells are associated with improved survival in immunotherapy-naive melanoma patients and expand significantly during anti-PD-1 treatment. Clin Cancer Res 2018;24:3036–45.
22. Djenidi F, Adam J, Goubar A, Durgeau A, Meurice G, de Montpreville V, et al. CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. J Immunol 2015;194:3475–86.
23. McGranahan N, Swanton C. Clonal heterogeneity and tumor evolution: past, present, and the future. Cell 2017;168:613–28.
24. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. Nature 2015;523:231–5.
25. Spranger S, Dai D, Horton B, Gajewski TF. Tumor-residing Batf3 dendritic cells are required for effector T cell trafficking and adoptive T cell therapy. Cancer Cell 2017;31:711–23.
26. Peranzoni E, Lemoine J, Vimeux L, Feuillet V, Barrin S, Kantari-Mimoun C, et al. Macrophages impede CD8 T cells from reaching tumor cells and limit the efficacy of anti-PD-1 treatment. Proc Natl Acad Sci U S A 2018;115:E4041–E50.

Chapter 2 Supplementary Tables and Figures

Supplementary Figure 1



Supplementary Table 1. Clinical charcteristics of patients in study

Clinical characteristics	Number of patient specimens n (%)			Number of patient specimens with genomic data available n (%)		
Demographic	186 (100)			129 (100)		
Male	116 (62)			77 (60)		
Female	70 (38)			52 (40)		
Age (median, years)	60			61.5		
Melanoma Subtype						
Cutaneous	151 (81)			97 (75)		
Acral	30 (16)			28 (22)		
Mucosal	5 (3)			4 (3)		
Specimen Type	*Total*	*Cutaneous* (%)	*Acral* (%)	*Total*	*Cutaneous* (%)	*Acral* (%)
Primary (P)	40 (22)	28 (19)	9 (30)	37 (28.5)	25 (26)	9 (32)
In-transit metastasis (ITM)	21 (11)	13 (7)	8 (27)	10 (8)	3 (3)	7 (25)
Local recurrence	4 (2)	4 (3)	0 (0)	1 (<1)	1 (1)	0 (0)
Regional node metastasis (RNM)	88 (47)	74 (49)	13 (43)	58 (45)	45 (46)	12 (43)
Regional field skin and soft tissue	6 (3)	6 (4)	0 (0)	5 (4)	5 (5)	0 (0)
Distant nodal metastasis (DNM)	7 (4)	7 (5)	0 (0)	4 (3)	4 (4)	0 (0)
Distant metastasis (DM)	20 (11)	19 (13)	0 (0)	14 (11)	14 (15)	0 (0)
Subtype of cutaneous melanoma						
Superficial spreading melanoma (SSM)	47 (31)			29 (30)		
Nodular melanoma (NM)	59 (39)			40 (41)		
Desmoplastic melanoma	11 (7)			7 (7)		
Lentigo maligna melanoma	4 (3)			2 (2)		
Occult	7 (5)			4 (4)		
Not-classifiable	11 (7)			5 (5)		
SSM with NM	12 (8)			10 (11)		
Molecular subtype						
BRAF	(-)			49 (38)		
NF1	(-)			16 (12)		
NRAS	(-)			35 (27)		
Triple wild-type	(-)			27 (21)		
HRAS	(-)			2 (1.5)		

Supplemetary Table 2. Spearman correlations between immune infiltrates and TMB by specimen type and site (cutaneous melanoma)

Analysis subtype (specimen type)	n	Y variable	X variable	p-value	Rank r
Primary melanoma	**25**	Mutation burden	CD8	0.66	0.09
		Mutation burden	CD103+ CD8+ Trm	0.44	0.16
		Mutation burden	PD-L1	0.4	0.18
		Mutation burden	CD45RO	0.86	-0.04
		Mutation burden	CD4+ T cells	0.75	-0.07
		Mutation burden	CD20	0.63	-0.1
		Mutation burden	CD68	0.93	0.02
		Mutation burden	CD11c	0.8	0.05
Regional node metastasis	**45**	Mutation burden	CD8	0.28	-0.16
		Mutation burden	CD103+ CD8+ Trm	0.4	-0.13
		Mutation burden	PD-L1	0.6	-0.08
		Mutation burden	CD45RO	0.42	-0.12
		Mutation burden	CD4+ T cells	0.31	-0.17
		Mutation burden	CD20	0.54	-0.11
		Mutation burden	CD68	0.42	-0.14
		Mutation burden	CD11c	0.65	0.08
Distant metastasis	**18**	Mutation burden	CD8	0.27	-0.27
		Mutation burden	CD103+ CD8+ Trm	0.14	-0.36
		Mutation burden	PD-L1	0.83	-0.06
		Mutation burden	CD45RO	0.61	-0.13
		Mutation burden	CD4+ T cells	0.99	0
		Mutation burden	CD20	0.87	0.04
		Mutation burden	CD68	0.33	0.25
		Mutation burden	CD11c	0.45	0.19
Analysis subtype (site of disease)		**Y variable**	**X variable**	**p-value**	**Rank r**
Nodal metastasis	**49**	Mutation burden	CD8	0.39	-0.12
		Mutation burden	CD103+ CD8+ Trm	0.5	-0.1
		Mutation burden	PD-L1	0.67	-0.06
		Mutation burden	CD45RO	0.51	-0.1
		Mutation burden	CD4+ T cells	0.4	-0.14
		Mutation burden	CD20	0.63	-0.08
		Mutation burden	CD68	0.5	-0.11
		Mutation burden	CD11c	0.49	0.11
Subcutaneous	**31**	Mutation burden	CD8	0.18	0.25
		Mutation burden	CD103+ CD8+ Trm	0.17	0.26
		Mutation burden	PD-L1	0.07	0.33
		Mutation burden	CD45RO	0.3	0.21
		Mutation burden	CD4+ T cells	0.31	0.19
		Mutation burden	CD20	0.58	0.1
		Mutation burden	CD68	0.22	0.23
		Mutation burden	CD11c	0.29	0.2
Bowel metastasis	**7**	Mutation burden	CD8	0.54	-0.29
		Mutation burden	CD103+ CD8+ Trm	0.18	-0.58
		Mutation burden	PD-L1	0.48	-0.46
		Mutation burden	CD45RO	0.39	-0.39
		Mutation burden	CD4+ T cells	0.18	-0.57
		Mutation burden	CD20	0.14	-0.61
		Mutation burden	CD68	0.94	-0.04
		Mutation burden	CD11c	0.76	0.14
Brain metastasis	**7**	Mutation burden	CD8	0.43	-0.36
		Mutation burden	CD103+ CD8+ Trm	0.94	0.04
		Mutation burden	PD-L1	0.09	0.68
		Mutation burden	CD45RO	1	0
		Mutation burden	CD4+ T cells	0.7	0.18
		Mutation burden	CD20	0.76	0.14
		Mutation burden	CD68	0.12	0.64
		Mutation burden	CD11c	0.25	0.5

Supplementary Table 3. Clinical charcteristics of patient specimens in validation cohort

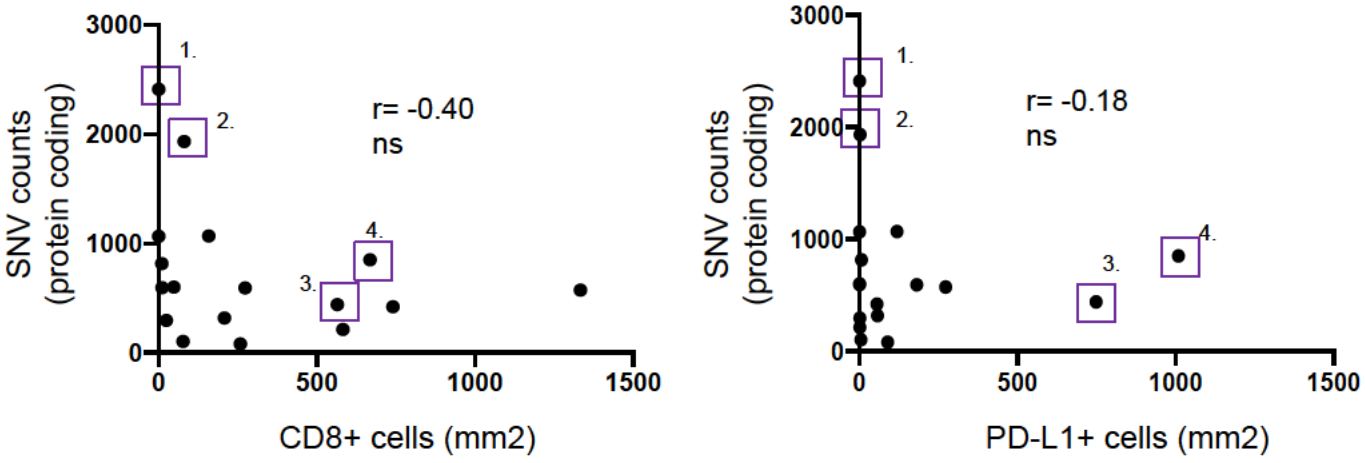
Clinical characteristics	Number of patient specimens n (%)
Total	67 (100)
Melanoma Subtype	
Cutaneous	56 (84)
Acral	8 (12)
Mucosal	3 (4)
Specimen Type	
Primary (P)	5 (7)
Local recurrence	3 (4)
Regional field skin and soft tissue	10 (15)
Metastasis (M)	46 (69)
Occult	3 (4)
Subtype of cutaneous melanoma	
Superficial spreading melanoma (SSM)	17 (30)
Nodular melanoma (NM)	19 (34)
SSM with NM	1 (2)
Desmoplastic melanoma	6 (11)
Lentigo maligna melanoma	2 (3.5)
Occult	7 (12.5)
Naevoid melanoma	2 (3.5)
Not classifiable	2 (3.5)

Supplementary Table 4. Mean and median SNV counts for patient groups defined as high or low for each variable

Patient groups	Mean SNV	Median SNV
TMB		
High	1494	979
Low	282	296
CD8		
High	848	616
Low	958	639
Trm		
High	891	619
Low	913	593
PD-L1		
High	870	619
Low	935	593

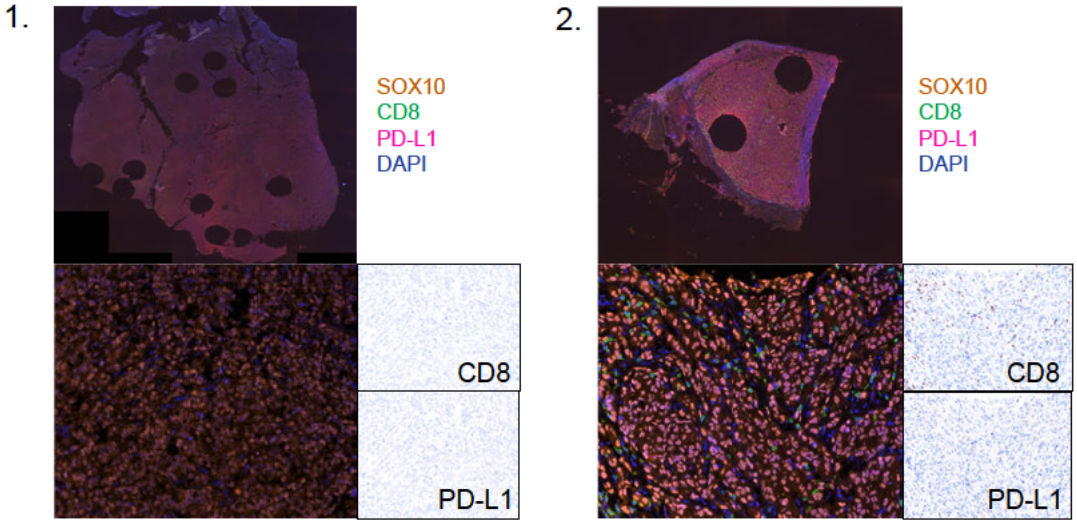
Supplementary Figure 2

A)

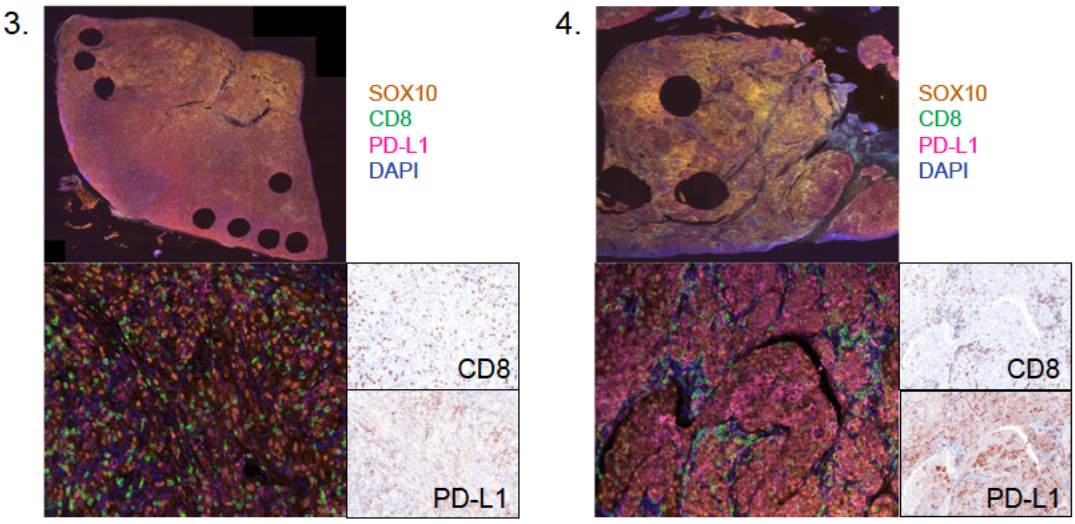


B)

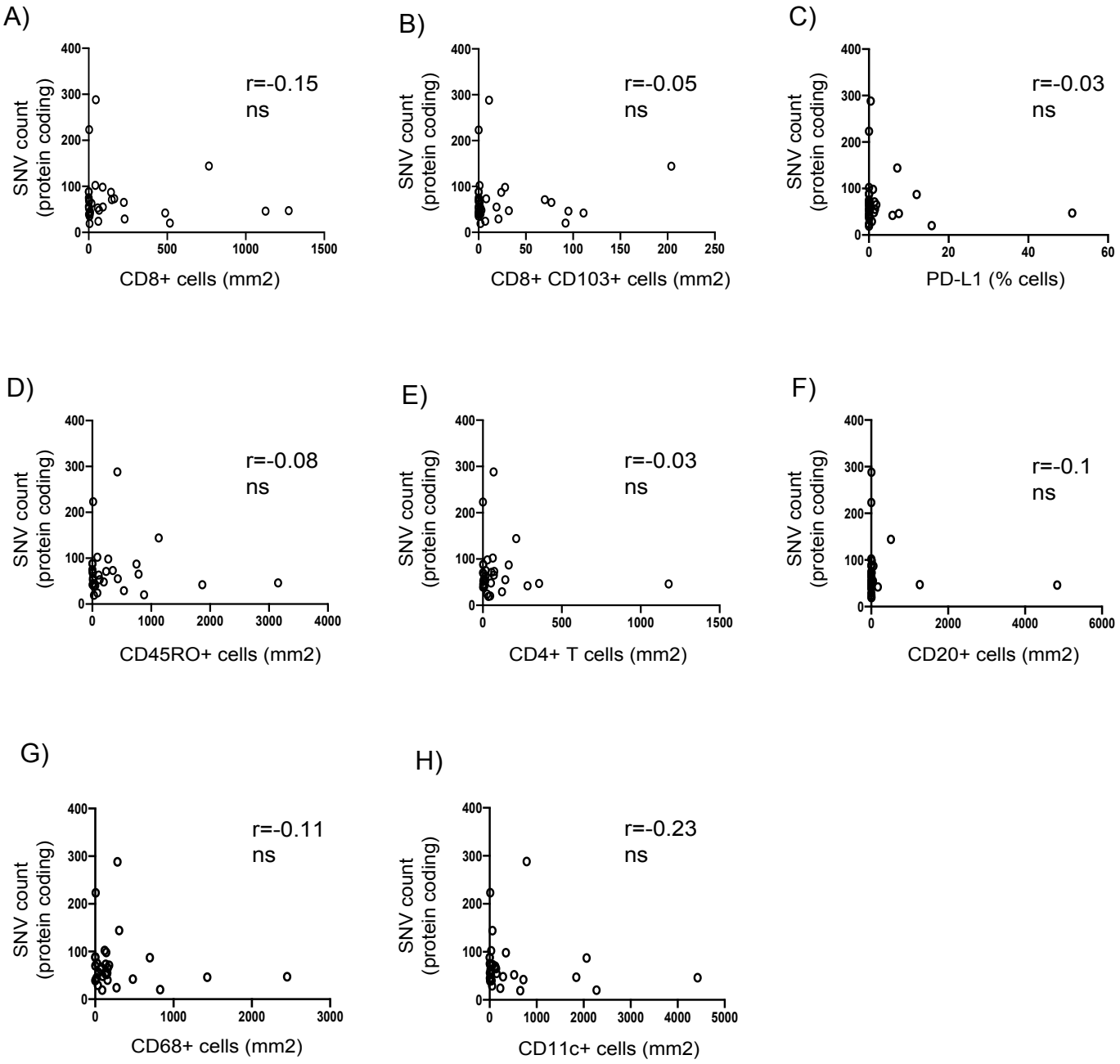
Cutaneous melanoma with high SNV



Cutaneous melanoma with relatively low SNV

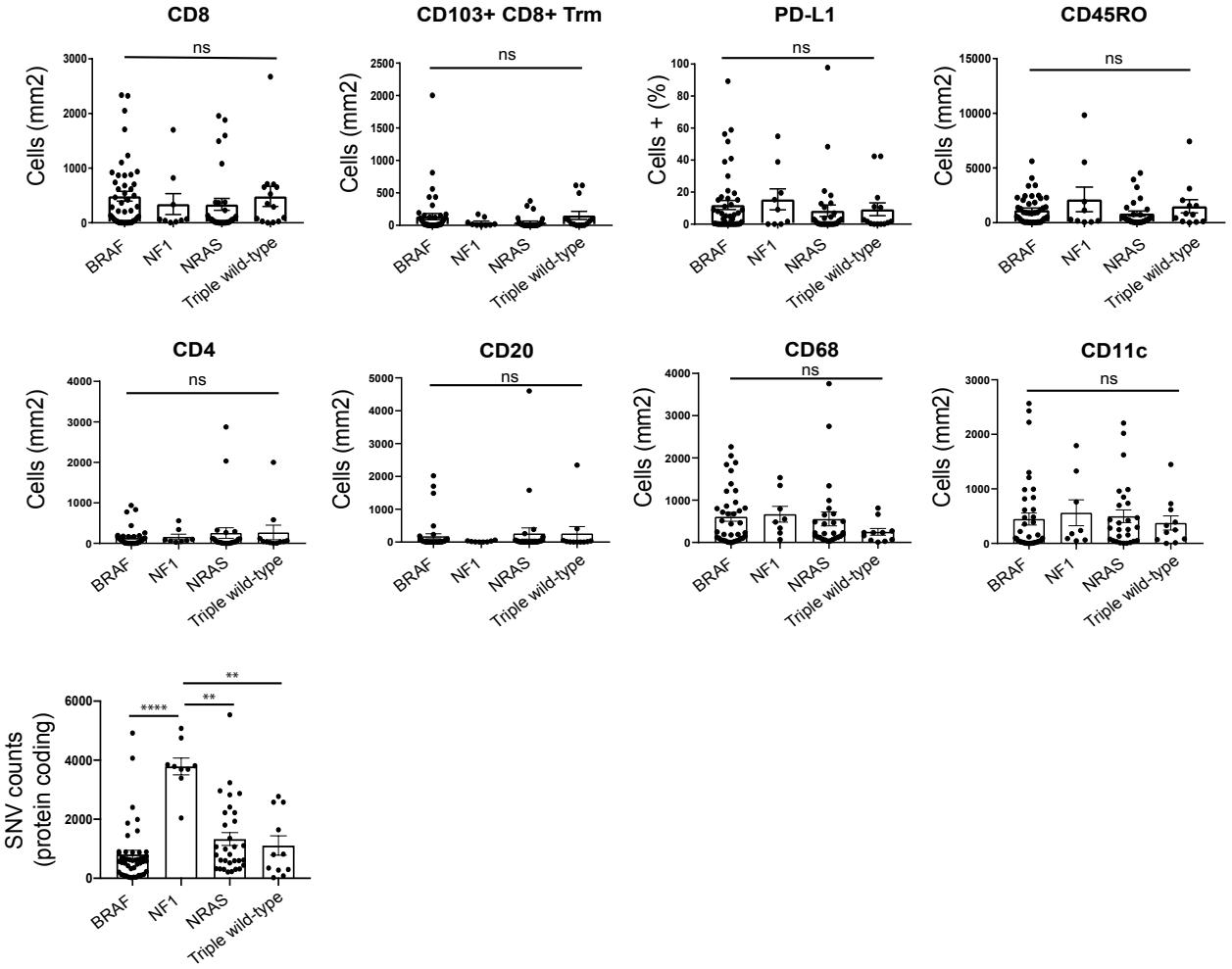


Supplementary Figure 3

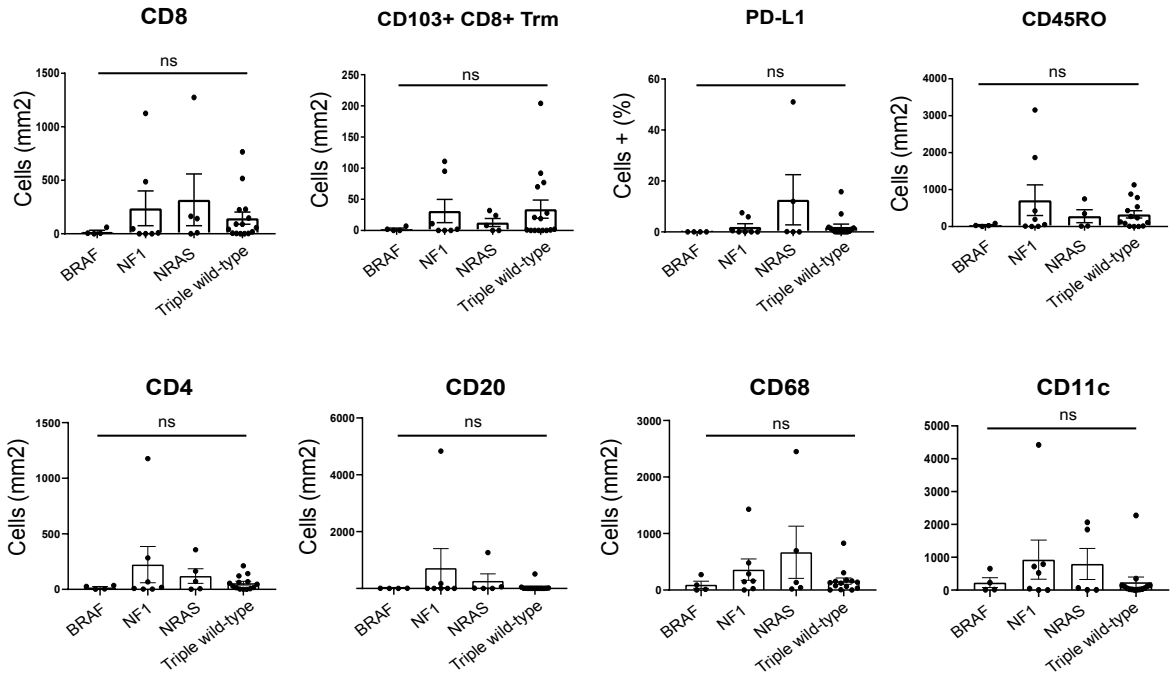


Supplementary Figure 4

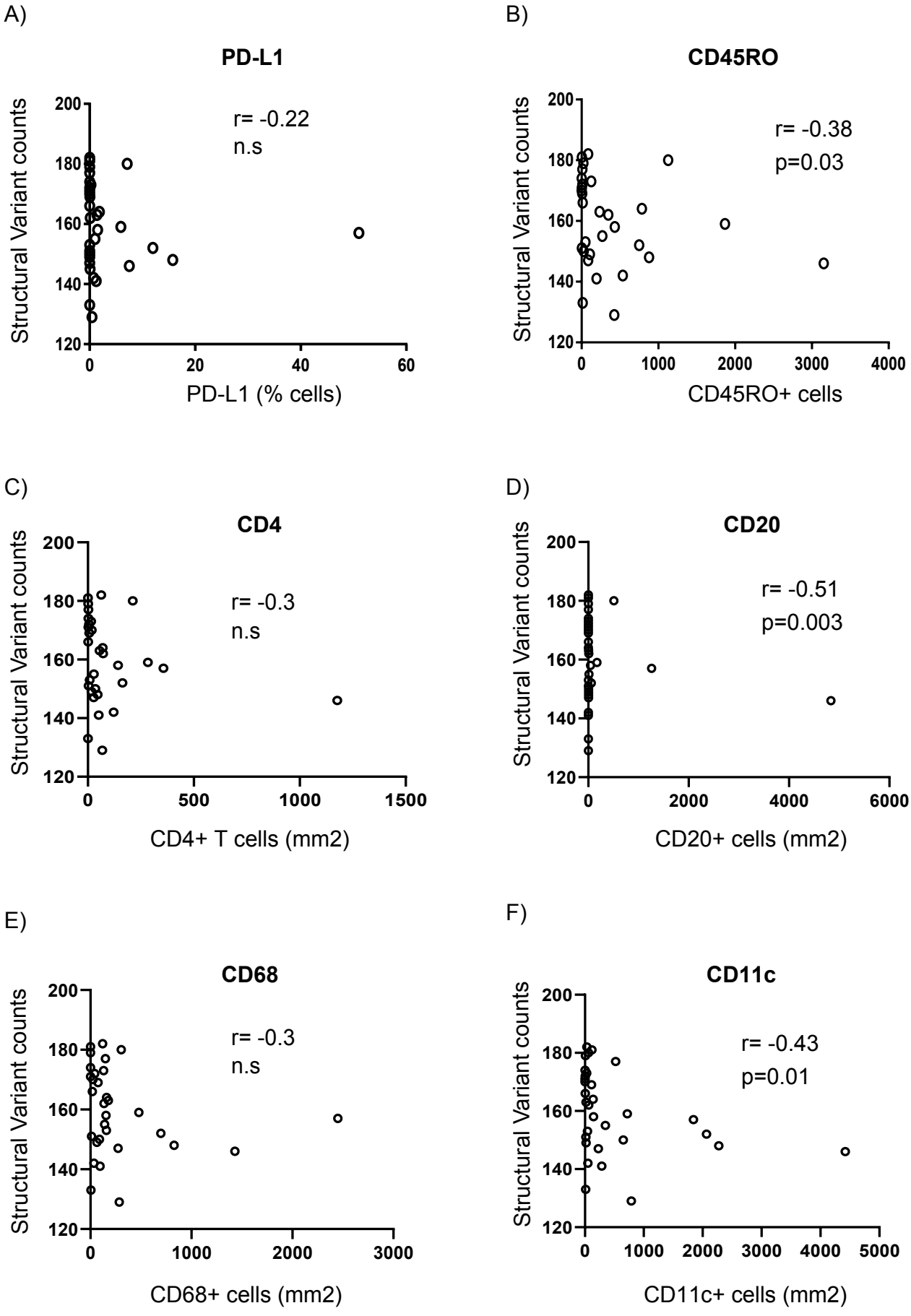
Cutaneous melanoma



Acral and mucosal melanoma

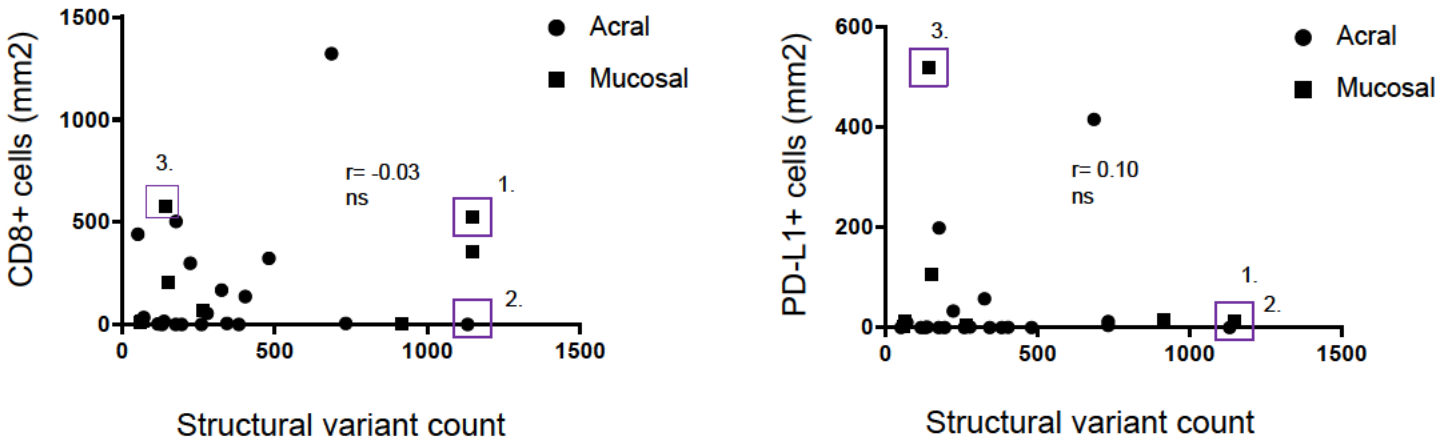


Supplementary Figure 5



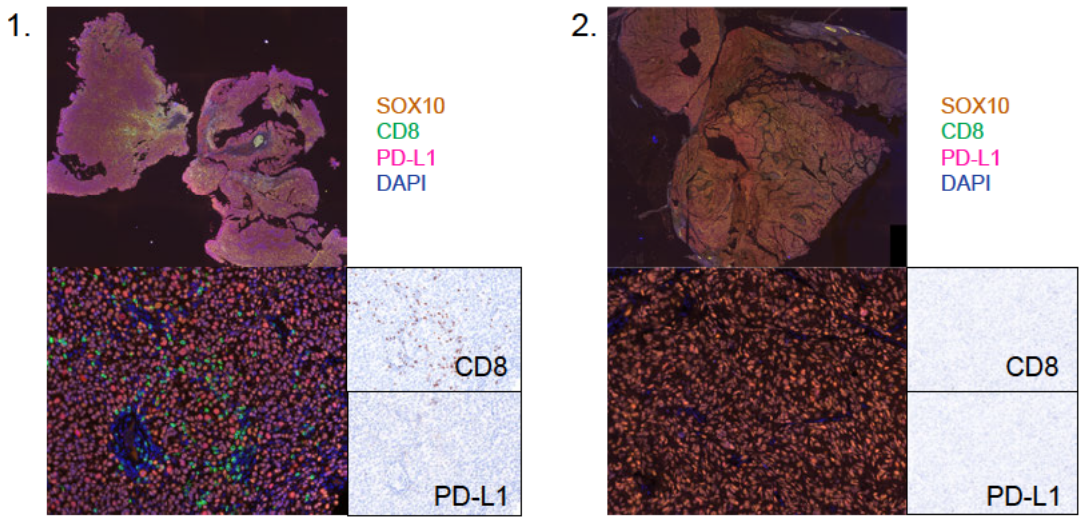
Supplementary Figure 6

A)

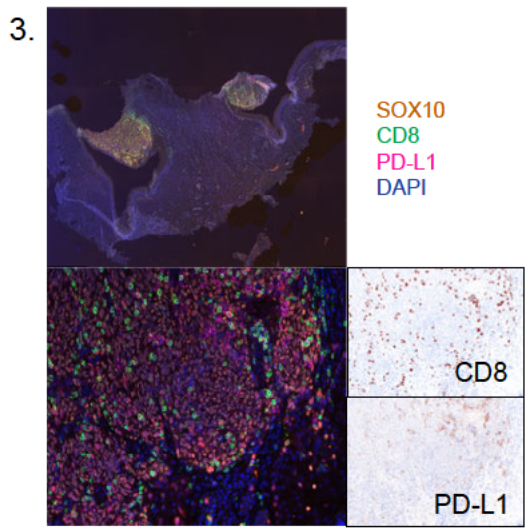


B)

Non-cutaneous melanoma with high Structural Variants

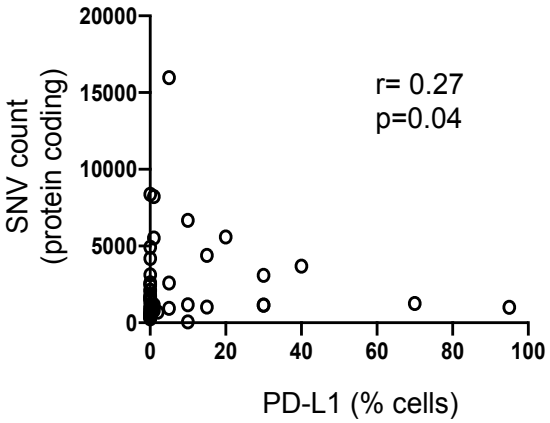


Non-cutaneous melanoma with low Structural Variants

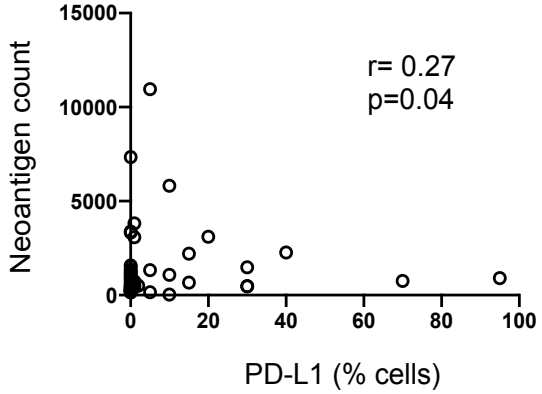


Supplementary Figure 7

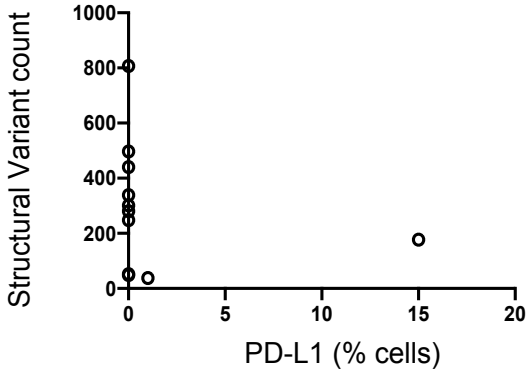
A)



B)

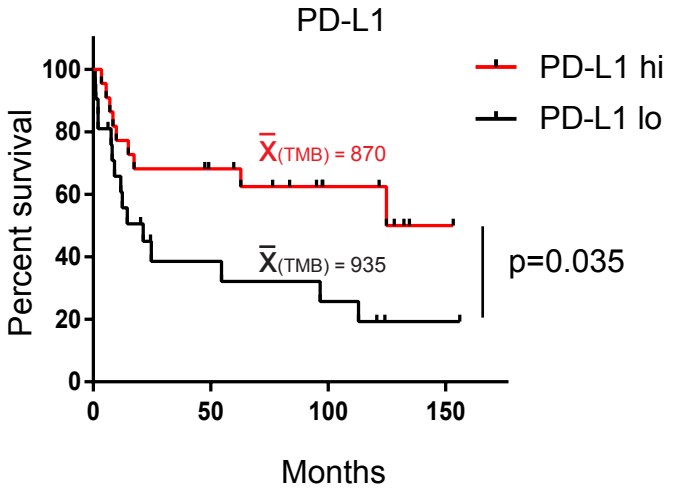


C)



Supplementary Figure 8

A)



# of subjects at risk				
PD-L1 hi	22	14	7	2
PD-L1 lo	21	7	3	2

Supplementary Figure Legends

Supplementary Figure 1

Abundance of immune markers across melanoma subtypes and correlation of immune cells and TMB in cutaneous melanoma A, Mean proportion of CD103+ CD8+ Trm in in cutaneous (n=151), acral (n=30) and mucosal (n=5) melanoma, B, Mean percentage of PD-L1 positive cells in the tumors of cutaneous (n=151), acral (n=30) and mucosal (n=5) melanoma. C, The mean number of CD4+ T cells per mm^2 of tumor in cutaneous (n=151), acral (n=30) and mucosal (n=5) melanomas. D, The mean number of CD20+ cells per mm^2 of tumor in cutaneous (n=151), acral (n=30) and mucosal (n=5) melanomas. E, The mean number of CD68+ cells per mm^2 of tumor in cutaneous (n=151), acral (n=30) and mucosal (n=5) melanomas. F, The mean number of CD11c+ cells per mm^2 of tumor in cutaneous (n=151), acral (n=30) and mucosal (n=5) melanomas. G, Spearman correlation (r) between single nucleotide variant (SNV) counts and percentage of PD-L1 positive cells in (n=97) cutaneous melanoma tumors. H, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD45RO positive lymphocytes per mm^2 of tumor in (n=97) cutaneous melanoma tumors. I, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD4+ T cells per mm^2 of tumor in (n=97) cutaneous melanoma tumors. J, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD20+ cells per mm^2 of tumor in (n=97) cutaneous melanoma tumors. K, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD68+ cells per mm^2 of tumor in (n=97) cutaneous melanoma tumors. L, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD11c+ cells per mm^2 of tumor in (n=97) cutaneous melanoma tumors. PD-L1 positivity was determined as the percentage of cells (total) in the tumor microenvironment (tumor cells and immune cells) positive for PD-L1 using a continuum scale from 0-100%. All error bars are displayed as ±1 standard error of the mean (SEM).

Supplementary Figure 2

Correlation of single nucleotide variant counts and CD8 T cells in whole slide sections of cutaneous melanoma. A, Spearman correlation (r) between single nucleotide variant (SNV) counts and CD8 positive lymphocytes (left) or PD-L1 positive staining (right) in n=17 cutaneous samples from seventeen patients. B, Multiplex fluorescence staining of marked cutaneous samples (1-4) from (A) with relatively high single nucleotide variant counts (top) and relatively low single nucleotide variant counts (bottom).

Supplementary Figure 3

Correlation between immune cells and single nucleotide variant counts in acral and mucosal melanomas. A, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD8 positive lymphocytes per mm^2 of tumor in (n=32) acral and mucosal melanoma tumors. B, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD8+ CD103+ tumor resident cells per mm^2 of tumor in (n=32) acral and mucosal melanoma tumors. C, Spearman correlation (r) between single nucleotide variant (SNV) counts and percentage of PD-L1 positive cells in (n=32) acral and mucosal melanoma tumors. D, Spearman correlation (r) between single nucleotide

variant (SNV) counts and number of CD45RO positive lymphocytes per mm^2 of tumor in (n=32) acral and mucosal melanoma tumors. E, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD4+ T cells per mm^2 of tumor in (n=32) acral and mucosal melanoma tumors. F, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD20+ cells per mm^2 of tumor in (n=32) acral and mucosal melanoma tumors. G, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD68+ cells per mm^2 of tumor in (n=32) acral and mucosal melanoma tumors. H, Spearman correlation (r) between single nucleotide variant (SNV) counts and number of CD11c+ cells per mm^2 of tumor in (n=32) acral and mucosal melanoma tumors. PD-L1 positivity was determined as the percentage of cells (total) in the tumor microenvironment (tumor cells and immune cells) positive for PD-L1 using a continuum scale from 0-100%. All error bars are displayed as ±1 standard error of the mean (SEM).

Supplementary Figure 4

Immune infiltration in cutaneous and acral/ mucosal melanoma of different melanoma molecular subtypes. A) Mean number of CD8+ T cells, CD103+ CD8+ tumor resident T cells, percentage of PD-L1 cells in tumor, CD45RO+ cells, CD4+ T cells, CD20+ cells, CD68+ cells, CD11c+ cells, and SNV counts in cutaneous melanoma (n=97) of different molecular subtypes (*BRAF*, *NF1*, *NRAS*, and wild-type). B) Mean number of CD8+ T cells, CD103+ CD8+ tumor resident T cells, percentage of PD-L1 cells in tumor, CD45RO+ cells, CD4+ T cells, CD20+ cells, CD68+ cells, and CD11c+ cells in acral/mucosal melanoma (n=32) of different molecular subtypes (*BRAF*, *NF1*, *NRAS*, and wild-type). All error bars are displayed as ±1 standard error of the mean (SEM).

Supplementary Figure 5

Correlation between immune cells and structural variant counts in acral and mucosal melanomas. A, Spearman correlation (r) between SVs counts and percentage of tumor positive for PD-L1 in acral and mucosal melanoma tumors (n=32). B, Spearman correlation (r) between SVs counts and number of CD45RO+ cells of tumor per mm^2 in acral and mucosal melanoma tumors (n=32). C, Spearman correlation (r) between SVs counts and number of CD4+ T cells of tumor per mm^2 in acral and mucosal melanoma tumors (n=32). D, Spearman correlation (r) between SVs counts and number of CD20+ cells of tumor per mm^2 in acral and mucosal melanoma tumors (n=32). E, Spearman correlation (r) between SVs counts and number of CD68+ cells of tumor per mm^2 in acral and mucosal melanoma tumors (n=32). E, Spearman correlation (r) between SVs counts and number of CD11c+ cells of tumor per mm^2 in acral and mucosal melanoma tumors (n=32).

Supplementary Figure 6

Correlation between structural variant counts and CD8+ T cells in whole slide sections of acral and mucosal melanomas. A, Spearman correlation (r) between structural variant (SVs) counts and CD8 positive lymphocytes (left) or PD-L1 positive staining (right) in n=21 acral and n=8 mucosal melanoma samples from twenty-nine patients. B, Multiplex fluorescence staining of marked cutaneous samples (1-4) from (A) with relatively high structural variant counts (top) and relatively low structural variant counts (bottom).

Supplementary Figure 7

Correlation between TMB, structural variant counts, and PD-L1 expression in validation cohort for cutaneous and acral/mucosal melanomas. A, Spearman correlation (r) between single nucleotide variant counts and PD-L1 positivity of tumor in n=56 cutaneous melanoma tumors. B, Spearman correlation (r) between neoantigen counts and PD-L1 positivity of tumor in n=56 cutaneous melanoma tumors. C, Spearman correlation (r) between structural variant counts and PD-L1 positivity of tumor in acral (n=8) and mucosal (n=3) melanoma (total n=11). PD-L1 positivity was determined as the percentage of cells (total) in the tumor microenvironment (tumor cells and immune cells) positive for PD-L1 using a continuum scale from 0-100%.

Supplementary Figure 8

Prognostic effect of PD-L1 expression in treatment-naïve melanoma patients. A, Kaplan-Meier curve for n= 43 stage III cutaneous melanoma patients with high PD-L1 expression (greater than the median (1%); red) and low PD-L1 expression (lower than median; black). The average TMB is given for PD-L1 high and PD-L1 low groups. PD-L1 positivity was determined as the percentage of cells (total) in the tumor microenvironment (tumor cells and immune cells) positive for PD-L1 using a continuum scale from 0-100%.

Chapter 2 – Concluding discussion

The purpose of this chapter was to understand the role of TMB on the immune infiltrate in melanoma tumors, and specifically, its effect on resident CD8 T cell populations. Our results, while somewhat surprising, have important implications for melanoma patients with relatively low tumor mutation burden. We hypothesized that patient tumors with higher TMB would be associated with a higher density of tumor resident CD8 T cells, given that resident CD8 T cells represent a tumor specific population and are strongly associated with protection. However, we showed that TMB, and by extension, the neoantigen load of tumors, have no effect on the presence or absence of tumor resident CD8 T cells in patient tumors. More importantly, we showed that the protective function of tumor resident CD8 T cells is independent of TMB in cutaneous melanoma tumors. Furthermore, we found that specific mutation types, including BRAF mutations and chromosomal structural aberrations, also have no apparent impact on resident CD8 T cell densities in other melanoma subtypes. These are important findings, because they suggest that patient melanoma tumors with relatively low TMB should not be expected to be devoid of protective immunity. Neither should relatively high TMB melanoma tumors be expected to have the presence of protective immunity. This is interesting, because as we have mentioned, the scientific community at the time assumed that TMB correlated with anti-tumor protective immunity, even within patients of the same cancer type. Our data unequivocally shows that this is not the case in melanoma and therefore other factors need to be considered to help boost resident CD8 T cell numbers. To be clear, neoantigens have a role in generating anti-tumoral responses, however, our data suggests that much fewer neoantigens are probably required than what is appreciated. In a relatively immunogenic cancer like cutaneous melanoma, it is possible that all patient tumors reach the minimum threshold of neoantigen numbers necessary to form a robust anti-tumoral response, after which, more dominant factors shape the immune response to dampen or strengthen, recruit, and retain.

More broadly, we have also shown that TMB is independent of other immune cell phenotypes, including biomarkers that have been associated with response to anti-PD-1 immunotherapy, namely PD-L1 and CD8 T cell densities. It is interesting that among the biomarkers of response to immunotherapy, TMB itself has been identified as a potential biomarker of anti-PD-1 and anti-CTLA-4 immunotherapy (Newell et al., 2021; Van Allen et al., 2015). Whatever the mechanism, CD8 T cell densities and TMB are likely to be independent factors that predict immunotherapy response rather than two interrelated markers. Certainly, the data presented in this chapter supports this notion. In another study, we performed multi-omic profiling of checkpoint inhibitor-treated melanoma and showed that TMB and the IFN-gamma signature are independent predictors of immunotherapy response (Newell et al., 2021), providing confirmatory findings that support this notion.

In conclusion, we have established that TMB and structural chromosomal aberrations are not important driving factors for the recruitment, retention, and persistence of protective immunity in cutaneous and acral/mucosal melanomas, respectively. The implication of this data is that within melanoma at least, other strategies, for example the cytokine milieu, might be more of an effective approach at boosting relevant targets of anti-PD-1 therapy in non-responding tumors.

Chapter 3

Prevalence and cellular distribution of novel immune checkpoint targets across longitudinal specimens in treatment-naïve melanoma patients: implications for clinical trials

Chapter 3 - Introduction

In the previous two chapters, we have provided a greater understanding of the immune cell targets of anti-PD-1 immunotherapy (resident CD8 T cells) as well as the factors that are likely (IL-15) and unlikely (tumor mutation burden) to influence the prevalence of this important population in melanoma tumors. These chapters provide unique insights into why patients respond, and in turn, fail anti-PD1 immunotherapy. These chapters also provide insights into opportunities to overcome resistance (by boosting critical immune populations), thereby increasing the patient pool that will benefit from durable immunotherapy responses. This is important given that most patients fail to respond to current checkpoint-based immunotherapies.

In subsequent chapters, we will continue to examine the tumor resident CD8 T cell phenotype but in the context of tumor progression and clinically driven therapeutic approaches to overcome resistance to anti-PD-1 therapy. At the time that this next body of work was written, a vast array of clinical trials was underway with various mechanisms of action being tested in melanoma patients failing immunotherapy. Among these, therapies targeting alternative co-inhibitory and co-stimulatory T cell receptors represented the overwhelming majority. As discussed, co-inhibitory receptors on T cells limit T cell activity and function by providing negative signals which dampen TCR sensitivity (Schnell, Bod, Madi, & Kuchroo, 2020). Co-stimulatory T cell receptors, on the other hand, provide positive signals that reinforce T cell activation signals and TCR sensitivity (Schnell et al., 2020). At the time of this writing, a plethora of co-inhibitory and co-stimulatory receptors had been discovered, and virtually all were being tested and targeted in some clinical trial by some pharmaceutical company. There was little doubt that this had become a race to find the next efficacious checkpoint inhibitor. Of course, this was partly because targeting co-inhibitory T cell receptors (anti-PD-1 and anti-CTLA-4) had already proven successful as a therapeutic strategy. Additionally, preliminary data from murine models suggested that dual checkpoint blockade with anti-PD1 and various other co-inhibitory T cell receptors demonstrated higher efficacy compared to single agent anti-PD-1 therapy alone (Johnston et al., 2014; Koyama et al., 2016; Sade-Feldman et al., 2018; Sakuishi et al., 2010; Woo et al., 2012). While these were exciting developments in the clinic, from a scientific standpoint, it was concerning just how little was actually known about the abundance and expression profiles of these various targets in human tumors. This limited understanding made it difficult to predict treatment outcomes as well as define potential biomarkers of response. Specifically, it was difficult to predict patient subgroups that would likely benefit from each of the various combination treatments in the context of anti-PD-1 failure. This was especially important in an era in which the heterogeneity of patient tumors was becoming more apparent and the need for personalized immunotherapies recognised. Another clinically relevant question was how the expression profile of these targets changed during the course of disease (from early to late stage) and by site of disease (organ), as patients with different disease presentation could theoretically respond differently if the expression profile of targets varied. Additionally, a greater understanding of what immune cell phenotypes in the TME expressed each of these receptors was also needed. At the time, it was thought that these novel therapies might target similar populations necessary for anti-PD-1 response. In this

context, tumor resident CD8 T cells, which express high levels of PD-1 and expand early during treatment with anti-PD-1, might also prove a target of these novel therapies. According to the alternative checkpoint hypothesis, because other co-inhibitory and co-stimulatory receptors on T cells could affect TCR activity (and hence effector function), it was thought that in some patients, anti-PD-1 alone was unlikely sufficient to restore T cell functionality and that the inhibition of other targets might help initiate response. Therefore, for CD8 T cell populations involved in anti-PD-1 response (including resident CD8 T cells), it was relevant to understand their expression of alternative targets too.

The aims and objectives of the current study were as follows:

1. To determine the prevalence and cellular distribution of novel co-inhibitory and co-stimulatory checkpoint targets in human melanoma patients.
2. To describe the changes in the abundance or distribution of targets with tumor progression and the impact of metastasis to different sites (organ type).
3. To describe which checkpoint targets are likely to be co-expressed together on cells, including on tumor resident CD8 T cells.
4. To provide a foundation for the rational selection of targets for patients and predictive biomarker development.

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Precision Medicine and Imaging

Clinical Cancer Research

Prevalence and Cellular Distribution of Novel Immune Checkpoint Targets Across Longitudinal Specimens in Treatment-naïve Melanoma Patients: Implications for Clinical Trials

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Abstract

Purpose: Immunotherapies targeting costimulating and coinhibitory checkpoint receptors beyond PD 1 and CTLA 4 have entered clinical trials. Little is known about the relative abundance, coexpression, and immune cells enriched for each specific drug target, limiting understanding of the biological basis of potential treatment outcomes and development of predictive biomarkers for personalized immunotherapy. We sought to assess the abundance of checkpoint receptors during melanoma disease progression and identify immune cells enriched for them.

Experimental Design: Multiplex immunofluorescence staining for immune checkpoint receptors (ICOS, GITR, OX40, PD 1, TIM 3, VISTA) was performed on 96 melano ma biopsies from 41 treatment naïve patients, including patient matched primary tumors, nodal metastases, and distant metastases. Mass cytometry was conducted on tumor dissociates from 18 treatment naïve melanoma metastases to explore immune subsets enriched for check point receptors.

Results: A small subset of tumor infiltrating leukocytes expressed checkpoint receptors at any stage of melanoma disease. GITR and OX40 were the least abundant checkpoint receptors, with <1% of intratumoral T cells expressing either marker. ICOS, PD 1, TIM 3, and VISTA were most abundant, with TIM 3 and VISTA mostly expressed on non T cells, and TIM 3 enriched on dendritic cells. Tumor resident T cells ($CD69^+/CD103^+/CD8^+$) were enriched for TIGIT (>70%) and other coinhibitory but not costimulatory receptors. The proportion of $GITR^+$ T cells decreased from primary melanoma (>5%) to lymph node (<1%, P 0.04) and distant metastases (<1%, P 0.0005).

Conclusions: This study provides the first comprehensive assessment of immune checkpoint receptor expression in any cancer and provides important data for rational selection of targets for trials and predictive biomarker development.

Introduction

Immunotherapy has now become an established pillar in the treatment of patients with many cancers, particularly melanoma and lung cancer, and is increasingly being used in patients with many other tumor types. With the advent of immune checkpoint inhibitors (ICI), targeting coinhibitory receptors on immune cells, the paradigm of translational cancer research has shifted to include not only the tumor itself, but also the immune system and tumor microenvironment. In particular, mAbs that target the receptors PD 1 and CTLA 4 have demonstrated unprecedented clinical efficacy and have revolutionized the treatment of mela noma in both the metastatic and adjuvant setting. Despite these incredible advances, the majority of patients treated with ICI therapy fails as a result of innate (primary) or acquired (second ary) resistance (1).

A number of resistance mechanisms for anti PD 1 and anti CTLA 4 therapy have been hypothesized. One such mechanism is the alternative immune checkpoint expression hypothesis, where by other checkpoint inhibitory receptors dampen T cell responses and contribute to severe T cell exhaustion (1), such that anti PD 1 alone is not enough to rescue T cell functionality. It has been suggested that in such a state, the additional targeting of other coinhibitory receptors, such as TIM 3, TIGIT, LAG 3, VISTA, and others (1 5), may achieve better clinical outcomes. In addition, one well described association with lack of response to immu notherapy occurs in tumors that lack adequate immune cell infiltration. Although such tumors are less responsive to ICI

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Translational Relevance

Costimulatory and coinhibitory receptors expressed on immune cells in tumor microenvironment represent promising therapeutic targets in patients with melanoma and other cancers, particularly those resistant to current anti PD 1 and anti CTLA 4 immunotherapies. However, little is known about the expression patterns of these receptors in human tumors, undermining the optimal selection of targets for clinical trials, interpretation of early phase trial data, and development of predictive biomarkers. Using a unique cohort of patients with melanoma with matched longitudinal biopsies, we quantified the prevalence and distribution of these receptors during disease progression, providing critical information about the potential efficacy of treatments targeting them. Furthermore, we performed mass cytometry (CyTOF) on tumor dissociates to dissect the immune subsets in melanoma tumors enriched for various targets, identifying immune subsets likely to be targeted by these therapies. These data are important for the field as we move toward the goal of providing personalized immunotherapies for patients with cancer.

therapy, it has been postulated that mAbs targeting costimulatory receptors (OX40, GITR, ICOS, and others) on T cells might work synergistically with checkpoint inhibitors to increase functional immune infiltrates specific for tumor antigens (1).

There are a growing number of clinical trials now underway involving these novel targets as single agent therapy or in combination with anti PD 1. A major confounding factor for developing novel strategies, rational selection of targets for testing in clinical trials, and clinical decision making, however, is the lack of understanding of the expression profile of most of these targets in the tumors of patients with cancer. For these reasons, it is critically important to understand the prevalence and expression profile of both costimulating and coinhibitory receptors in human malignancies, how they change in the course of disease progression, and what immune cells (or tumor cells) these receptors are expressed on and enriched for. Such knowledge will provide the field with the necessary foundation to develop tailored therapies for individual patients in the future.

To address these questions, we analyzed the protein expression profile of coinhibitory receptors (PD 1, TIM 3, VISTA) and costimulatory receptors (GITR, ICOS, OX 40) by multiplex immunofluorescence staining in 96 tumor biopsies from 41 patients with melanoma, including matched biopsies for primary tumors, lymph node metastases, and distant metastases from the same patient. We then performed mass cytometry on leukocytes isolated from 18 fresh baseline melanoma tumor samples to evaluate the expression of checkpoint receptors (ICOS, GITR, OX40, PD 1, CTLA 4, TIGIT, TIM 3, PD L1, and PD L2) on innate and adaptive immune infiltrates.

Materials and Methods

Patients and cohort

Ninety six tumor biopsies from 41 patients with melanoma, including matched biopsies for the primary tumors, lymph node, metastases, and distant metastases from the same patient were used to ascertain costimulatory and coinhibitory expression by multiplex IHC staining. The Melanoma Institute Australia database and archival files of the Department of Tissue Pathology and Diagnostic Oncology at the Royal Prince Alfred Hospital (Sydney, Australia) were used to identify patients with stage IIIC/IV melanoma with multiple formalin fixed paraffin embedded (FFPE) melanoma specimens available at various stages of disease progression, as described previously (6, 7). In patients with a history of multiple primary melanomas, the culprit primary melanoma was selected using a previously defined algorithm (8). An additional cohort of 18 fresh tumor biopsies taken from 18 patients with either stage III or stage IV melanoma were used to generate the mass cytometry data. Details of these patients and the site of biopsy are presented in Supplementary Table S3. The study was undertaken with Human Ethics Review Committee approval (protocol no X17 0312) and patients' informed consent. No patients received any prior systemic treatment and the site from which the biopsy specimens were obtained had not been previously treated at any time with radiotherapy or with topical or intralesional therapy.

Multiplexed immunofluorescence staining

All immunofluorescence staining was carried out on 4 μm thick sections using an Autostainer Plus (Dako, Agilent Technologies) or on the bench with appropriate positive and negative controls. Opal Multiplex IHC Assay Kit (PerkinElmer) was used as per the manufacturer's protocol. Briefly, FFPE tumor specimens were cut at 4 μm and air dried overnight. Specimens were then baked at 65°C for 30 minutes, deparaffinized, and rehydrated by xylene and ethanol. Heat induced antigen retrieval was performed in a Decloaking Chamber (Biocare), which heated samples at 95°C for 20 minutes in pH 9 antigen retrieval (AR) buffer (PerkinElmer). The primary antibody panels targeting (i) GITR (1/1,000; CST D919D), ICOS (1/3,000; CST D1K2T), OX40 (1/3,000; CST E9U7O), CD3 (1/1,000; CM103R95); (ii) PD 1 (1/100; Cell Marque NAT205), TIM 3 (1/500; CST D5D5R), VISTA (1/2,000; CST D1L2G), CD3 (1/1,000) (CM103R95); or (iii) CD14 (1/100; Sigma, polyclonal), CD68 (1/1,000; Kp 1), and CD8 (1/800; Ab4055) were incubated for 30 45 minutes. Primary antibodies were detected using either Opal Polymer HRP (GITR, ICOS, OX40, TIM 3, VISTA, CD3, CD14, CD68, CD8) (Perkin Elmer) or MACH 3 HRP polymer (PD 1; Biocare) for 30 or 10 minutes, respectively, and then visualized using Tyramide Signal Amplification for 10 minutes (Opal 7 Colour IHC, PerkinElmer). Between subsequent staining runs, tissues were boiled in pH 9 AR buffer for 15 minutes to strip the primary antibody complex from the sample. On the last staining run, DAPI was added to the sample for 5 minutes. The samples were cover slipped using Vectashield (H 1400) and left overnight to the dry at 4°C.

Imaging and statistical analyses

Vectra 3 multispectral slide scanner was used in conjunction with Vectra 3.3 and Phenochart 1.0.4 software to image samples. Images were then unmixed and analyzed using inForm 2.3.0 software (PerkinElmer) to phenotype and quantify the expression of each of the markers on individual cells. Because endogenous microenvironment in lymph node metastases is formed by lymphoid tissue and is therefore unique compared with other sites of metastases, an expert pathologist reviewed the images and annotated any lymph node associated structures/cells not associated

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with the tumor out of downstream analysis. Quantitative analysis was conducted using TIBCO Spotfire 3.3.1. Graphical and statistical analyses were performed using Prism version 6.0f (GraphPad Software) or TIBCO Spotfire 3.3.1. *P* values between matched primary and distant metastasis groups were determined using a Wilcoxon paired matched test (nonparametric). All other *P* values were determined using a nonparametric Kruskal Wallis test and Dunn multiple comparisons test, where appropriate. Correlation plots for each of the checkpoint receptors were performed using a linear regression analysis in TIBCO Spotfire 3.3.1. *P* values less than 0.05 were considered significant. All variability in the data is shown as the SEM.

Dissociation of leukocytes from fresh melanoma tumors

Fresh tumor biopsies were collected and placed in RPMI1640 (Thermo Fisher Scientific) at 4°C before being subjected to a mechanical/enzymatic dissociation system (GentleMACS, Miltenyi Biotec). Dissociation was performed according to the manufacturer's protocol. Briefly, the tumor was cut into small fragments (<3 mm) and incubated in a C tube (Miltenyi Biotec) with enzymes H, R, and A, made up to 5 mL with RPMI1640. The C tube and contents were then placed upside down onto the GentleMACS Dissociator with heating elements and then subjected to a mechanical disaggregation step followed by 30 to 60 minute incubation at 37°C (program 37C h TDK 1). After dissociation, tissue was passed through 70 μm strainer and washed with RPMI1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 25 mmol/L HEPES, and 50 μg/mL gentamicin at 1,800 rpm for 10 minutes before being resuspended in FCS supplemented with 10% dimethyl sulfoxide. Samples were slow cooled to 80°C and cryopreserved for future analysis.

Antibody staining

A total of 2×10^6 cells were stained for mass cytometry analyses, as described previously (9, 10). Briefly, cells were stained with 1.25 μmol/L Cell IDTM Cisplatin in PBS (Fluidigm, catalog no. 201064) for 3 minutes at room temperature and quenched by rapid addition of FBS. Cells were then washed twice in FACS buffer, then stained with a fluorophore conjugated antibody cocktail for 20 minutes at 4°C. Following washing with FACS buffer, cells were stained with a metal conjugated surface stain antibody cocktail for 20 minutes at 4°C. Cells were then fixed and permeabilized using the FOXP3 Transcription Factor Staining Buffer Set, according to the manufacturer's protocol (eBiosciences, catalog no 00 5523 00). Cells were subsequently stained with a metal conjugated intracellular antibody cocktail for 40 minutes at 4°C. Cells were then washed twice, once in Perm/Wash buffer and once in FACS buffer. Next, the cells were fixed overnight in 4% paraformaldehyde solution containing DNA Intercalator (0.125 μmol/L iridium 191/193; Fluidigm, catalog no. 201192B).

Mass cytometry data acquisition and analysis

Prior to acquisition, cells were washed once in FACS buffer and twice in dH_2O. Cells were then diluted to 8×10^5 cells/mL in dH_2O containing 10% EQ Four Element Calibration Beads (Fluidigm, catalog no. 201078) and filtered. Cells were acquired at a rate of 200 400 cells/second using a CYTOF 2 Helios Upgraded Mass Cytometer (Fluidigm). Flow cytometry standard (FCS) files were normalized to EQ bead signal and were then analyzed using FlowJo v10.2 (Tree Star). TD leukocytes were debarcoded manually in FlowJo. For manual gating of immune subsets, a minimum threshold of 50 cells was set to analyze the checkpoint expression on a particular immune subpopulation within patients. Subpopulations with fewer than 50 cells were excluded from the analysis.

Isolation of CD45$^+$ leukocytes from tumor dissociates

Prior to staining, CD45$^+$ leukocytes were isolated from TD samples by magnetic labeling with CD45 MicroBeads (Miltenyi Biotec, catalog no. 130 045 802) and subsequent separation using the autoMACS Pro Separator (Miltenyi Biotec, catalog no. 120 092 545), according to the manufacturer's instructions. To ensure that the CD45 MicroBeads did not prevent binding of the CD45 antibody, cells were simultaneously stained with metal conjugated anti CD45 (Pd104). The timing of anti CD45 addition was determined by previous experiments. Isolated CD45$^+$ cells were then counted and washed in FACS media.

CD45 barcoding and PBMC spike in

Tumor dissociates with yield $<2 \times 10^6$ leukocytes after separation were spiked with donor peripheral blood mononuclear cells (PBMC) to increase pellet bulk. To differentiate between tumor dissociate leukocytes and PBMCs, a CD45 based barcoding approach was used prior to spike in, as described previously (11). Tumor dissociate leukocytes were labeled with CD45 Pd104 during CD45$^+$ isolation PBMCs were stained with CD45 Pd110 for 20 minutes at 4°C and then washed twice in FACS buffer. Labeled PBMCs were then added to tumor samples (if needed) up to a final count of 2×10^6 cells.

Mass cytometry antibodies

Metal conjugated antibodies used in CyTOF analysis are presented in Supplementary Table S5 (cell surface) and Supplementary Table S6 (intracellular). For some markers, fluorophore conjugated antibodies were used as primary antibodies (Supplementary Table S7), followed by secondary labeling with anti fluorophore metal conjugated antibodies. Antibodies were either purchased from Fluidigm or conjugated in house using MaxPar X8 reagent kits (Fluidigm), according to the manufacturer's protocol. The concentration of each antibody was assessed using a NanoDrop (Thermo Scientific) and was then adjusted to 200 mg/mL in BioStab Antibody Stabilizer (Sigma Aldrich, catalog no. 55514). Conjugated antibodies were titrated for optimal concentration prior to use. Surface and intracellular antibody staining cocktail master mixes were prepared prior to each experiment. This protocol was carried out by the Ramaciotti Facility for Human Systems Biology, Sydney, Australia.

Flow cytometry

Cryopreserved single cell isolates from tumor samples obtained from patients with metastatic melanoma (isolated as described previously) were thawed, washed, and counted. Tumor cells were stained at 4°C for 30 minutes with saturating concentrations of the following extracellular mAbs: anti CD3, anti CD4, anti CD11c, anti CD13, anti CD14, anti HLA DR, (all from BD Biosciences), anti CD8, anti CD19, anti CD45 (BioLegend), and anti VISTA antibody (R&D Systems). Stained sample acquisition was performed on a 5 laser Fortessa flow cytometer (BD Biosciences) and acquisition was performed using FACS DIVA. Data were analyzed on FlowJo software.

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t SNE analysis

t Distributed Stochastic Neighbor Embedding (t SNE) analysis was performed using Cytobank (www.cytobank.org) on 6×10^4 T cells from a total of 18 tumor dissociates. Analysis was performed using the following settings: Iterations 1000, Perplexity 30, Theta 0.5, and Seed "random." Clustering was performed using the checkpoint receptor channels (ICOS, GITR, OX40, PD 1, CTLA 4, TIGIT, TIM 3, PD L1, and PD L2) so as to promote tight clustering of each checkpoint receptor and hence quick visualization of overlapping/distinct checkpoint receptor positive T cells.

Results

Abundance of checkpoint receptors and colocalization with CD3 in melanoma tumor tissue in humans

Ninety six melanoma biopsies from 41 patients (Table 1) were stained for ICOS, GITR, OX40, PD 1, TIM 3, and VISTA using multiplex immunofluorescence IHC. Representative staining for each of the markers are shown in Fig. 1A. The costimulatory receptor ICOS and the coinhibitory receptors PD 1, TIM 3, and VISTA were the most abundant (mean expression of 303 ± 94 cells/mm^2, 166 ± 47 cells/mm^2, 122 ± 36 cells/mm^2 and 286 ± 87 cells/mm^2, respectively), while GITR and OX40 were the least abundant ($P < 0.001$, mean expression of 13 ± 4 and 16 ± 6 cells/mm^2 respectively; Fig. 1B; Supplementary Table S1). When using a positive threshold of either >1 or >5 positive cells per 1 mm^2 to distinguish positive from negative biopsies for a particular marker, OX40 was the least common in patient tumors compared with other costimulatory and coinhibitory receptors (Supplementary Table S1).

Table 1. Clinical characteristics of cohort used in study

Clinical characteristics	Clinical characteristics by number of specimens ($N = 96$)	Clinical characteristics by number of patients
Demographic, *n* (%)	96 (100)	41 (100)
Male	53 (55)	21 (51)
Female	43 (45)	20 (49)
Specimen type		
Primary (P)	29 (30)	29 (71)
In transit metastasis (ITM)	10 (11)	6 (15)
Lymph node metastasis (LN)	26 (27)	25 (61)
Distant Metastasis (DM)	31 (32)	22 (54)
Metastasis site		
Subcutaneous	10 (11)	9 (22)
Brain	11 (12)	11 (27)
Small bowel	2 (2)	2 (5)
Other visceral	5 (5)	3 (7)
Bone	3 (3)	2 (5)
Subtype of melanoma primary		
Nodular melanoma		13 (32)
Desmoplastic		3 (7)
Not classified		1 (2)
Superficial spreading melanoma		6 (15)
Acral lentiginous		4 (10)
Unknown		4 (10)
BRAF status		
Wild type		12 (29)
Mutation		3 (7)
Matching specimens in same patient at different stages		
Primary Lymph node		20 (49)
Primary Distant Metastasis		14 (34)
Lymph node Distant Metastasis		10 (24)

We then examined the coexpression of CD3 and each of the various checkpoint receptors in the tumor environment. We first determined the proportion of T cells that were positive for each of the markers per patient (n 41) in all samples. Melanoma associated T cells in patients were ICOS$^+$ CD3$^+$ in 0% to 34% (average 12%) and PD 1$^+$ CD3$^+$ in 0% to 55% (average 10%; Fig. 1C). A smaller proportion of T cells expressed VISTA (average 7% ± 2%), TIM 3 (4% ± 1%), GITR (2% ± 1%), and OX40 (<1% ± 1%; Fig. 1C).

We next sought to determine the proportion of checkpoint receptor positive cells that were T cells. A high proportion of ICOS, GITR, and PD 1 positive cells were T cells (88% ± 2%, 64% ± 5%, and 77% ± 4%, respectively), while significantly less TIM 3 (36% ± 4%) and VISTA (24% ± 3%) positive cells were T cells (Fig. 1D, $P < 0.005$). The cytomorphology characteristics (larger cell size, lower nucleus: cytoplasm ratio and irregular shape) of TIM 3 and VISTA expressing non T cell populations suggested that these cells were myeloid derived cells such as macrophages or dendritic cells. In line with this, we observed colocalization between VISTA, CD68, and CD14 (Supplementary Fig. S1A). OX 40 displayed a high degree of interpatient variability, with a mean of 42% ± 7% of OX40 positive cells being T cells across the patients, but 30% of patients expressed OX40$^+$ cells that were all CD3$^-$ (Fig. 1D). Nevertheless, 70% of OX40$^+$ cells were found to be T cells when all positive cells from all patients and samples were combined (Supplementary Fig. S1B). In addition, VISTA was occasionally expressed on tumor cells (Supplementary Fig. S1C), although this was not observed for any of the other checkpoint receptors. Furthermore, the number of all checkpoint receptor expressing cells was significantly correlated with the CD3 numbers (Supplementary Table S2).

Because tumors lacking T cell infiltrate or PD 1/PD L1 expression represent a broad class of patients generally less responsive to anti PD 1 therapy, we examined PD 1 negative tumors (≤1 cell/mm^2) to investigate the expression of alternative checkpoint markers. In these tumors, the abundance of alternative checkpoint receptors was low (generally fewer than 50 cells/mm^2), with VISTA showing relatively higher expression compared with GITR ($P < 0.05$) and OX40 ($P < 0.01$; Supplementary Fig. S1D). The mean expression of ICOS, TIM 3, and VISTA checkpoint receptors in PD 1 negative tumors was negligible when compared with their expression in PD 1 positive tumors ($P < 0.05$; Supplementary Fig. S1E).

Together, these results show that only a small subset of T cells in the tumor express any given checkpoint receptor, and therefore only a select subpopulation of the total infiltrating lymphocytes are likely to be targeted by any given checkpoint therapy. Furthermore, some checkpoint targeting therapies such as TIM 3 and VISTA are likely to function predominately through non T cell populations.

Proportion of GITR$^+$ T cells decreases between matched primary and metastatic melanoma patient biopsies

To determine whether the expression pattern of any of the checkpoint receptors changes during the progression of melanoma disease, we stratified the patient biopsies according to the stage of disease; primary (P), in transit, metastasis (ITM), regional lymph node metastasis (LN), or distant metastasis (DM). GITR had a higher density of positive cells in primary

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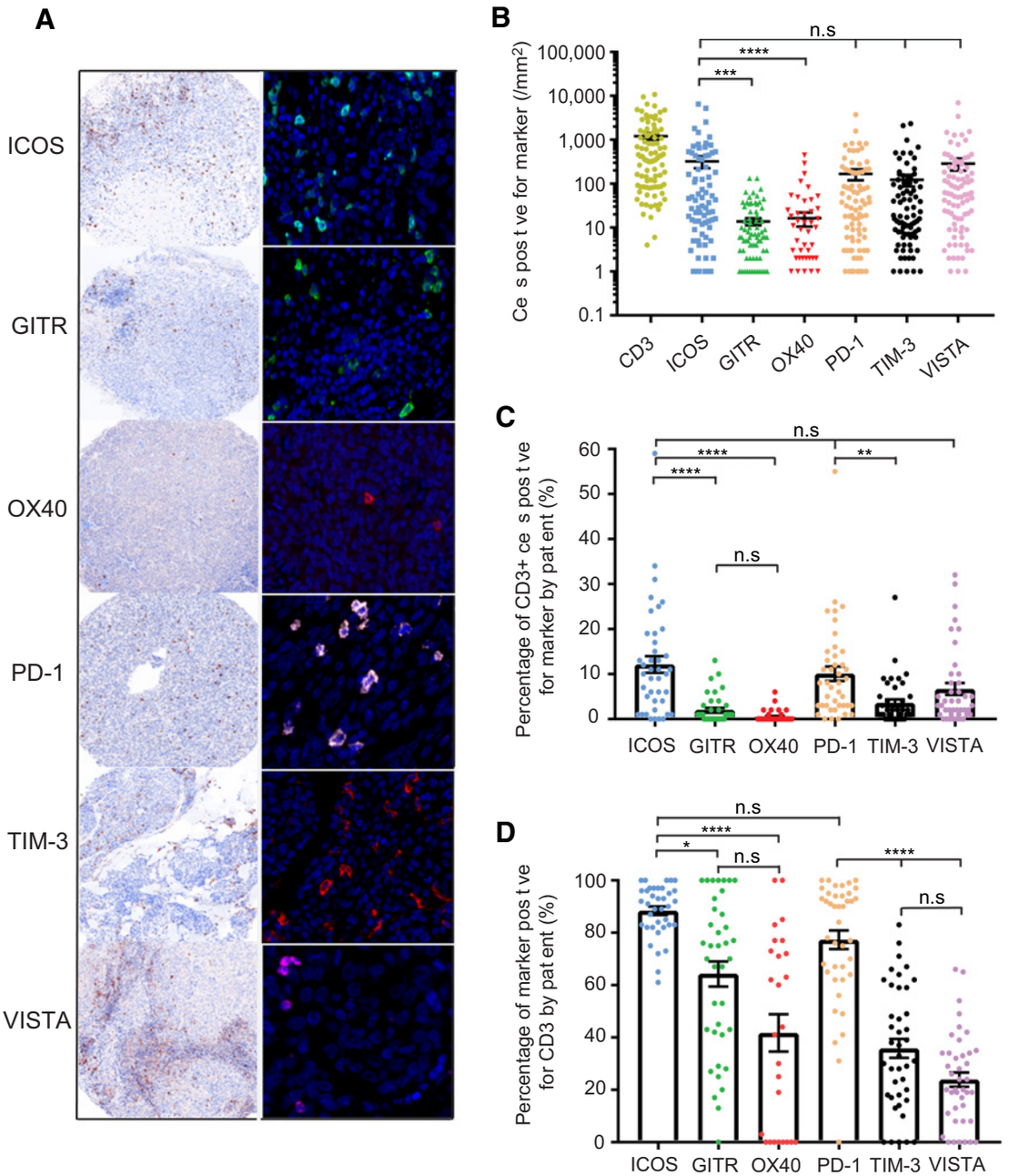

Figure 1.

Relative abundance of checkpoint receptors and their colocalization with CD3 in melanoma tissue. **A,** Representative images of costimulatory receptors (ICOS, GITR, and OX40) and coinhibitory receptors (PD 1, TIM 3, and VISTA) in melanoma tumor. Low power pseudo IHC images are shown on the left for each marker, while high power fluorescent images taken from the same region are shown on the right. **B,** Abundance of checkpoint receptors. The number of cells positive for each marker per 1 mm^2 of melanoma tumor, where each point represents a single melanoma biopsy (total, $n = 96$). The data are displayed using a logarithmic scale and normalized to CD3. **C,** Proportion of $CD3^+$ tumor infiltrating T cells expressing costimulatory and coinhibitory receptors, where each point represents a single patient (total, $n = 41$). In cases where a patient had multiple biopsies, the average was used. **D,** Proportion of costimulatory or coinhibitory positive cells in tumor also positive for CD3. Each point represents a single patient (total, $n = 41$). In cases where a patient had multiple biopsies the average was used. All error bars displayed represent the SEM.

biopsies compared to in transit metastases (P 0.016) and distant metastases (P 0.003), but not regional lymph node metastases (Fig. 2A; Supplementary Fig. S2A). No pattern in the densities of any of the other checkpoint receptors between the various stages of disease was observed (one way ANOVA per receptor).

We next sought to examine the proportion of T cells expressing a given checkpoint receptor during disease progression. Given that

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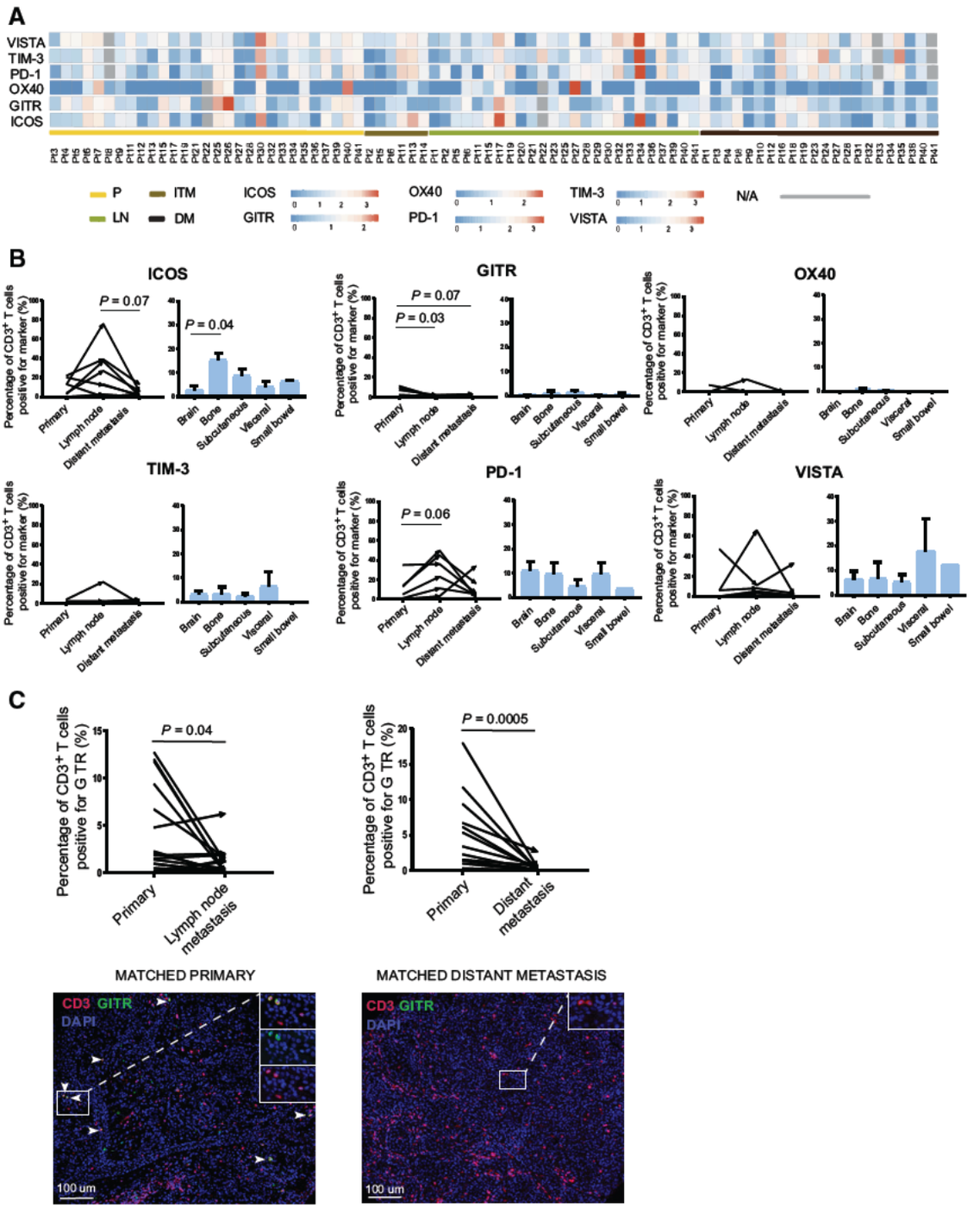

Figure 2.

Expression profile of checkpoint receptors at different stages of melanoma disease and site of disease. **A,** Heatmap of costimulatory (ICOS, GITR, and OX40) and coinhibitory receptor (PD 1, TIM 3, and VISTA) expression (the logged value of cells positive per 1 mm^2) in melanoma at primary (P), in transit metastasis (ITM), regional lymph node metastasis (LN), and distant metastatic (DM) stage of disease. Each column represents a single biopsy matched to a patient identification number. In cases where two biopsies from the same stage and patient were present, the average was calculated and used. Data normalized to CD3. Each row displays the relative expression for that marker. **B,** The proportion of intratumoral T cells expressing each checkpoint receptor in longitudinal patient matched biopsies from the primary tumor, regional lymph node, metastases, and distant metastases (left), and in all distant metastatic biopsies grouped by site of disease (right), brain ($n = 11$), bone ($n = 3$), subcutaneous ($n = 10$), visceral ($n = 5$), small bowel ($n = 1$ and 2 for costimulatory and coinhibitory receptors, respectively). **C,** The proportion of intratumoral $GITR^+$ T cells in matched primary and regional lymph node ($n = 18$) and primary and distant metastatic melanoma specimens from the same patient ($n = 14$). Representative images are shown below and arrows indicate examples of $GITR^+CD3^+$ cells.

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within our cohort of patient biopsies, there was a subgroup of patients (n 6) with matched specimens for primary, regional lymph node, and distant metastases, we were able to explore this in a model that paralleled melanoma disease progression in patients. Interestingly, although a very small percentage of intratumoral T cells expressed GITR, there were strong trends for decrease in the proportion of GITR$^+$ T cells between primary and regional lymph node (P 0.03) and primary and distant metastases (P 0.07) in individual patients (Fig. 2B). PD 1 and ICOS were expressed on a high proportion of T cells, relative to other checkpoint receptors at all stages of disease progression, with trends for higher percentages in regional lymph node metastases compared with primary (PD 1, P 0.06) or distant metastases (ICOS, P 0.07; Fig. 2B), possibly reflecting the unique microenvironment of the lymph node.

We examined checkpoint expression changes in a larger cohort (from within the 96 biopsies) of patients with matched biopsies between primary and lymph node metastases (n 20) or primary and distant metastases (n 14). This confirmed that the proportion of intratumoral GITR$^+$ T cells were higher in primary tumors (~5%) compared with matched regional lymph node (<1%, P 0.04) and distant metastases (<0.5%, P 0.0005; Fig. 2C), indicating that the proportion of intratumoral GITR$^+$ T cells decreases during disease progression. No other statistically significant differences were observed for any of the other checkpoint receptors (Supplementary Fig. S2B and S2C). Our results suggest that GITR therapy may theoretically be more effective as an upfront therapy in patients with locally advanced but clinically localized primary melanoma rather than those with occult nodal or distant metastases.

Immune checkpoint expression at different sites of distant metastasis

To determine whether the site of tumor influenced the expression pattern of checkpoint receptors, we examined the percentages of T cells expressing a given checkpoint receptor in all distant metastatic biopsies by site of disease. Interestingly, no statistically significant differences were observed for most of the checkpoint receptors expressed on intratumoral T cells except higher ICOS expression in bone metastases relative to brain metastases (Fig. 2B).

Distribution and enrichment of checkpoint receptors on intratumoral immune subsets

As potential drug targets, it is imperative that there is an understanding of the distribution of checkpoint receptors in the tumor microenvironment, particularly, the various immune cells enriched for each of the costimulatory and coinhibitory receptors so as to anticipate the immune cells each checkpoint antibody may affect. Therefore, we performed 43 parameter mass cytometry (CyTOF cytometry by time of flight), on treatment naïve melanoma tumor dissociates (n 18) from 18 stage III or stage IV patients with melanoma (Supplementary Table S3) that had undergone CD45 isolation and enrichment. The mass cytometry antibody panel analyzed checkpoint expression on a broad range of immune cell populations, including central (Tcm), effector (Tem), and tissue resident (Trm) memory subsets of CD8$^+$ and CD4$^+$ T cells (defined by the expression of CD69 and CD103 in CD8 T cells; ref. 12), T regulatory cells (Treg), natural killer (NK) cells, conventional dendritic cells (cDC1 and cDC2), monocytes, CD14±, macrophages (Mφ CD68+ CD14±), and B cells (See Supplementary Fig. S3A for full gating strategy). The percentage of each immune population positive for each of the checkpoint receptors ICOS, GITR, OX40, PD 1, CTLA 4, TIGIT, TIM 3, PD L1, and PD L2 was determined by manual gating and the results are summarized in a heatmap (Fig. 3A) as well as in column graphs for each marker (Fig. 3B).

Our results revealed that costimulatory and coinhibitory receptor expression varied widely between different immune populations, and indeed within subsets of memory T cells. PD 1 and TIGIT were expressed largely on T cell populations, with PD 1 being enriched on CD8$^+$ Trm (mean >70% in all patients), and TIGIT being enriched on CD8$^+$ Trm (>70%) and Tregs cells (>90%; Fig. 3A and B). Innate populations generally expressed relatively little TIGIT except for NK cells and monocytes, which expressed >15% and >10%, respectively (Fig. 3B). TIM 3 expression on T cells was largely restricted to the CD8$^+$ Trm phenotype (>10%) and Tregs (~9%) with minimal expression observed for other CD8 and CD4 memory populations (Fig. 3A and B). However, within the innate populations, TIM 3 was expressed highly on dendritic cells, most notably CD141$^+$ cDC1 cells (~20%) and to a lesser extent on NK cells (6%). These results are in line with our IHC data in which TIM 3 was largely expressed on non T cell populations. The CyTOF data also revealed ICOS, OX40, and CTLA 4 were expressed most highly on Treg cells (Fig. 3A and B; Supplementary Table S4). However, a small subset of non Treg CD4$^+$ T cells, and to a lesser extent, CD8$^+$ T cells, also expressed ICOS and CTLA 4. In line with our IHC data, GITR expression was very low on all immune populations in these melanoma tumor dissociates. Interestingly, GITR expression was highest on B cells (~2%) in these melanoma tumor dissociates relative to T cell populations (<1%; $P < 0.01$) and was minimally expressed on other innate immune populations (Fig. 3A; Supplementary Table S4). As expected, PD L1 was largely expressed on dendritic cells, macrophages, and monocytes, with the highest expression on conventional dendritic cells type II (cDC2) and CD14$^+$ CD68$^+$ macrophages (both ~10%). However, we found that PD L2 was highly expressed on monocytes (~25%). To identify the immune cells expressing VISTA, we assessed its expression on immune infiltrates in melanoma tumors on a separate cohort by flow cytometry. Our results showed that VISTA was predominately expressed on (CD3$^-$CD19$^-$CD45$^+$ HLA DR$^+$) myeloid populations, such as CD11c$^+$ CD14$^+$ macrophages, CD11c$^+$ CD14$^-$ dendritic cells, monocytes, and also on B cells (Supplementary Fig. S3B), in line with what we observed for IHC.

Coexpression of checkpoint receptors on T cells and myeloid cells

In order to further assess the distribution and coexpression of checkpoint receptors on T cells at the single cell level, we performed tSNE analysis on 60,000 T cells from our collective 18 melanoma tumor dissociates, clustering based on costimulatory/coinhibitory expression. The regions positive for each costimulatory and coinhibitory receptor are shown in Fig. 4A. In addition to this, we also explored cooccurrence and pairwise associations for each of the checkpoint receptors on T cells and myeloid cells by analyzing the correlation in the expression for each marker pair. The strength of the relationship between each checkpoint pair for each of the cell types is summarized in Fig. 4B and C, respectively, with the highest correlating pair in each cell type displayed as a scatter plot.

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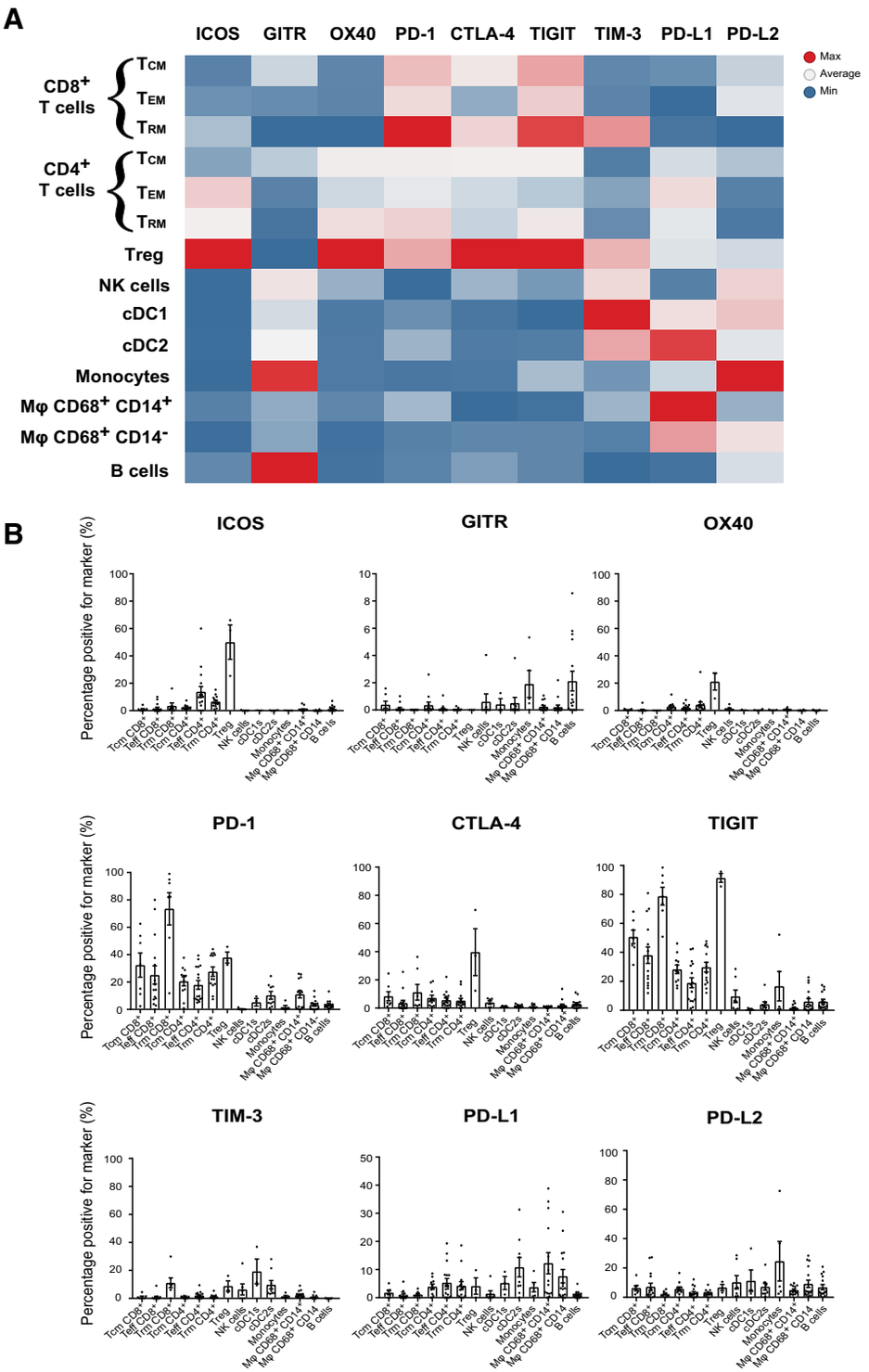

Figure 3.

Expression and distribution of checkpoint receptors on immune cells in melanoma tumor. **A,** Heatmap showing the average expression for each of the checkpoint receptors on manually gated immune populations from $n = 18$ patient tumor dissociates. Strong red indicates enrichment for a particular receptor relative to other immune populations, while dark blue indicates relatively low levels. **B,** Distribution of checkpoint receptors on immune populations shown as the percentage of a population positive for that marker. Each dot represents a single tumor dissociate run through CyTOF. Immune subpopulations with <50 cells in a sample were excluded for that particular sample in the analysis.

The results provided by tSNE analysis and cooccurrence heat map (T cells) showed that the checkpoint receptors ICOS, PD 1, TIGIT, CTLA 4, and TIM 3 were highly coexpressed on cells (Fig. 4A and B). T cells expressing either PD 1 or TIGIT were largely seen to overlap with one another (Fig. 4A) and correlation of the expression between the two receptors was moderately strong (R 0.58; $P < 0.0001$), suggesting that T cell populations targeted by PD 1 or TIGIT agonists are likely to be very similar (Fig. 4A and B). These T cells were mostly EOMES$^+$ and CD69$^+$ (Supplementary Fig. S4A), which we previously demonstrated to represent a tissue resident memory cell type associated with response to anti PD 1 ± anti CTLA 4 immunotherapy (12, 13).

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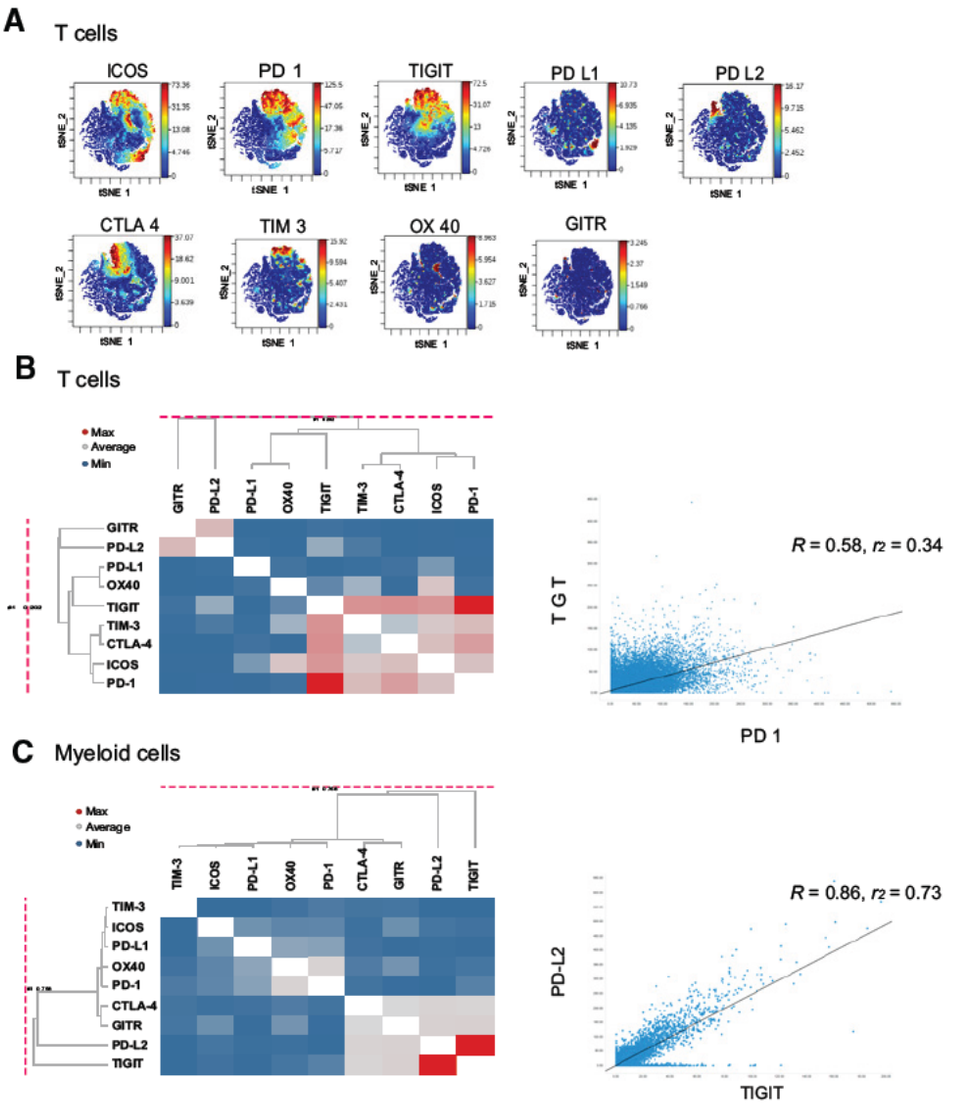

Figure 4.

Coexpression and pairwise associations of checkpoint receptors on T cells and myeloid cells. **A,** Tsne plots generated on CD3 positive T cells from all patients (60,000 events, concatenated). The distribution of each checkpoint receptor is represented in each of the plots (red indicates high expression). Regions that are positive for more than one marker indicate coexpression on cells. **B,** Heatmap summarizing pairwise associations (Linear regression correlation) for each of the checkpoint receptors on (60,000) $CD3^+$ T cells, where red indicates strong correlation (max, $R = 0.58$) and dark blue indicates mutual exclusivity (min, $R = 0$). **C,** Heatmap summarizing pairwise associations (Linear regression correlation) for each of the checkpoint receptors on (61,500) HLA DR^+ $CD11c^+$ myeloid cells, where red indicates strong correlation (max, $R = 0.86$) and dark blue indicates mutual exclusivity (min, $R = 0$).

It is also interesting to note that ICOS, although a costimulatory receptor, had a degree of coexpression with many of the coinhibitory receptors on this $EOMES^+$ $CD69^+$ subpopulation, indicating that the expression of many inhibitory receptors does not necessarily preclude the expression of costimulatory receptors on the same population of cells (Fig. 4A; Supplementary Fig. S4A). In line with this, a weak correlation was observed between ICOS and many of the coinhibitory checkpoint receptors, including TIGIT (R 0.38, $P < 0.0001$), TIM 3 (R 0.29, $P < 0.0001$), CTLA 4 (R 0.28, $P < 0.0001$), and PD 1 (R 0.29, $P < 0.0001$; Fig. 4B). Interestingly, there was no correlation observed between ICOS and other costimulatory receptors, except for ICOS and OX40, which showed a weak correlation between the two (R 0.27, $P < 0.0001$), likely representing Treg and CD4 populations of T cells based on the expression of FOXP3 and CD4 (Fig. 4A and B; Supplementary Fig. S4A).

On myeloid cells, pairwise association analysis revealed that the expression of costimulatory and coinhibitory receptors were largely unassociated with one another, except for PD L2 and TIGIT, which showed a very strong correlative relationship

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(R 0.86, $P < 0.0001$; Fig. 4C). Our enrichment analysis demonstrated that monocytes express relatively high levels of both TIGIT and PD L2 in the myeloid compartment. Our pairwise association analysis indicates, however, that there was a relationship between these two receptors in the myeloid compartment generally. It is also interesting to note that PD L1 and PD L2 expression on myeloid cells lack any correlative relationship (Fig. 4C), suggesting divergent subsets, and therefore their inhibition may have distinct functions on this immune compartment.

Discussion

The targeting of various novel costimulatory and coinhibitory receptors in tumors is focused on providing alternative treatment options to patients refractory to current anti PD 1/anti CTLA 4 therapies, and represents a step forward in the path toward personalization of immunotherapy treatments. Still, little is known about the actual mechanistic basis of these new therapies, which immune cells they are likely to target and modulate in the cancer setting, the relative prevalence of the targets in human malignancies, how they change during the course of the disease, and consequently, which patients are likely to benefit from them. Understanding these issues is critical to drug development, determining whether the target expression has any correlation with drug activity and ultimate clinical decision making; to select the best immunomodulatory therapeutic regimen at the optimal time for individual patients based upon robust predictive biomarkers of response and resistance to therapy. For these reasons, we analyzed the prevalence and cellular distribution of novel immunotherapy targets in patients with treatment naïve melanoma with primary melanomas and corresponding metastases, including longitudinal cohorts from individual patients that parallel melanoma disease progression. Our collective data demonstrate that only a small proportion of intratumoral lymphocytes express any given target and that some of these checkpoint receptors (TIM 3 and VISTA) are likely to be targeted on non T cell populations. In addition, we showed that while the majority of the checkpoint receptors demonstrate high interpatient variability with no significant patterns during disease progression (globally and matched), the proportion of GITR expressing T lymphocytes consistently decreases from primary tumor to nodal and distant metastases. We have also demonstrated that while relative abundance profile of novel checkpoint receptors is similar in PD 1 positive and PD 1 negative tumors, their expression is drastically reduced in PD 1 negative tumors compared with PD 1 positive tumors, indicating that tumors lacking T cell infiltration or PD 1 expression are less likely to have alternative novel targets in the tumor. Finally, we have detailed the immune populations enriched for each of the checkpoint receptors, including immune populations that are likely to be the same targets of multiple therapies.

Importantly, our data show that the costimulatory receptors OX40 and GITR are far less abundant in melanoma tumors at all stages of disease compared with other costimulatory (ICOS) and coinhibitory (PD 1, TIM 3, TIGIT, VISTA, CTLA 4) receptors, with overall less than 1% 2% of intratumoral T cells on average expressing either marker. While it is unknown whether these differences will translate into differences in the clinical efficacy of agents targeting these molecules, undoubtedly the data will have important implications for the field of personalized immunotherapy where quantitation of expression of each marker in treatment naïve patients is necessary for the evaluation of their role as robust predictive biomarkers. Our results are in line with a study that demonstrated similar average counts of $OX40^+$ cells in melanoma by IHC (14), and corroborates the finding of another study, which reported GITR expression to be drastically lower in patients with cancer compared with cancer in murine models (15). Concerning the immune subsets enriched for these receptors, the expression of OX40 on T cells was largely restricted to Tregs (~21% expressing the OX40 receptor), while GITR was expressed on a higher proportion of B cells compared with T cell populations, despite the majority of $GITR^+$ cells being T cells in melanoma. This is not surprising as GITR is known to be expressed by activated B cells, NK cells, and other innate immune cell populations (16, 17); however, their exact function in melanoma remains unknown. Our data does suggest, however, that any benefit observed in patients from the use of GITR and OX40 targeting therapies is likely to incorporate immune subsets other than simply $CD8^+$ effector T cells in the tumor, particularly Tregs in the case of OX40 targeting therapy in human melanoma.

Mechanistically, antibodies targeting OX40 and GITR have been shown to increase T cell proliferation, effector function, and survival for effector populations expressing the target receptor, while depleting target positive Tregs or dampening their function (18 22). However, given that such a small percentage of intratumoral T cells express GITR or OX40, this calls into question the likely effectiveness of these treatments and whether antibodies targeting these receptors are likely to have much mechanistic effect in nodal or distant metastases, as appears from early clinical trial data (23, 24). It is known that GITR and OX40 are intermediately expressed 24 72 hours after T cell activation and then decrease days later (16, 18). It is possible that GITR and OX40 are expressed on a greater proportion of T cells, when T cells are initially primed against tumor antigen. In line with this, we found that the percentage of T cells expressing GITR is higher at the early stages of melanoma (primary) compared with later stages (nodal and distant metastases) in matched specimens from the same patient. One limitation to the interpretation of our data, however, is that the tissue microarrays were used. Because some tumors are heterogeneous in their cellular composition, it is possible that our analysis is not representative of other areas of tumor. Nevertheless, the consistency and strong statistical trends in matched and global analyses for GITR between primary and distant metastases, strongly argues against this being a spurious result. This would suggest that GITR targeting antibodies could be biologically relevant at the very early stage of immune interaction with melanoma, but less effective for advanced disease. However, systemic immunotherapy is unlikely to be clinically relevant for patients with localized primary disease. In addition, our results indicate that comparative analysis for GITR expression between primary and distant metastases in predicting response to GITR therapies may be unsuitable and ineffective.

We also highlight that the majority of intratumoral TIM 3 and VISTA expression in melanoma is found on non T cell populations, including tumor cells (VISTA). Our cytometry data revealed that dendritic cells in the tumor, including cDC1 cells (defined by CD141 expression), and cDC2 cells, were enriched for the TIM 3 receptor. In murine models of breast cancer, cross presenting $CD103^+$ dendritic cells express TIM 3 and upon cotherapy with a TIM 3 targeting antibody, they upregulate CXCL9, resulting in the activation of T cells, reduction in tumor burden, and increased survival compared with the single agent paclitaxel therapy control

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arm (25). Such mechanisms may also exist in patients with melanoma treated with TIM 3 targeting antibodies, as cDC1 and cDC2 dendritic cells express TIM 3 and CD141$^+$ cDC1 represent the human equivalent of cross presenting CD103$^+$ DCs in murine models (26). Although we were unable to explore VISTA expression by mass cytometry, our combined flow cytometry and IHC data showed that VISTA was predominately expressed on myeloid cells and B cells in melanoma tumors. Indeed, there are reports that VISTA can be expressed strongly on myeloid populations in addition to Tregs, and that mAb treatment targeting VISTA can decrease the number of myeloid derived suppressive cells in melanoma tumors (27). Previously, we have shown that VISTA expression is increased in the melanoma biopsies of patients progressing on anti PD 1 therapies (28). Therefore, the functional effects of targeting VISTA warrant further investigation and testing of its efficacy in clinical trials.

To the best of our knowledge, this is the first study to explore the expression of TIGIT extensively on immune subsets in melanoma patient tumor dissociates. We demonstrate that innate cells generally express minimal TIGIT compared with T cells, with natural killer cells and monocytes, showing a trend for higher TIGIT expression within the innate compartment. Most importantly, however, when we investigated TIGIT expression on T cell subsets, TIGIT was highly expressed on CD69$^+$CD103$^+$CD8$^+$ tissue resident memory T cells (>70%) and on Treg cells (>90%). This has important implications as we have previously shown that CD103$^+$ CD8$^+$ resident T cells are a critical population for melanoma control (that make up approximately 30% of tumor infiltrating CD8$^+$ T cells) and are the likely targets and responders to anti PD 1 therapy (12, 13). Our study and others have shown that CD103$^+$ CD8$^+$ resident memory T cells are enriched for PD 1 and TIM 3 (12, 29), and together this suggests that TIGIT, PD 1, and TIM 3 inhibitors could all be primarily involved in reinvigorating the same important population of T cells in tumors. In line with this, our pairwise association analysis revealed that many of the coinhibitory receptors on T cells were correlated with one another, particularly PD 1 and TIGIT, further supporting this notion. Indeed, the expression profile of PD 1 and TIGIT on the immune subsets in melanoma was similar. Therefore, combining anti PD 1 and anti TIGIT may target the same T cell subsets and it remains to be determined whether the addition of TIGIT inhibition in patients with advanced melanoma has an additive or redundant effect on disease control and its effect on the frequency of immune related adverse events. Nevertheless, our data indicate that targeting TIGIT is likely to only benefit patients already responsive to anti PD 1 therapy (i.e., patients with PD 1 expression on tumor infiltrating lymphocytes in tumor) and therefore may have a purpose in a small percentage of patients who develop acquired resistance to anti PD 1. Given the high expression of TIGIT on Treg cells, however, it is likely that anti TIGIT therapy would have a dual role in depleting TIGIT$^+$ Tregs, the more potent suppressive cells, as others have demonstrated (30, 31).

We have also shown that ICOS is a relatively abundant costimulatory receptor on T cells in melanoma, accounting for approximately 12% of tumor infiltrating T cells. ICOS was enriched on Treg cells, but also expressed on CD4, and to a lesser extent, CD8 memory populations, including on populations positive for coinhibitory receptors. We also demonstrated that ICOS expression weakly correlated with coinhibitory receptor expression on T cells. In the past, the ICOS/ICOSL pathway has been shown to be important in the efficacy of anti CTLA 4 therapy (32, 33). Recently, a study in mice demonstrated that ICOS$^+$ Th1 like CD4 effector populations are the likely targets of anti CTLA 4 therapy (34). Given the abundance of ICOS in melanoma tumors, it is possible that targeting the ICOS/ICOSL pathway may have an effect on a significant subset of infiltrating T cells. Indeed, a recent study demonstrated that direct targeting of the ICOS/ICOSL pathway via an oncolytic virus helped increase activated T cell infiltrates in the treated tumor and untreated tumors at distant sites (35). Future studies will need to explore the exact contribution of ICOS positive CD4 and CD8 populations in melanoma disease.

The number of distant metastatic biopsies from specific sites in our study was limited. However, our results suggest that while the majority of checkpoint receptor expression did not change between sites, it is possible that site of disease may be an important factor in the expression of some checkpoint receptors. This should be validated and further examined in larger cohorts.

In conclusion, our study highlights significant differences in the prevalence and cellular distribution of expression of the major immune checkpoint receptors that are being targeted in current or soon to be commenced clinical trials in patients with melanoma. Furthermore, the interpatient expression of these potential drug targets was highly variable, suggesting that personalized treatment decisions based on predictive biomarker evaluation may be required to maximize treatment efficacy of novel drug combinations. In addition, the low abundance of some receptors (OX40 and GITR) and the coexpression of others (PD 1 and TIGIT) will need to be considered when designing clinical trials with these agents in melanoma.

Disclosure of Potential Conflicts of Interest

R. P. M. Saw reports receiving speakers bureau honoraria from Bristol-Myers Squibb, and is a consultant/advisory board member for Novartis, MSD, and Amgen. J. F. Thompson is a consultant/advisory board member for GlaxoSmithKline, Bristol-Myers Squibb, Merck Sharp Dohme, and Provectus. A. M. Menzies is a consultant/advisory board member for Bristol-Myers Squibb, MSD, Novartis, Roche, and Pierre-Fabre. G. Long is a consultant/advisory board member for Bristol-Myers Squibb, Merck, Novartis, Aduor, Mass Array, and Pierre-Fabre. R. A. Scolyer is a consultant/advisory board member for Merck Sharp & Dohme, Novartis, Myriad, and NeraCare. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.J. Edwards, A. Tasker, M. Batten, A.L. Ferguson, R. Allen, R.P.M. Saw, J.F. Thompson, A.M. Menzies, J.S. Wilmott, G. Long, R.A. Scolyer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.J. Edwards, A. Tasker, I. Silva, C. Quek, M. Batten, A.L. Ferguson, R. Allen, R.P.M. Saw, J.S. Wilmott, G. Long, R.A. Scolyer

Writing, review, and/or revision of the manuscript: J.J. Edwards, I. Silva, B.M. Allanson, J.F. Thompson, A.M. Menzies, U. Palendira, J.S. Wilmott, G. Long, R.A. Scolyer

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References

1. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, adaptive, and acquired resistance to cancer immunotherapy. Cell 2017;168:707–23.
2. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. Cancer Cell 2014;26:923–37.
3. Koyama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. Nat Commun 2016;7:10501.
4. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. J Exp Med 2010;207:2187–94.
5. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. Cancer Res 2012;72:917–27.
6. Menzies AM, Lum T, Wilmott JS, Hyman J, Kefford RF, Thompson JF, et al. Intrapatient homogeneity of BRAFV600E expression in melanoma. Am J Surg Pathol 2014;38:377–82.
7. Madore J, Vilain RE, Menzies AM, Kakavand H, Wilmott JS, Hyman J, et al. PD-L1 expression in melanoma shows marked heterogeneity within and between patients: implications for anti-PD-1/PD-L1 clinical trials. Pigment Cell Melanoma Res 2015;28:245–53.
8. Murali R, Brown PT, Kefford RF, Scolyer RA, Thompson JF, Atkins MB, et al. Number of primary melanomas is an independent predictor of survival in patients with metastatic melanoma. Cancer 2012;118:4519–29.
9. Bendall SC, Simonds EF, Qiu P, Amir el AD, Krutzik PO, Finck R, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science 2011;332:687–96.
10. Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. Immunity 2012;36:142–52.
11. Lai L, Ong R, Li J, Albani S. A CD45-based barcoding approach to multiplex mass-cytometry (CyTOF). Cytometry A 2015;87:369–74.
12. Edwards J, Wilmott JS, Madore J, Gide TN, Quek C, Tasker A, et al. CD103(+) tumor-resident CD8(+) T cells are associated with improved survival in immunotherapy-naive melanoma patients and expand significantly during anti-PD-1 treatment. Clin Cancer Res 2018;24:3036–45.
13. Gide TN, Quek C, Menzies AM, Tasker AT, Shang P, Holst J, et al. Distinct immune cell populations define response to anti-PD-1 monotherapy and anti-PD-1/anti-CTLA-4 combined therapy. Cancer Cell 2019;35:238–55.e6.
14. Ladanyi A, Somlai B, Gilde K, Fejos Z, Gaudi I, Timar J. T-cell activation marker expression on tumor-infiltrating lymphocytes as prognostic factor in cutaneous malignant melanoma. Clin Cancer Res 2004;10:521–30.
15. Sukumar S, Wilson DC, Yu Y, Wong J, Naravula S, Ermakov G, et al. Characterization of MK-4166, a clinical agonistic antibody that targets human GITR and inhibits the generation and suppressive effects of T regulatory cells. Cancer Res 2017;77:4378–88.
16. Schaer DA, Murphy JT, Wolchok JD. Modulation of GITR for cancer immunotherapy. Curr Opin Immunol 2012;24:217–24.
17. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol 2002;3:135–42.
18. Aspeslagh S, Postel-Vinay S, Rusakiewicz S, Soria JC, Zitvogel L, Marabelle A. Rationale for anti-OX40 cancer immunotherapy. Eur J Cancer 2016;52:50–66.
19. Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, et al. Activating Fc gamma receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. J Exp Med 2013;210:1685–93.
20. Gough MJ, Ruby CE, Redmond WL, Dhungel B, Brown A, Weinberg AD. OX40 agonist therapy enhances CD8 infiltration and decreases immune suppression in the tumor. Cancer Res 2008;68:5206–15.
21. Knee DA, Hewes B, Brogdon JL. Rationale for anti-GITR cancer immunotherapy. Eur J Cancer 2016;67:1–10.
22. Ronchetti S, Nocentini G, Bianchini R, Krausz LT, Migliorati G, Riccardi C. Glucocorticoid-induced TNFR-related protein lowers the threshold of CD28 costimulation in CD8+ T cells. J Immunol 2007;179:5916–26.
23. Tran B, Carvajal RD, Marabelle A, Patel SP, LoRusso PM, Rasmussen E, et al. Dose escalation results from a first-in-human, phase 1 study of glucocorticoid-induced TNF receptor–related protein agonist AMG 228 in patients with advanced solid tumors. J Immunother Cancer 2018;6:93.
24. Curti BD, Kovacsovics-Bankowski M, Morris N, Walker E, Chisholm L, Floyd K, et al. OX40 is a potent immune-stimulating target in late-stage cancer patients. Cancer Res 2013;73:7189–98.
25. de Mingo Pulido A, Gardner A, Hiebler S, Soliman H, Rugo HS, Krummel MF, et al. TIM-3 regulates CD103(+) dendritic cell function and response to chemotherapy in breast cancer. Cancer Cell 2018;33:60–74.
26. Haniffa M, Shin A, Bigley V, McGovern N, Teo P, See P, et al. Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. Immunity 2012;37:60–73.
27. Le Mercier I, Chen W, Lines JL, Day M, Li J, Sergent P, et al. VISTA regulates the development of protective antitumor immunity. Cancer Res 2014;74:1933–44.
28. Kakavand H, Jackett LA, Menzies AM, Gide TN, Carlino MS, Saw RPM, et al. Negative immune checkpoint regulation by VISTA: a mechanism of acquired resistance to anti-PD-1 therapy in metastatic melanoma patients. Mod Pathol 2017;30:1666–76.
29. Boddupalli CS, Bar N, Kadaveru K, Krauthammer M, Pornputtapong N, Mai Z, et al. Interlesional diversity of T cell receptors in melanoma with immune checkpoints enriched in tissue-resident memory T cells. JCI Insight 2016;1:e88955.
30. Blake SJ, Dougall WC, Miles JJ, Teng MW, Smyth MJ. Molecular pathways: targeting CD96 and TIGIT for cancer immunotherapy. Clin Cancer Res 2016;22:5183–8.
31. Kurtulus S, Sakuishi K, Ngiow SF, Joller N, Tan DJ, Teng MW, et al. TIGIT predominantly regulates the immune response via regulatory T cells. J Clin Invest 2015;125:4053–62.
32. Fan X, Quezada SA, Sepulveda MA, Sharma P, Allison JP. Engagement of the ICOS pathway markedly enhances efficacy of CTLA-4 blockade in cancer immunotherapy. J Exp Med 2014;211:715–25.
33. Fu T, He Q, Sharma P. The ICOS/ICOSL pathway is required for optimal antitumor responses mediated by anti-CTLA-4 therapy. Cancer Res 2011;71:5445–54.
34. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAS, Andrews MC, et al. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. Cell 2017;170:1120–33.
35. Zamarin D, Holmgaard RB, Ricca J, Plitt T, Palese P, Sharma P, et al. Intratumoral modulation of the inducible co-stimulator ICOS by recombinant oncolytic virus promotes systemic anti-tumour immunity. Nat Commun 2017;8:14340.

Chapter 3 Supplementary Tables and Figures

Supplementary Table 1. The mean and median values for checkpoint receptor expression in melanoma (positive cells per 1 mm2) for all biopsies (total, n=96). The proportion of biopsies positive for each marker is also presented using a cut-off of >1 cell and >5 cells per 1mm2 to define positive.

Marker	CD3	ICOS	GITR	OX40	PD-1	TIM-3	VISTA
Mean (cells positive for marker per mm2)	1101	303	13	16	166	122	286
Median (Cells positive for marker per mm2)	349	22	4	0	19	13	51
Proportion of biopsies with >1 positive cell/mm2 for marker (%)	98	78	61	36	80	81	90
Proportion of biopsies with >5 positive cells/mm2 for marker (%)	97	70	46	23	70	70	78

Supplementary Table 2. Correlation between CD3 and checkpoint receptors from IHC data

Markers	p-value	Rank R squared	Rank R
CD3 vs. ICOS	5.60E-28	0.79	0.89
CD3 vs. TIM-3	3.11E-14	0.54	0.73
CD3 vs. PD-1	2.24E-10	0.42	0.65
CD3 vs. OX40	3.23E-08	0.33	0.57
CD3 vs. GITR	7.53E-08	0.31	0.56
CD3 vs. VISTA	3.21E-05	0.21	0.46

Supplementray Table 3. Clinical characteristics of melanoma biopsies used in CyTOF

Patient number	Bx Site	LN or Distant Met
1	Right medial thigh (involving LN)	Distant
2	Left frontal brain	Brain Distant
3	Right parotid lymph node	LN
4	Abdomen	Distant
5	Left shoulder	Distant
6	Left flank	Distant
7	Skin right axilla	LN
8	Right frontal intraventricular tumour	Distant
9	Right axilla and pectoral lymph node	LN
10	Rectum and Anus (involving LN)	Distant
11	Right arm	Distant
12	Left parotid gland	LN
13	Left buttock	Distant
14	Right Thigh	Distant
15	Anterior chest, thorax	In-transit
16	Right anterior chest, thorax	Distant
17	Brain	Distant
18	Brain	Distant

LN = Lymph node metastasis
Distant = Distant metastasis

Supplementary Table 4. Statistical summary of immune populations from CYTOF data

	ICOS		GITR		OX40		PD-1		CTLA-4		TIM-3		TIGIT		PD-L1		PD-L2	
	Summary	*P Value*	*Summary*	*P Value*	*Summary*	*P Value*	*Summary*	*P Value*	*Summary*	*P Value*	*Summary*	*P Value*	*Summary*	*P Value*	*Summary*	*P Value*	*Summary*	*P Value*
CD8+ Tcm vs. CD8+ Teff	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 5678	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Tcm vs. CD8+ Trm	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 398	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Tcm vs. CD4+ Tcm	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Tcm vs. CD4+ Teff	ns	0 7767	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Tcm vs. CD4+ Trm	ns	0 442	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Tcm vs. Treg	ns	0 4374	ns	0 9999	ns	0 9489	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Tcm vs. NK cells	ns	0 9999	ns	0 9999	ns	0 9999	**	0 0029	ns	0 9999	ns	0 9999	ns	0 824	ns	0 9999	ns	0 9999
CD8+ Tcm vs. cDC1s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 3377	*	0 0275	ns	0 9999	ns	0 9999
CD8+ Tcm vs. cDC2s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 038	**	0 0047	ns	0 9999	ns	0 9999
CD8+ Tcm vs. Monocytes	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 0979	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Tcm vs. CD68+ CD14+	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 025	ns	0 9999	****	<0 000	ns	0 9999	ns	0 9999
CD8+ Tcm vs. CD68+ CD14	ns	0 9999	ns	0 9999	ns	0 9999	*	0 0283	ns	0 4846	ns	0 9999	**	0 00	ns	0 9999	ns	0 9999
CD8+ Tcm vs. B cells	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 097	ns	0 9999	ns	0 9999	**	0 0062	ns	0 9999	ns	0 9999
CD8+ Teff vs. CD8+ Trm	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	*	0 0 27	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Teff vs. CD4+ Tcm	ns	0 9999	ns	0 9999	**	0 009	ns	0 9999	ns	0 696	ns	0 9999	ns	0 9999	ns	0 5 4	ns	0 9999
CD8+ Teff vs. CD4+ Teff	*	0 0255	ns	0 9999	ns	0 693	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 2977	ns	0 9999
CD8+ Teff vs. CD4+ Trm	*	0 0 24	ns	0 9999	***	0 0009	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Teff vs. Treg	ns	0 027	ns	0 9999	*	0 0 66	ns	0 9999	ns	0 0785	ns	0 904	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Teff vs. NK cells	ns	0 9999	ns	0 9999	ns	0 9999	**	0 0058	ns	0 9999	ns	0 9999	ns	0 72 4	ns	0 9999	ns	0 9999
CD8+ Teff vs. cDC1s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	*	0 0402	ns	0 038	ns	0 9999	ns	0 9999
CD8+ Teff vs. cDC2s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	***	0 0009	*	0 0 28	*	0 0 83	ns	0 9999
CD8+ Teff vs. Monocytes	ns	0 9999	ns	0 4279	ns	0 9999	ns	0 29 9	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Teff vs. CD68+ CD14+	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 2 56	****	<0 000	**	0 0099	ns	0 9999
CD8+ Teff vs. CD68+ CD14	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 0502	ns	0 9999	ns	0 9999	**	0 00 5	ns	0 3 4	ns	0 9999
CD8+ Teff vs. B cells	ns	0 9999	*	0 04 7	ns	0 9999	ns	0 225	ns	0 9999	ns	0 9999	*	0 0 32	ns	0 9999	ns	0 9999
CD8+ Trm vs. CD4+ Tcm	ns	0 9999	ns	0 9999	**	0 0072	ns	0 9999	ns	0 9999	ns	0 88	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Trm vs. CD4+ Teff	ns	0 9999	ns	0 9999	ns	0 098	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 678	ns	0 9999	ns	0 9999
CD8+ Trm vs. CD4+ Trm	ns	0 9999	ns	0 9999	**	0 00 3	ns	0 9999	ns	0 9999	ns	0 85 2	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Trm vs. Treg	ns	0 9999	ns	0 9999	**	0 0069	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD8+ Trm vs. NK cells	ns	0 9999	ns	0 9999	ns	0 9999	****	<0 000	ns	0 9999	ns	0 9999	*	0 0 74	ns	0 9999	ns	0 9999
CD8+ Trm vs. cDC1s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 3 79	ns	0 9999	ns	0 9999	**	0 0036	ns	0 9999	ns	0 9999
CD8+ Trm vs. cDC2s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 804	ns	0 9999	ns	0 9999	***	0 0002	ns	0 9999	ns	0 9999
CD8+ Trm vs. Monocytes	ns	0 9999	ns	0 439	ns	0 9999	***	0 00	ns	0 9999	ns	0 9999	ns	0 2 6	ns	0 9999	ns	0 9999
CD8+ Trm vs. CD68+ CD14+	ns	0 9999	ns	0 9999	ns	0 9999	*	0 0397	ns	0 7726	ns	0 9999	****	<0 000	ns	0 9999	ns	0 9999
CD8+ Trm vs. CD68+ CD14	ns	0 9999	ns	0 9999	ns	0 9999	****	<0 000	ns	0 9999	ns	0 0687	****	<0 000	ns	0 9999	ns	0 9999
CD8+ Trm vs. B cells	ns	0 9999	*	0 024	ns	0 9999	***	0 0002	ns	0 9999	***	0 0004	***	0 0002	ns	0 9999	ns	0 9999
CD4+ Tcm vs. CD4+ Teff	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Tcm vs. CD4+ Trm	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Tcm vs. Treg	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 8535	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Tcm vs. NK cells	ns	0 687	ns	0 9999	ns	0 9999	**	0 0056	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Tcm vs. cDC1s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 2284	ns	0 3456	ns	0 9999	ns	0 9999
CD4+ Tcm vs. cDC2s	ns	0 4467	ns	0 9999	ns	0 8074	ns	0 9999	ns	0 9999	*	0 0322	ns	0 305	ns	0 9999	ns	0 9999
CD4+ Tcm vs. Monocytes	ns	0 5 5	ns	0 9999	ns	0 9999	ns	0 232	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Tcm vs. CD68+ CD14+	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	*	0 0 96	ns	0 9999	**	0 00 7	ns	0 9999	ns	0 9999
CD4+ Tcm vs. CD68+ CD14	*	0 0 72	ns	0 9999	**	0 0034	ns	0 053	ns	0 335	ns	0 9999	*	0 0408	ns	0 9999	ns	0 9999
CD4+ Tcm vs. B cells	ns	0 9999	ns	0 9999	*	0 0 35	ns	0 2082	ns	0 9999	ns	0 9999	ns	0 2008	ns	0 9999	ns	0 9999
CD4+ Teff vs. CD4+ Trm	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Teff vs. Treg	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Teff vs. NK cells	**	0 0037	ns	0 9999	ns	0 9999	**	0 0052	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Teff vs. cDC1s	ns	0 5243	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Teff vs. cDC2s	*	0 0 3	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 35	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Teff vs. Monocytes	*	0 0284	ns	0 662	ns	0 9999	ns	0 2856	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 8437
CD4+ Teff vs. CD68+ CD14+	ns	0 2 23	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 372	ns	0 9999	ns	0 0508	ns	0 9999	ns	0 9999
CD4+ Teff vs. CD68+ CD14	****	<0 000	ns	0 9999	ns	0 07	*	0 0444	ns	0 8677	ns	0 9999	ns	0 8858	ns	0 9999	ns	0 9999
CD4+ Teff vs. B cells	ns	0 354	**	0 0073	ns	0 244	ns	0 2073	ns	0 9999	ns	0 4297	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Trm vs. Treg	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Trm vs. NK cells	**	0 00 9	ns	0 9999	ns	0 9999	****	<0 000	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Trm vs. cDC1s	ns	0 3329	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 7457	ns	0 24 5	ns	0 9999	ns	0 9999
CD4+ Trm vs. cDC2s	**	0 0057	ns	0 9999	ns	0 2777	ns	0 9999	ns	0 9999	ns	0 835	ns	0 0578	ns	0 9999	ns	0 9999
CD4+ Trm vs. Monocytes	*	0 0 55	ns	0 3606	ns	0 7424	*	0 0 9	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD4+ Trm vs. CD68+ CD14+	ns	0 075	ns	0 9999	ns	0 9999	ns	0 5 44	ns	0 387	ns	0 9999	***	0 0003	ns	0 9999	ns	0 9999
CD4+ Trm vs. CD68+ CD14	****	<0 000	ns	0 9999	***	0 0003	***	0 0003	ns	0 8355	ns	0 9999	*	0 0 7	ns	0 9999	ns	0 9999
CD4+ Trm vs. B cells	ns	0 0675	*	0 0345	**	0 00 5	**	0 002	ns	0 9999	ns	0 9999	ns	0 0758	ns	0 9999	ns	0 9999
Treg vs. NK cells	*	0 0 39	ns	0 9999	ns	0 9999	**	0 0089	ns	0 9999	ns	0 9999	ns	0 2264	ns	0 9999	ns	0 9999
Treg vs. cDC1s	ns	0 2076	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	*	0 0303	ns	0 9999	ns	0 9999
Treg vs. cDC2s	*	0 0327	ns	0 9999	ns	0 2383	ns	0 9999	ns	0 2977	ns	0 9999	*	0 0 84	ns	0 9999	ns	0 9999
Treg vs. Monocytes	*	0 0336	ns	0 9999	ns	0 363	ns	0 0 9	ns	0 2292	ns	0 9999	ns	0 9254	ns	0 9999	ns	0 9999
Treg vs. CD68+ CD14+	ns	0 3025	ns	0 9999	ns	0 8684	ns	0 9999	*	0 0 85	ns	0 9999	**	0 00 2	ns	0 9999	ns	0 9999
Treg vs. CD68+ CD14	**	0 004	ns	0 9999	**	0 0098	ns	0 0772	ns	0 0692	ns	0 5446	*	0 0	ns	0 9999	ns	0 9999
Treg vs. B cells	ns	0 2478	ns	0 754	*	0 02 3	ns	0 739	ns	0 839	*	0 0242	*	0 0322	ns	0 9999	ns	0 9999
NK cells vs. cDC1s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
NK cells vs. cDC2s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 7767	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 4285	ns	0 9999
NK cells vs. Monocytes	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
NK cells vs. CD68+ CD14+	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 6056	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 468	ns	0 9999
NK cells vs. CD68+ CD14	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
NK cells vs. B cells	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
cDC1s vs. cDC2s	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
cDC1s vs. Monocytes	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
cDC1s vs. CD68+ CD14+	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
cDC1s vs. CD68+ CD14	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 304	ns	0 9999	ns	0 9999	ns	0 9999
cDC1s vs. B cells	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	**	0 004	ns	0 9999	ns	0 9999	ns	0 9999
cDC2s vs. Monocytes	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
cDC2s vs. CD68+ CD14+	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
cDC2s vs. CD68+ CD14	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	**	0 0073	ns	0 9999	ns	0 9999	ns	0 9999
cDC2s vs. B cells	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	****	<0 000	ns	0 9999	ns	0 734	ns	0 9999
Monocytes vs. CD68+ CD14+	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
Monocytes vs. CD68+ CD14	ns	0 9999	ns	0 7 38	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
Monocytes vs. B cells	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD68+ CD14+ vs. CD68+ CD14	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999
CD68+ CD14+ vs. B cells	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	**	0 0056	ns	0 9999	ns	0 364	ns	0 9999
CD68+ CD14 vs. B cells	ns	0 9999	ns	0 09 6	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999	ns	0 9999

Supplementary Table 5. Cell surface antibodies used in CyTOF

Label	Specificity	Clone	Manufacturer
89	CD8A	RPA-T8	Biolegend
104	CD45	HI30	BD
110	CD45	HI30	BD
113	CD56	REA196	Miltenyi Biotech
115	CD11c	Bu16	Biolegend
139	CD206	15-Feb	Biolegend
141	CD27	M-T271	BD
142	CD19	HIB19	BD
143	CD45RA	HI100	BD
144	CD69	FN50	Biolegend
145	CD4	RPA-T4	BD
146	CD1c	L161	Biolegend
147	CD45RO	UCHL1	BD
148	CD16	3GB	BD
149	CD366 (TIM3)	7D3	BD
150	Biotin	1D4-C5	Biolegend
151	CD278 (ICOS)	DX29	BD
152	CD13	WM15	Fluidigm*
154	CD3	UCHT1	BD
156	CD86	IT2.2	BD
158	CD33	WM53	BD
159	CD197 (CCR7)	G043H7	Biolegend
161	CD141 (BDCA3)	AD5-14H12	Miltenyi Biotech
163	CD223 (Lag3)	11C3C65	BD
164	CD274 (PD-L1)	MIH1	Biolegend
165	PE	PE001	Biolegend
166	TIGIT	MBSA43	RIZO
167	APC	APC003	Biolegend
168	CD273 (PD-L2)	24F.10C12	Biolegend
169	CD25	M-A251	BD
170	CD152 (CTLA-4)	14D3	eBiosciences
172	CD134 (OX40)	ACT35	Biolegend
173	CD14	M5E2	Biolegend
174	HLA-DR	G46-6	BD
175	CD279 (PD-1)	EH12.1H7	Biolegend
176	CD127	A019D5	BD
209	CD57	NK-1	BD

* Fluidigm antibodies purchased metal conjugated. Remainder antibodies were conjugated in-house.

Supplementary Table 6: Intracellular antibodies used in CyTOF

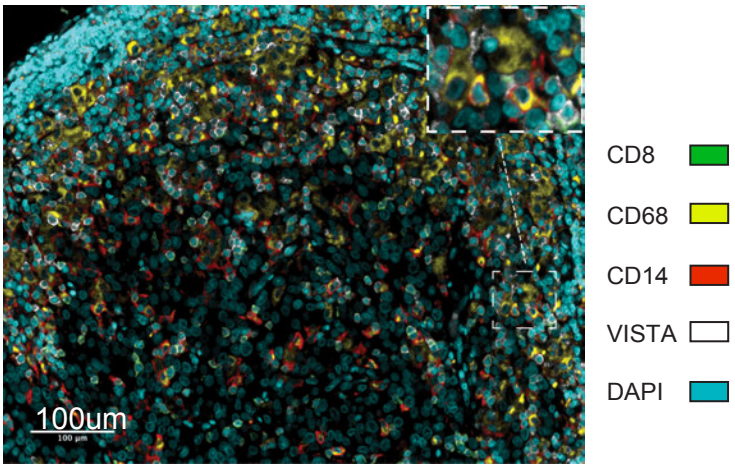
Label	Specificity	Clone	Manufacturer
153	CD68	KP1	Biolegend
155	EOMES	WD1928	eBiosciences
160	T-bet	4B10	Biolegend
162	FoxP3	PCH101	eBiosciences

Supplementary Table 7. Fluorophore-conjugated Cell Surface antibodies

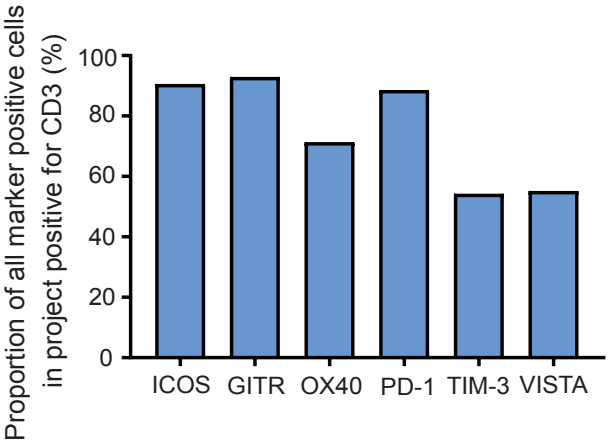
Label	Specificity	Clone	Manufacturer
Biotin	CD103	Ber-ACT8	Biolegend
PE	CD357 (GITR)	621	Biolegend

Supplementary Figure 1

A)



B)



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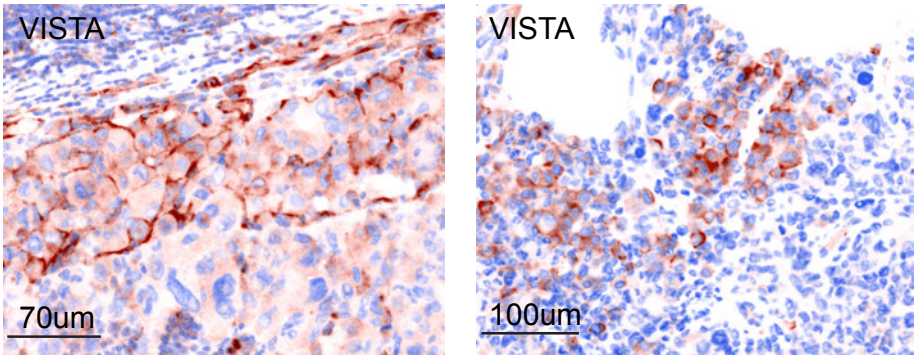

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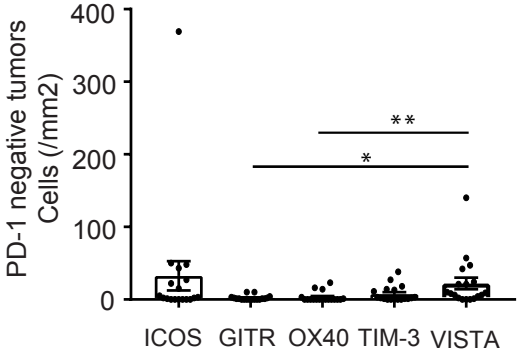

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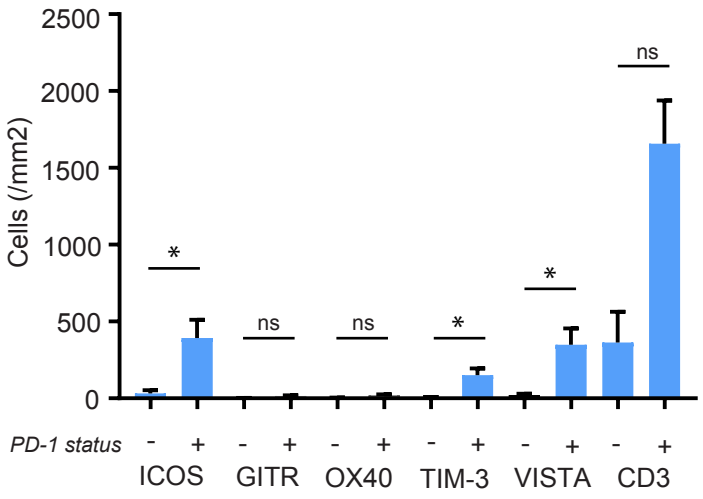
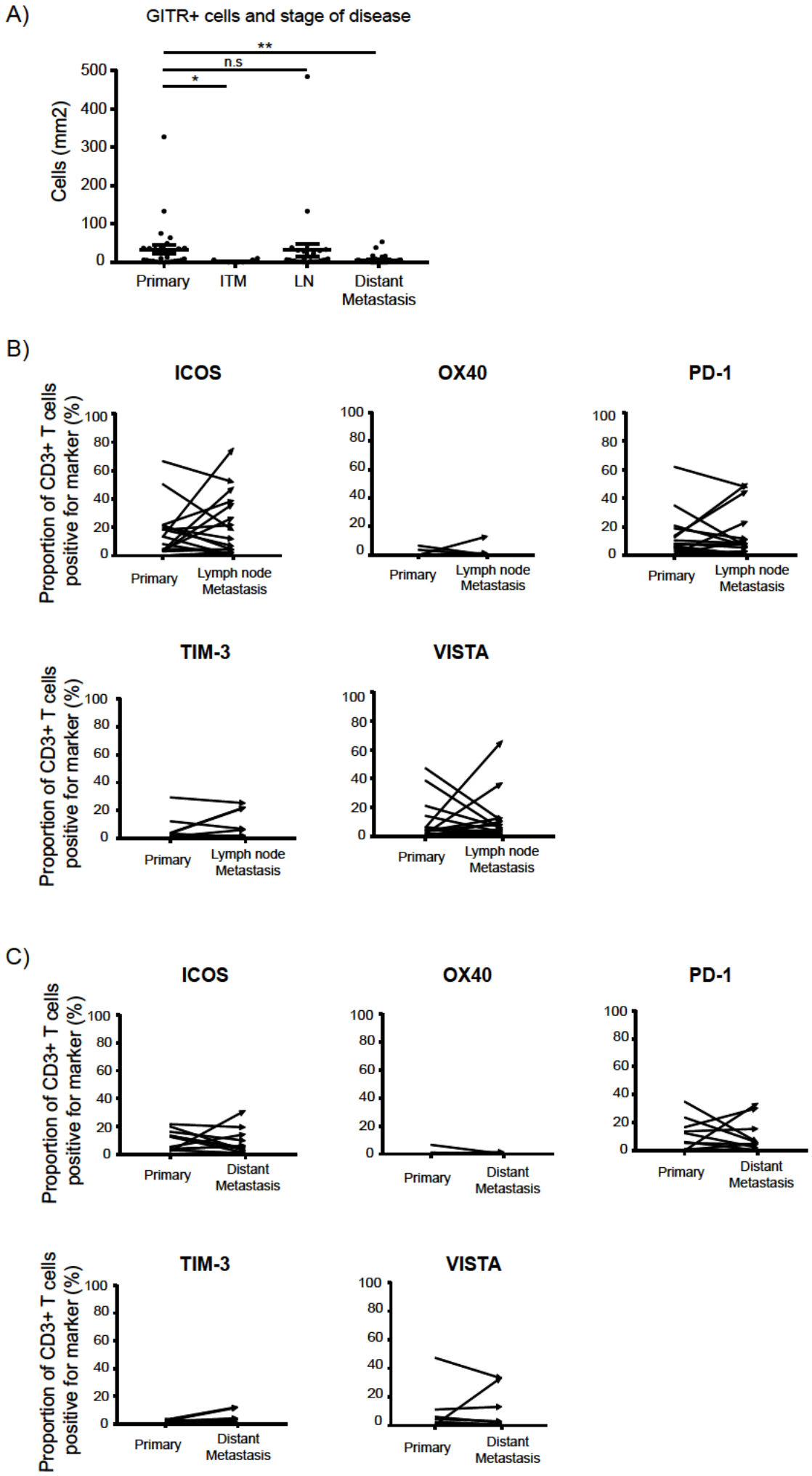
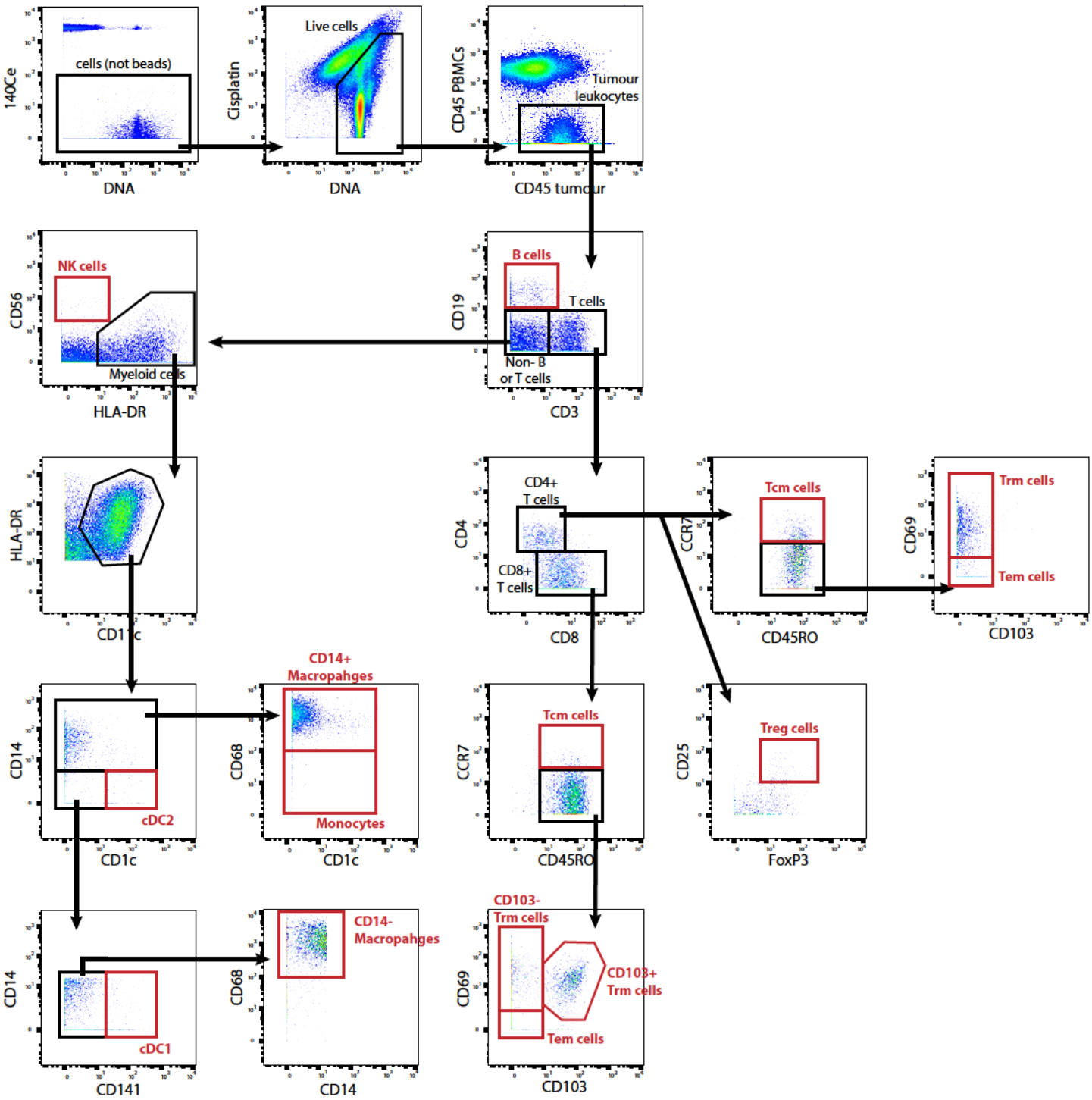


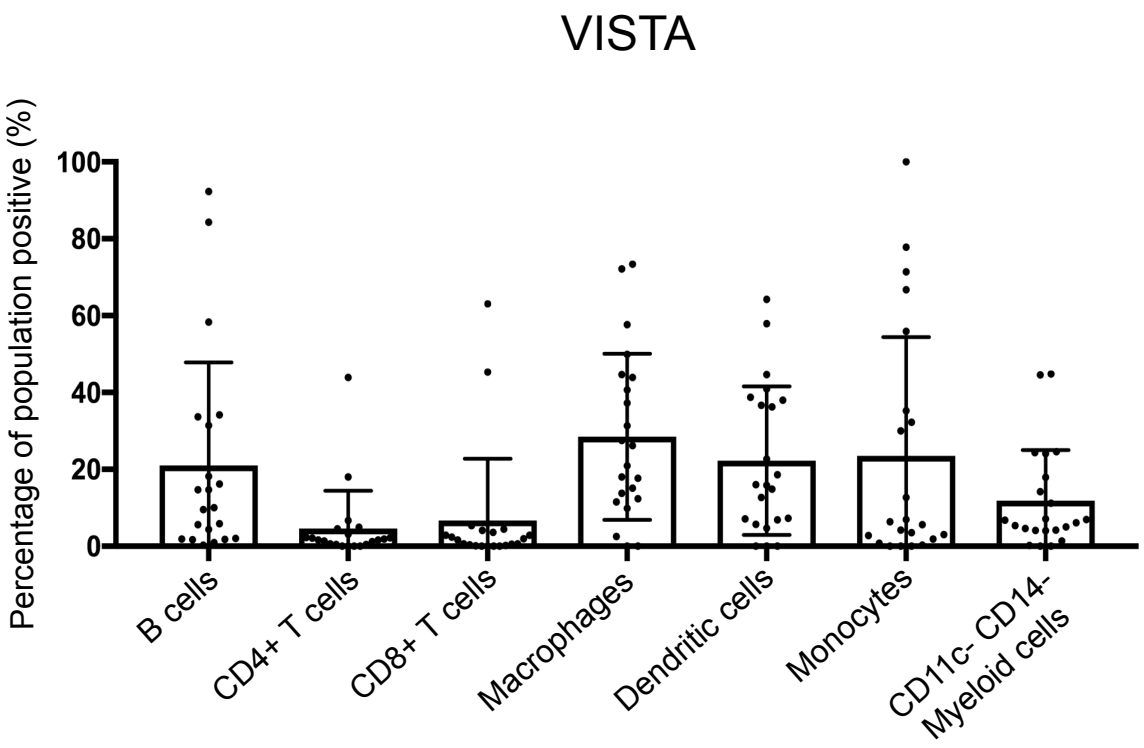
Supplementary Figure 2



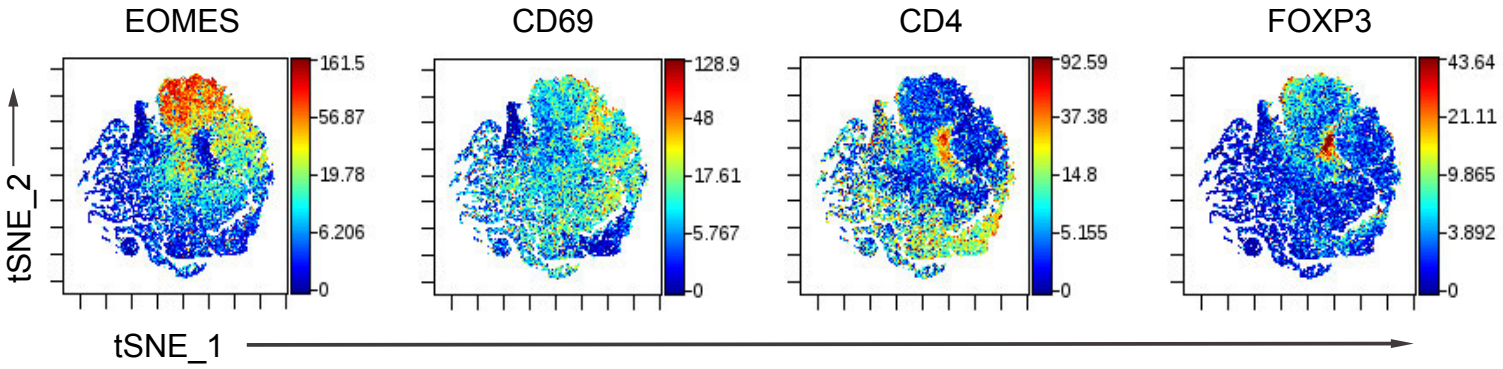
Supplementary Figure 3A



Supplementary Figure 3B



Supplementary Figure 4A



Chapter 3 - Supplementary Figure Legends

Supplementary Figure 1.

Expression of co-stimulatory and co-inhibitory markers in tumors and their expression in PD-1 negative biopsies. A, Representative staining showing co-localization of VISTA with CD68, CD14, and CD8 in melanoma tumor. B, The number of positive cells for each marker from all biopsies (n=96) were summed and the percentage expressing CD3 was calculated. C, Representative images of VISTA expression on melanoma tumor cells in two individual patient biopsies. D, The number of positive cells for each marker per mm^2 in PD-1 negative (≤1 cell/mm^2) (n=18) tumors. E, Mean comparison in the expression of alternative checkpoint markers in PD-1 negative (≤1 cell/mm^2) and PD-1 positive tumors (>1 cell/ mm^2).

Supplementary Figure 2.

Expression profile of checkpoint receptors at different stages of melanoma disease and site of disease in unmatched and matched patient specimens. A, The number of GITR positive cells per $1mm^2$ in primary, in-transit metastases, lymph node metastases and distant metastases (unmatched). B, The proportion of intra-tumoral T cells expressing co-stimulatory (ICOS and OX4O) and co-inhibitory (PD-1, TIM-3, and VISTA) receptors per $1mm^2$ between matched primary and regional lymph node melanoma samples from the same patient (n=20). C, The proportion of intra-tumoral T cells expressing co-stimulatory (ICOS and OX4O) and co-inhibitory (PD-1, TIM-3, and VISTA) receptors per $1mm^2$ between matched primary and distant metastatic melanoma samples from the same patient (n=14).

Supplementary Figure 3A.

Gating strategy for the identification of T cell and other immune cell subsets from CyTOF dataset.

Supplementary Figure 3B.

Expression and distribution of VISTA on immune cells in melanoma tumor. Distribution of VISTA on manually gated immune populations from n=20 patient tumor dissociates. Data is shown as the percentage of a population positive for that marker. Each dot represents a single tumor dissociate run through flow cytometry.

Supplementary Figure 4A.

Tsne plots generated on CD3 positive T cells from all patients (60,000 events, concatenated) for EOMES, CD69, FOXP3, and CD4.

Letter to the Editor

Clinical Cancer Research

Novel Immune Targets in Melanoma—Letter

Giuseppe Nocentini, Luigi Cari, and Carlo Riccardi

We have read with great interest the article by Edwards and colleagues, recently published in *Clinical Cancer Research* (1). However, we have concerns about the conclusions on GITR expression in tumor infiltrating cells.

Figure 1D of the article depicts the expression of T cell markers in the melanoma microenvironment. The images reveal that ICOS, PD 1, and GITR are mainly expressed by T cells in all or the majority of the patients, and OX40 and Tim 3 are mainly expressed by T cells in less than half of the patients. On the basis of the information in Fig. 1C, the markers can be divided into two groups: (i) GITR, OX40, and Tim 3 (expressed by a low number of T cells, e.g., regulatory T cells) and (ii) ICOS and PD 1 (expressed by a high number of T cells). According to the data in Figure 3B, this is true for all markers, with the exception of GITR, which shows the highest expression levels in monocytes and B cells (in disagreement with the data in Figure 1D) and is not expressed at all by regulatory T cells (in disagreement with the literature; ref. 2).

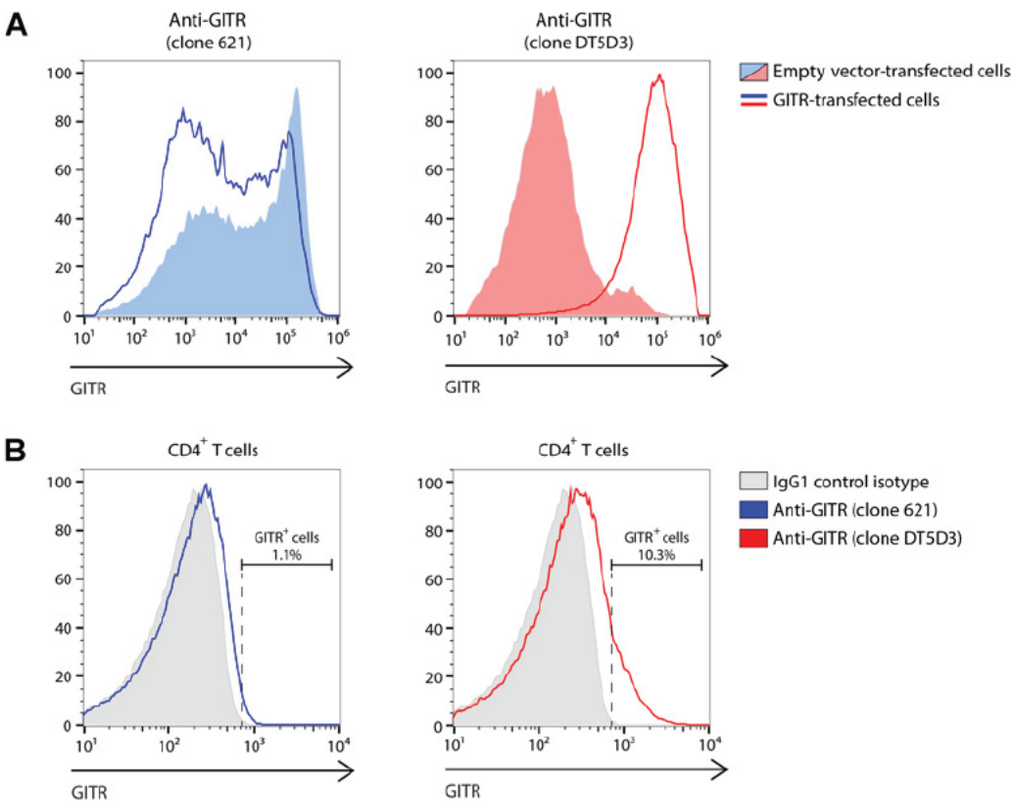


Figure 1.

Difference between staining of GITR by anti GITR, clone 621 and anti GITR, clone DT5D3. (**A**) HEK FT cell line was transfected with the pcDNA 3.1 vector (empty vector transfected cells) or the vector containing the ORF of GITR (NM 004195.3)(GITR transfected cells). Cell clones were selected by Kanamycin and GITR overexpression was checked by real time PCR. An empty vector transfected cell clone and a GITR$^+$ clone were stained with anti GITR, clone 621 (left) and anti GITR, clone DT5D3 (right). Similar results were obtained with two more clones. (**B**) CD4$^+$ cells purified from PBMC of a healthy donor were stained by the IgG1 isotype control (BD Biosciences) or anti GITR, clone 621 (left) and the IgG1 isotype control or anti GITR, clone DT5D3 (right).

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We have evidence that the anti GITR antibody clone 621 (621 mAb), which was used in the experiments in Figure 3B, but not those in Figure 1 of the Edwards and colleagues' study, does not bind GITR, or, at least, its principal isoform. Indeed, a cell line transfected with human GITR (NP 004186.1) was confirmed to overexpress GITR by the anti GITR clone DT5D3 mAb (Miltenyi Biotec) and not by 621 mAb (Figure 1A shown here). On the contrary, comparison of GITR staining by the two mAbs in purified $CD4^+$ T cells (Figure 1B shown here) may only indicate that 621 mAb has a relatively low affinity to GITR.

In conclusion, Figure 1A shown here and Figure 1D of the Edwards and colleagues manuscript contradict the data pub lished by the Edwards and colleagues study in Figure 3B. Thus, we propose that the anti GITR 621 mAb is not reliable for assessing GITR expression, as suggested by another group (3). It is unclear whether this is a result of the aspecific binding of 621 mAb or its specific binding to another protein or to shedded GITR bound to its transmembrane ligand. Nonetheless, we are of the opinion that the conclusions drawn by Edwards and colleagues and other similar studies using this antibody (such as refs. 3 5) must be reconsidered.

Disclosure of Potential Conflicts of Interest

G. Nocentini is a consultant for Pieris Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

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References

1. Edwards JJ, Tasker A, Silva I, Quek C, Batten M, Ferguson AL, et al. Prevalence and cellular distribution of novel immune checkpoint targets across longitudinal specimens in treatment-naïve melanoma: implications for clinical trials. Clin Cancer Res 2019.
2. Ronchetti S, Ricci E, Petrillo MG, Cari L, Migliorati G, Nocentini G, et al. Glucocorticoid-induced tumour necrosis factor receptor-related protein: a key marker of functional regulatory T cells. J Immunol Res 2015;2015:171520.
3. Schiavinato JLDS, Haddad R, Saldanha-Araujo F, Baiochi J, Araujo AG, Santos Scheucher P, et al. TGF-beta/atRA-induced Tregs express a selected set of microRNAs involved in the repression of transcripts related to Th17 differentiation. Sci Rep 2017;7:3627.
4. Nocentini G, Alunno A, Petrillo MG, Bistoni O, Bartoloni E, Caterbi S, et al. Expansion of regulatory GITR+CD25low/-CD4+ T cells in systemic lupus erythematosus patients. Arthritis Res Ther 2014;16:444.
5. Mason GM, Lowe K, Melchiotti R, Ellis R, de Rinaldis E, Peakman M, et al. Phenotypic complexity of the human regulatory T cell compartment revealed by mass cytometry. J Immunol 2015;195:2030–7.

Clinical Cancer Research

Novel Immune Targets in Melanoma—Response

Jarem Edwards[1,2,3,4], Marcel Batten[1,4], Angela Ferguson[2,3,4], Umaimainthan Palendira[2,3,4], James S. Wilmott[1,2,4], Georgina V. Long[1,2,4,5,6], and Richard A. Scolyer[1,2,4,7]

We thank Nocentini and colleagues for their interest in our recent work in which we reported on the prevalence and distribution of the expression of novel checkpoint targets in melanoma, as published in *Clinical Cancer Research* (1). In their letter, the authors state that anti GITR clone 621, an antibody used in our CyTOF analysis, but not immunohistochemical experiments, may not be as sensitive for detecting GITR as other clones. To address possible differences in the sensitivity of

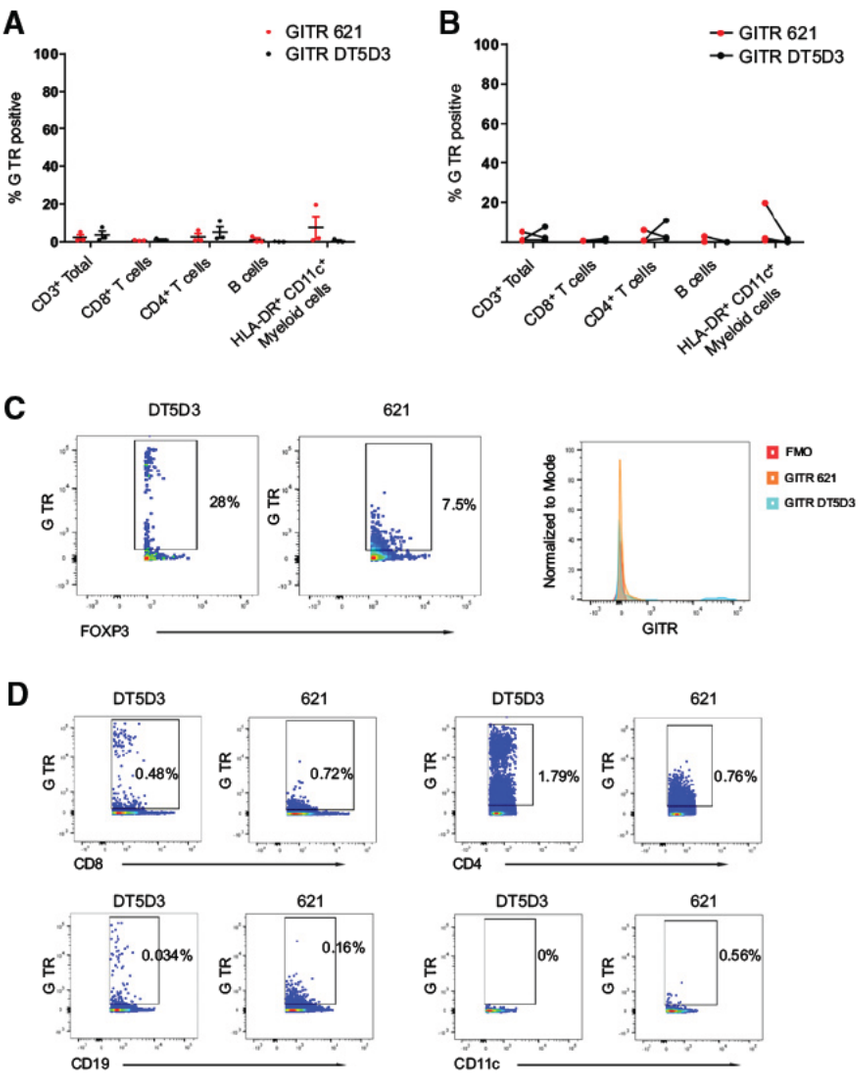

Figure 1.
Difference between GITR staining by anti GITR clone 621 and anti GITR clone DT5D3 on immune cells in human melanoma tumors. **A,** Percent average of immune cell subsets positive for GITR as detected by anti GITR clone 621 (red) and anti GITR clone DT5D3 (black) from $n = 3$ melanoma tumor dissociates. **B,** GITR expression on immune cell subsets as detected by anti GITR clone 621 (red) and anti GITR clone DT5D3 (black) from $n = 3$ melanoma tumor dissociates (matched by patient sample). **C,** GITR expression on FOXP3^{+} CD25^{hi} T regulatory cells in one melanoma tumor dissociate as detected by anti GITR clone DT5D3 (left) anti GITR clone 621 (right). Histogram plot with FMO control is also given on far right. **D,** Representative dot plots comparing the separation of GITR positive CD8 T cells (top left), CD4 T cells (top right), CD19^{+} B cells (bottom left) and HLA DR^{+} CD1c^{+} myeloid cells (bottom right) by anti GITR clone DT5D3 and anti GITR clone 621.

different anti GITR antibodies, we performed a direct comparison of GITR staining by anti GITR clone 621 and anti GITR clone DT5D3 (the clone recommended by Nocentini and colleagues) using flow cytometry and compared the levels of GITR detected on immune cells in three human melanoma tumor dissociates.

We found that the average levels of GITR detected on immune cell subsets was comparable between the two clones, albeit with 2% average increase in GITR being detected on $CD4^+$ T cells by clone DT5D3, and elevated levels of GITR seen on myeloid cells with clone 621 (Fig. 1A). We note, however, that there were differences in the level of GITR detected by the two clones within the same tumor sample in some cases (Fig. 1B). Nonetheless, the results provided from anti GITR clone DT5D3 suggest that <1% of CD8 T cells, approximately 2% 3% of CD4 T cells, and approximately 1% 2% of total T cells in the tumor microenvironment express GITR (Fig. 1A), reaffirming our previous conclusion that GITR is minimally expressed on T cells in the tumor microenvironment. While we previously reported an average 2% expression of GITR on B cells and monocytes with anti GITR clone 621, results utilizing clone DT5D3 show that GITR expression on these cells was <1%.

In one of the three melanoma tumor dissociates, we found sufficient numbers of $FoxP3^+$ $CD25^{hi}$ T regulatory cells to compare levels of GITR detected by anti GITR clone DT5D3 and anti GITR clone 621. In this sample, the anti GITR clone DT5D3 did detect a significantly higher proportion of $GITR^+$ regulatory T cells than with the anti GITR clone 621, and with better resolution of $GITR^+$ cells (Fig. 1C). Indeed, anti GITR clone DT5D3 resolved GITR positive cells better than anti GITR clone 621 on all immune cell subsets examined (Fig. 1D).

In conclusion, while differences in GITR expression levels were observed between anti GITR clone DT5D3 and anti GITR clone 621, the overall results from both antibodies were comparable, with the notable exception of GITR staining on regulatory T cells. The results provided by anti GITR clone DT5D3 reaffirm our conclusions on low level GITR expression in the tumor microenvironment in melanoma, with the exception that a higher proportion of regulatory T cells are likely to be positive for GITR, previously undetected by anti GITR clone 621.

Disclosure of Potential Conflicts of Interest

G. V. Long is a consultant/advisory board member for Aduro, Amgen, Array Biopharma, Bristol-Myers Squibb, Merck Sharpe & Dohme, Novartis, Oncosec, Pierre Fabre, and Roche. R.A. Scolyer is a consultant/advisory board member for Merck Sharp & Dohme, Novartis, Myriad, and NeraCare. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

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doi: 10.1158/1078 0432.CCR 19 1866

Reference

1. Edwards J, Tasker A, Pires da Silva I, Quek C, Batten M, Ferguson A, et al. Prevalence and cellular distribution of novel immune checkpoint targets across longitudinal specimens in treatment-naive melanoma patients: implications for clinical trials. Clin Cancer Res 2019.

Chapter 3 – Concluding discussion

To conclude this chapter, we have provided the first comprehensive study of the prevalence and distribution of co-inhibitory and co-stimulatory receptors in melanoma. Our results, while descriptive in nature, provide an important foundation for the evaluation of these markers in the clinic and their correlation with treatment outcomes to novel immunotherapies in anti-PD-1 refractory patients. Overall, our study highlights the heterogeneity of patient tumors and the differences in checkpoint receptor expression profiles between patients, reinforcing the likely role that personalised therapies will have in the future. Additionally, we showed that the expression of alternative checkpoint receptors is only found on a subset of tumor infiltrating lymphocytes, suggesting that only a small proportion of lymphocytes are likely to be the targets of these therapies. It is interesting that the expression of co-inhibitory receptors (TIM-3, VISTA, and TIGIT) was more prevalent than co-stimulating receptors (OX-40 and GITR) in melanoma tumors, except for ICOS. Currently, only co-inhibitory receptors (PD-1 and CTLA-4) have proven to be clinically efficacious targets in cancer, and it remains to be determined whether targeting co-stimulatory receptors will also prove efficacious. A limited expression profile would challenge the idea that costimulatory receptors can be an effective target, given that the abundance of a target generally associates with therapy response. This has certainly been true for BRAFV600 staining and response to BRAF inhibitors (Chapman et al., 2011; G. V. Long et al., 2015), as well as PD-L1 staining and response to anti-PD-1 (Doroshow et al., 2021). However, it is also important to recognise that this is not always the case. The prevalence of CTLA-4 in melanoma tumors, for example, does not correlate with response to anti-CTLA-4 therapy, and could be partly due to the fact that anti-CTLA-4 therapy is thought to exert its mechanism of action outside of the tumor microenvironment (Waldman et al., 2020). Thus, if therapies like anti-OX40 or anti-GITR do prove efficacious, the lack of their abundance in the tumor microenvironment might warrant an investigation into their mechanism of action outside of the tumor, something that we did not explore, but may be important for developing biomarkers to these therapies. Additionally, it is important to recognize that our data is reflective of treatment-naïve patients and may not necessarily reflect co-stimulatory checkpoint expression early during anti-PD-1 therapy. One study showed that OX-40 and GITR expression increased on responding T cells during anti-PD-1 immunotherapy (Elliot et al., 2021). Thus, sequencing and timing of such therapies may be important factors for increasing clinical response to anti-PD-1. One of the key questions surrounding the targeting of alternative checkpoint receptors, is whether or not they will prove redundant in the context of anti-PD-1 therapy failure. In other words, will their efficacy be restricted to patient populations that would otherwise already be responsive to anti-PD-1 therapy? Within murine models, dual checkpoint blockade with anti-PD-1 and novel targets (TIM-3, LAG-3, VISTA and TIGIT) has shown superior efficacy compared to anti-PD-1 alone (Johnston et al., 2014; Koyama et al., 2016; Sade-Feldman et al., 2018; Sakuishi et al., 2010; Woo et al., 2012). In humans, dual checkpoint blockade with anti-CLTA-4 and anti-PD-1 is known to be more efficacious in melanoma than single agent anti-PD-1 blockade (Larkin et al., 2015). Recently, the results of a phase 3 clinical trial testing Relatlimab (an anti-LAG-3 antibody), showed that progression free-survival in metastatic melanoma patients was longer with dual therapy (anti-PD-1+ anti-LAG-3) compared to single agent-anti-PD-1 therapy alone (Lipson et al., 2021). The clinical data, therefore, suggests that there is an added benefit when targeting these alternative checkpoint receptors – the question is how much of an added benefit? The evidence so far suggests that the improvements in efficacy are incremental. Indeed, the results from our study also seem to provide a rationale for why this might be the case. Firstly, in tumors that lack PD-1 expression, we found a significant reduction in the presence of other co-stimulatory and co-inhibitory receptors compared to tumors with positive PD-1 expression. Secondly, when we analysed the

expression profile of checkpoint receptors on T cells, we found that many of the alternative checkpoint receptors were found on the same T cell populations expressing PD-1. Tumor resident memory T cells, for example, which express the highest levels of PD-1, were also highest for TIGIT, TIM-3 and LAG-3. These data suggest that patients failing anti-PD-1 are less likely to have the appropriate targets or immune cell populations required for response to alternative checkpoint-based immunotherapies when compared to anti-PD-1 responding individuals. If true, this would have drastic implications, because the problem at hand is developing suitable therapies for anti-PD-1 refractory patients and not for patients that respond to anti-PD1 therapy. It is possible that any efficacy observed from novel checkpoint-based therapies may be due to the rescuing of patient populations which initially respond (often partially) but later develop acquired resistance to anti-PD-1. Certainly, this is a problem that needs to be addressed, though it is a problem that reflects a small proportion of patients when compared to those with innate resistance to anti-PD-1 therapy (P. Sharma et al., 2017). In this way, targeting additional co-inhibitory/co-stimulatory receptors may act to restore TCR sensitivity and help activate immune cells in those that upregulate alternative checkpoint inhibitors during acquired resistance, as has been observed (Koyama et al., 2016). It remains to be determined from mature clinical trial data what specific patient populations, if any, benefit from these novel therapies. Our data, nonetheless, provides an important foundation to interpret clinical findings when they do become available.

Lastly, the decreased expression of GITR in metastatic patient biopsies compared to primary patient biopsies is intriguing but difficult to explain. Additionally, the mechanism and biological reason for this differential expression was outside the scope of this study, but perhaps would be appropriate as a future direction for this research. It is possible that this is a timing phenomenon. As previously discussed, the costimulatory receptor GITR is known to be transiently expressed at the early stages of T cells activation. Primary tumors represent early-stage disease when compared to late-stage metastatic disease. It is therefore possible that resected primary tumors represent early stage anti-tumoral immunity (and therefore more GITR expressing T cells). Another possible explanation is that the biology of primary tumors and metastatic tumors are not always the same. Indeed, some have reported discordance between immune infiltrates and other biomarkers between primary and metastatic samples in other cancers (Dagenborg et al., 2021). Therefore, distinct biology between these two tumor groups might also explain differences in GITR expression.

Overall, this chapter provides an important contribution to the broad field of anti-PD-1 resistance by providing a thorough and detailed expression profile of alternative checkpoint targets in melanoma patient biopsies. While it is too early to comment on whether this therapeutic approach will move the field forward significantly in overcoming the problem of anti-PD-1 resistance, it cannot be denied that this is one of the most invested areas of research. If the alternate checkpoint hypothesis is true in the context of resistance, then it is likely that tumor resident CD8 T cells remain the key population of interest given this population's relatively high expression of alternative checkpoint inhibitors within the overall CD8 T cell population.

Chapter 4

Characterization and immune profiling of patient melanoma tumors unresponsive to single agent anti-PD-1 therapy but responsive to combination anti-PD-1 + anti-CTLA-4 therapy

Chapter 4 - Introduction

Thus far, we have approached the problem of resistance to anti-PD-1 therapy in two ways. Firstly, we have explored and deepened our understanding of the mechanisms of anti-PD-1 response, specifically the immune cells that are required for immunotherapy response. This has enabled us to investigate approaches for boosting critical immune cell populations in patient tumors that lack them. Secondly, we have investigated the expression profile of alternative checkpoint targets in melanoma patient tumors in an effort to 1. understand what populations (same or distinct from anti-PD-1) are likely targets based on expression, and 2. provide a foundation for the development of biomarkers to novel therapies currently being explored in clinical trials. In this last chapter, we turn our attention to a very unique and specific clinical cohort of patients that have actually derived benefit from the addition of one of these checkpoint inhibitors, namely patients that have derived benefit from combination anti-CTLA-4 + anti-PD-1 but not from single agent anti-PD-1 therapy. In clinical practice, this cohort of patients represent a very real way in which resistance to anti-PD-1 therapy is being overcome right now in some individuals. As we have already discussed, it is a well-known fact that combination anti-CTLA-4 + anti-PD-1 therapy yields higher efficacy rates in melanoma than single agent anti-PD-1 therapy (Larkin et al., 2015). However, the reality is that combination therapy is not always administered upfront because it also represents the most toxic regimen. Particularly in the context of patient-related factors, such as a history of autoimmunity, elderly age/fragility, and the patient's own risk appetite, single agent anti-PD-1 therapy may preferentially be decided as the first line of treatment. However, upon failing anti-PD-1, and having no option with BRAF/MEK inhibitors (tumor is BRAF WT), combination therapy may then be decided. Retrospective clinical data suggests that approximately 30% of patients failing anti-PD-1 therapy will derive clinical benefit from second-line combination anti-CTLA-4 + anti-PD-1 (Pires da Silva et al., 2021). Therefore, second-line combination therapy after first-line anti-PD-1 failure remains a valid and utilized treatment algorithm for the treatment of metastatic melanoma patients resistant to anti-PD-1 therapy. The responsibility of the clinician is to carefully manage the risks and benefits of these therapies to patients. In this context then, one of the clinical goals is to limit the toxicity of combination anti-CTLA-4 + anti-PD-1 therapy to those patients that actually require and are likely to benefit from it. The problem, however, is that there are currently no biomarkers for predicting who these patients are. This complicates the clinical decision-making process. If a biomarker was available, patients and clinicians alike would be more willing to go through with combination therapy (despite added risks) if there was an expectation of response. Likewise, if a biomarker indicated that a patient would be exposed to greater toxicity with very little chance of response, perhaps risk appetites would be subdued. While biomarkers have been described for both single agent anti-PD-1 and combination anti-PD-1 + anti-CTLA-4, very little has been explored with regards to the tumor biology of patients who are unlikely to benefit from anti-PD-1 monotherapy but are likely to benefit from combination anti-PD-1 + anti-CTLA-4 therapy. Additionally, such cohorts are rare, particularly patient backgrounds with "clean" treatment algorithms that allow scientists to make direct associations with tumor biology and clinical outcome due to specific treatment types. In this final chapter, we make use of a unique cohort of metastatic melanoma patients

from the Melanoma Institute of Australia, who were treated with single agent anti-PD-1 therapy, failed, and subsequently received combination anti-CLTA-4 + anti-PD-1 therapy. We utilized tumor samples from patients that both responded and did not respond to subsequent combination treatment and hypothesised that there would be biological differences between responding and non-responding patient tumors. In particular, we investigated the immune composition of melanoma tumors by focusing our investigations on immune cell phenotypes that had previously been implicated in response to anti-PD-1 or anti-CTLA-4 checkpoint immunotherapy. Given the importance of tumor resident CD8 T cells in anti-tumoral immunity and given their likely role as a target of anti-PD-1 immunotherapy, we chose to investigate this population and its various phenotypes, including the CD39+ tumor resident CD8 T cell subset, which had recently been described by others as a more tumor antigen specific resident phenotype (Duhen et al., 2018). Additionally, among other immune phenotypes, we also investigated another population of CD8 T cells defined by their expression of TCF7. At the time that this next body of work was written, a group from Harvard had performed single cell transcriptomics from a number of melanoma patient tumor samples and found that the proportion of TCF7+ CD8 T cells was predictive of response to anti-PD1 immunotherapy (Sade-Feldman et al., 2018). By focusing our immune phenotyping on immune cells that had already been implicated in immunotherapy response, we hoped to build upon the work of ours and others to identify potential predictive biomarkers in this cohort of patients, as well as work towards better understanding the mechanisms of response to combination anti-CTLA-4 + anti-PD-1 therapy and anti-PD-1 monotherapy failure.

The aims and objectives of the current study were as follows:

1. To describe and determine any biological differences in the tumors of melanoma patients responsive and non-responsive to subsequent combination anti-CTLA-4 + anti-PD-1 therapy after anti-PD1 failure.
2. Determine biomarkers of response to combination anti-CTLA-4 + anti-PD-1 therapy after anti-PD1 failure.
3. To better understand the mechanism of action for response to combination anti-CTLA-4 + anti-PD-1 therapy.
4. To better understand the mechanisms of resistance involved in anti-PD-1 therapy failure.

Characterization and immune profiling of patient melanoma tumors unresponsive to single agent anti-PD-1 therapy but responsive to combination anti-PD-1 + anti-CTLA-4 therapy

Abstract

Background: A proportion of patients that fail single agent anti-PD-1 therapy will subsequently receive and respond to combination anti-PD-1 and anti-CTLA-4 immunotherapy. The biological characteristics of these patient tumors remain poorly defined and as such there are presently no biomarkers to distinguish patients requiring the addition of anti-CTLA-4 in the context of anti-PD-1 therapy. **Clinical cohort and methods**: We performed histology, immunofluorescence-IHC, and RNA sequencing on formalin-fixed paraffin embedded (FFPE) tumors at 3 different timepoints from n=34 metastatic melanoma patients that failed anti-PD-1 therapy and went on to receive combination immunotherapy (18 non-responders; 16 responders). **Results**: The proportion of CD4 T cells (of CD3) and TCF7+ CD8 T cells were strongly correlated with response to combination therapy after anti-PD-1 failure, ($p<0.006$ and $p<0.0004$, respectively) and were independent of patient and disease related factors. RNA differential gene expression analysis revealed higher expression of cancer testis antigens (CTAs) in responding patient tumors. TCF7+ CD8 T cells were strongly correlated with the proportion of CD4 T cells, were closer to CD4 T cells and T-regulatory T cells in the tumor microenvironment and were located more distantly from tumor cells compared to TCF7- CD8 T cells. **Conclusion:** Our data provides the first comprehensive assessment of patient tumors that are unresponsive to single agent anti-PD-1 therapy but responsive to combination anti-CTLA-4 + anti-PD-1 therapy. The proportion of TCF7+ CD8 T cells at baseline may serve as a useful biomarker to determine patients likely to respond to subsequent combination therapy after anti-PD-1 failure.

Introduction

Anti-PD-1 and anti-CTLA-4 immunotherapy checkpoint inhibitors have truly revolutionized patient treatment and improved patient long-term outcome in many solid malignancies, most notably melanoma [1–3]. While anti-PD-1 inhibitors have stronger efficacy compared to anti-CTLA-4 inhibitors, clinical studies have shown that the combination of anti-PD-1 + anti-CTLA-4 yields the highest efficacy and durability in melanoma [4]. Even within melanoma patients that have failed first-line anti-PD-1 treatment, subsequent combination anti-PD-1 + anti-CTLA-4 therapy has shown to improve multiple patient outcomes compared to anti-CTLA-4 therapy alone, pointing to a clear synergistic effect between the two therapies [5]. Despite this, not all patients receive combination treatment in clinical practice, primarily due to the fact that anti-PD-1 + anti-CTLA-4 immunotherapy represents the most toxic regimen [4]. Thus, in clinical practice the benefits and risks of anti-PD-1 monotherapy vs combination therapy must be evaluated for each patient in the context of individual disease and patient-related factors. For example, some melanoma subtypes have been described as being more responsive to anti-PD-1 therapy, such as desmoplastic melanoma[6], while others, like acral and mucosal melanomas, generally require combination therapy to achieve meaningful clinical benefit [7,8]. Likewise, patients with low volume of disease or disease restricted to certain organs such as the lung or subcutaneous areas are known to be more responsive to checkpoint blockade than patients with high volume of disease or disease that has metastasized to the liver, bone, or brain [9]. Patient-related factors, including the patient's own risk appetite, age and fragility,

comorbidities, and pre-existing autoimmune conditions may also be important in determining whether a patient should receive anti-PD-1 monotherapy or combination therapy upfront [10]. One of the challenges in this clinical decision-making process is that very little is known about the tumor biology of patients who are unlikely to benefit from anti-PD-1 monotherapy but are likely to benefit from combination anti-PD-1 + anti-CTLA-4 therapy. A greater understanding and characterization of these patient tumors may lead to predictive biomarkers that can be used to assist clinicians in their clinical decision-making and limit the toxicity of combination therapy to patients that actually require and are likely to benefit from it. While there is a breadth of work that has described response and non-response to anti-PD-1 monotherapy or combination anti-PD1 + anti-CTLA-4 immunotherapy [11–13], few studies have described the unique group of patients that require the addition of anti-CTLA-4 to obtain some clinical benefit from anti-PD-1 inhibitors. Emerging studies are beginning to explore the clinical characteristics of these patients [5,14], but no scientific studies have investigated the biological characteristics of these patient tumors. Part of the challenge involves limited access to suitable patient cohorts that will address these questions. Additionally, such patient cohorts are rare. Consequently, no biomarkers presently exist and therefore this research represents an area of unmet clinical need.

The aim of this study, therefore, was to characterise and profile the tumors of patients unresponsive to anti-PD-1 monotherapy but responsive to combination anti-PD-1 + anti-CTLA-4 immunotherapy, and in so doing, provide a foundation for the discovery of suitable biomarkers as well as provide insights that could help rationalize their failure to anti-PD-1 and response to combination treatment. To achieve this, histopathological, multi-fluorescence-IHC, and RNA sequencing analyses were performed on tumor biopsies at three different timepoints (1. Pre-anti-PD-1, 2. Pre-anti-PD-1+ anti-CTLA-4, and 3. Post anti-PD-1 + anti-CTLA-4) in n= (16) patients who had failed first-line anti-PD-1 monotherapy but responded to subsequent combination anti-PD-1 + anti-CTLA-4 immunotherapy. To identify biological characteristics that were clinically meaningful, comparisons were made with patients that had undergone the same treatment algorithm but who had failed both first line anti-PD-1 and subsequent combination anti-PD-1 + anti-CTLA-4 immunotherapy, n= (18). We hypothesized that there would be differences in the tumor characteristics of responding and non-responding patients to combination therapy in the context of anti-PD-1 failure.

Methods

Patients

Metastatic melanoma patients who had been treated with first-line anti-PD-1 therapy, failed, and subsequently received combination anti-PD-1 + anti-CTLA-4 therapy, were retrospectively identified from patients treated at the Melanoma Institute of Australia and Westmead hospital. Within this cohort, patients with formalin-fixed paraffin-embedded (FFPE) tissue available at pre-treatment (timepoint 1), post anti-PD-1 failure (timepoint 2), or post combination anti-PD-1 + anti-CTLA-4 (timepoint 3) were included in the study. Patients who had been treated with systemic therapy prior to commencement on anti-PD-1 immunotherapy were excluded. Patient response to subsequent combination anti-PD-1 + anti-CTLA-4 was determined using the RECIST 1.1 criteria[15], as previously described [12]. Briefly, patients having fulfilled criteria for CR, PR, or SD of greater than 6 months with no progression, were classified as responders, while patients with PD, or SD for less than or equal to 6 months before disease progression were classified as non-responders. The study was conducted in accordance with the National Health and Medical Research Council of Australia's National

Statement on Ethical Conduct in Human Research. The study was undertaken with institutional Human Ethics Review Committee approval and patient's written informed consent.

Hematoxylin and eosin (H&E) staining

H&E staining was carried out on 4-µm thick formalin-fixed paraffin-embedded (FFPE) sections. Briefly, FFPE tumor specimens were baked at 65°C for 30 minutes, then deparaffinized and rehydrated by xylene and an ethanol gradient (100%, 95%, and 70%; Sigma-Aldrich). Sections were then washed in distilled water before undergoing Mayers hematoxylin staining (Sigma-Aldrich) for 10 minutes at room temperature. After which, sections were washed with conventional tap water for 5 mins and stained with 0.1% Eosin for 5 seconds. Sections were then dehydrated by ethanol (70%, 95% and 100%; Sigma Aldrich) and xylene, and then cover slipped and dried at room temperature.

Multiplexed immunofluorescence staining

Immunofluorescence staining was carried out on 4-µm thick sections using an Autostainer Plus (Dako – Agilent Technologies) and Opal Multiplex IHC Assay Kit (Akoya Biosciences) with appropriate positive and negative controls, as reported previously [16]. Briefly, FFPE tumor specimens were baked at 65°C for 30 minutes, then deparaffinized and rehydrated by xylene and an ethanol gradient (100%, 95%, and 70%; Sigma-Aldrich). Heat-induced antigen retrieval (AR) was performed at 95°C for 20 minutes in pH 9 AR Buffer (Akoya Biosciences). Sections were then cooled and incubated with 3% Hydrogen Peroxide (Sigma-Aldrich) for 10 minutes at room temperature, followed by incubation with a single primary antibody against CD8 (1:1500; SP16, Cell Marque), CD103 (1:1500; EPR4166, Abcam), CD39 (1:2000; EPR20627), TCF7 (1:200; CST-C63D9), FOXP3 (1:1000), Tbet (1:1000; CST-D6N8B), ICOS (1:3000 CST-D1 K2T), CD3 (1:2000; MRQ-39), CD16 (1:500; SP175 Cell Marque), CD68 (1:500; Kp-1, Cell Marque), HLA-ABC (1:15000; ab70328), MAGE-A (1:500; 6C1 Santa Cruz), or SOX10 (1:300; BC34, Biocare Medical) for 30 minutes at room temperature. Following this, samples were either incubated with Opal Polymer HRP (Akoya Biosciences) for 30 minutes (CD8, CD3, CD103, CD39, TCF7, FOXP3, CD68, MAGE-A, CD16) or 45 minutes (ICOS), or incubated with the MACH3 Probe/HRP-Polymer Kit (Biocare Medical) for 5 minutes (Tbet). Finally, sections were incubated with opal fluorophores at 1:100 dilution made up in Tyramide Signal Amplification Reagent (Opal 7-Color IHC, Akoya Biosciences). The AR step was repeated for subsequent stains on the same slide. On the last staining run, DAPI was added to the sample for 5 minutes. All samples were cover slipped using Vectashield (H-1400) and left overnight to dry at 4 °C. Three separate panels were designed as follows: 1. CD8, CD103, CD39, TCF7, FOXP3, SOX10, 2. CD8, Tbet, ICOS, TCF7, CD3, SOX10, and 3. CD16, CD68, HLA-ABC, MAGE, SOX10.

Imaging and staining quantification

The Vectra 3 multispectral slide scanner was used in conjunction with Vectra 3.3 and Phenochart 1.0.4 Software (Akoya Biosciences) to image immunofluorescence staining of whole slide sections at 20X high power field view (HPF). Images were then unmixed using inForm 2.3.0 Software (Akoya Biosciences) and analyzed and stitched together using HALO Image Analysis version 2.3. Briefly, images of whole slide sections were annotated to exclude non-tumor tissue, while marker thresholds were determined to appropriately identify immune phenotypes in tumors. Lymphocyte/leukocyte densities and HLA-ABC/MAGE-A expression was assessed within tumor only (defined by the presence of SOX10-positive staining), given

the well-recognised importance and prognosis of tumor infiltrating lymphocytes (and TILs) in anti-tumor function and survival [17–19]. Intratumoral areas were identified with the assistance of a trained pathologist (P.M. Ferguson). In nodal metastases, any residual lymph node tissue, associated structures, and/or cells were annotated out and excluded from downstream analyses. All images were checked individually to ensure correct identification of negative and positive staining for each individual marker. Quantification of positive markers were conducted in HALO and exported for subsequent quantitative analysis using the TIBCO Spotfire 3.3.1 from TIBCO. Tumor-resident CD8 T cells were quantified using the colocalization of CD103 on CD8+ T cells. CD4+ T cells were quantified as CD8- CD3+ cells. Treg cells were quantified as FoxP3+ CD8- cells. For scoring of histopathological markers, P.M. Ferguson reviewed each individual case using a conventional upright brightfield microscope and assigned a score to each (where 0, absent; 1, sparse; 2, moderate; and 3, dense) based on the density of intratumoral lymphocytes, peritumoral lymphocytes, plasma cells, neutrophils and peritumoral fibrosis. Necrosis and intratumoral fibrosis were measured as a percentage (0-100%) of the tumor.

Spatial analysis

Nearest neighbourhood analysis was performed using HALO Image Analysis version 2.3 to determine the average distance between two immune cell phenotypes within the tumor. Briefly, immune phenotypes of interest were identified and mapped onto the tumor image after staining quantification had been performed in HALO. Immune cell phenotypes were defined based on positivity (0, negative; 1, positive) of each marker. Neighbourhood analysis between TCF7+/TCF7- CD8 T cells and melanoma SOX10+ cells were calculated based on the average distance of TCF7+/TCF7- CD8 T cells to nearest SOX10+ cell. Neighbourhood analysis of CD4 T cells and Treg cells to TCF7+/TCF7- CD8 T cells was calculated based on the average distance of TCF7+/TCF7- CD8 T cells to nearest CD4 or Treg cell, respectively. Average distance was measured in micrometres.

RNA isolation and sequencing

All samples were pathologically assessed for melanoma (P.F) before RNA isolation and sequencing. Samples with greater than 80% tumour content were included. Samples requiring tumour enrichment underwent macrodissection or frozen tissue coring (Cryoxtract, Woburn, Massachusetts, USA) using a marked haematoxylin and eosin slide as a reference. RNA isolation and sequencing was performed as previously described [12]. Briefly, RNA from formalin-fixed paraffin-embedded (FFPE) sections was isolated using AllPrep DNA/RNA FFPE kit (Qiagen) according to manufacturer's instructions. RNA quantity was assessed on Qubit, and RNA integrity was assessed using the RNA 6000 Nano kit and run on the Agilent 2100 Bioanalyzer (Agilent Technologies). The mRNA samples were fragmented in preparation for cDNA synthesis and library construction using the TruSeq RNA Access Library Prep Kit (Illumina) according to the manufacturer's protocol. Library quality was assessed on an Agilent 2100 Bioanalyzer using a DNA 1000 chip prior to sequencing by the Ramaciotti Centre.

Differentially expressed gene analysis

The R packages, DESeq2[20] and HTSeq version 0.6.1[21], were used respectively to perform gene count and differential gene expression analysis. Aligned reads that mapped to the human gene annotation from Ensembl were identified using HTSeq with the default htseq-count functionality. To measure differential expression between responders and non-responders, the count-based expression profiling was performed using model count data based on negative

binomial distribution and shrinkage estimator for distribution's variance to assess the quantitated reads. Read counts were normalized using the *DESeq()* function[20]. To determine fold change differences, the log of ratio of expression levels for each gene between conditions being tested was computed. The normalized expression values were displayed as counts per million (cpm). Differential expression was visualized on SeqMonk with a corrected p value, and significantly differentially expressed genes were identified as those with an adjusted (adj.) p value of < 0.05 with Benjamini-Hochberg (BH) multiple testing correction at 5% false-discovery rate (FDR).

Statistical analyses

Statistical analyses were performed using Prism version 9.1f (GraphPad Software) or TIBCO Spotfire 3.3.1. Patient characteristics were summarized using frequencies and percentages. P values were determined using a nonparametric Mann-Whitney test for comparisons between responders vs. non-responders, Spearman rho test for correlation analyses, and non-parametric Wilcoxon test of change for differences between two time-points in paired samples or in spatial analysis data, where appropriate. Univariate logistic regression analysis was performed for determining the significance of clinical factors on objective responses in patients. Dunn's multiple comparisons tests were performed to evaluate differences in CD8 densities and TCF7+ CD8 T cell proportions in this and other studies. P values less than 0.05 were considered significant. Variability in all data was expressed in terms of 1 standard error of the mean (SEM).

Results

Patient characteristics and histological analysis of patient tumors receiving combination anti-PD-1+ anti-CTLA-4 therapy post anti-PD-1 monotherapy failure.

A total of n=34 metastatic melanoma patients were identified from the Melanoma Institute of Australia that had received first-line anti-PD-1 monotherapy (pembrolizumab/nivolumab), failed, and subsequently received combination anti-PD-1+ anti-CTLA-4 therapy (Figure S1A). Of these, 79% were BRAF WT, and 70% demonstrated innate resistance to first line anti-PD-1 therapy (30% acquired) with a median time to progression of 2.7 months (1.2-23.1) (Table 1). Of the 34 patients who failed first-line anti-PD-1 monotherapy, n= 16 (47%) were responders to subsequent combination anti-PD-1 + anti-CTLA-4 therapy. Other key demographic and clinical characteristics at start of anti-PD-1 or anti-PD-1+ anti-CTLA-4 treatment can be found in Table 1. Patient tumor biopsies were taken at three different timepoints in the treatment algorithm; 1. Pre-anti-PD-1 (n=31) (91%), 2. Post anti-PD-1 monotherapy and Pre-anti-PD-1+ anti-CTLA-4 (n=14) (41%), and 3. Post anti-PD-1 + anti-CTLA-4 (n=9) (26%) (Table 1 and Figure S1A). We first performed histological analyses on all patient tumors from all 3 timepoints to characterize broad histological parameters that have previously been associated with response to either anti-PD-1 or anti-CTLA-4 therapy. No statistical differences were observed in the density of tumor infiltrating lymphocytes (TILs), peritumoral lymphocytes, neutrophils or plasma cells between responders and non-responders to second-line anti-PD-1+ anti-CTLA-4 therapy (Figure S1B). However, peritumoral fibrosis was higher in non-responders (1.12 ±0.2) at timepoint 1 compared to responders (0.38 ±0.13) (p=0.013) and approached significance for other histological parameters, including intra-tumoral necrosis and fibrosis (p=0.06) (Figure S1C). A trend towards higher intra-tumoral fibrosis percentages after combination therapy was observed in responders (p=0.08) (Figure

S1C), in line with what has been observed early during treatment in neoadjuvant treatment with anti-PD-1 + anti-CTLA-4 [22].

The proportion of CD4 T cells and TCF7+ CD8 T cells in patient tumors are strongly associated with response to combination anti-PD-1+ anti-CTLA-4 therapy after anti-PD-1 failure.

To examine more comprehensively the T cell make-up and immune profile of these tumors, we performed multi-fluorescence-IHC with three panels that enabled the identification of key immune cells known to be implicated in the response to anti-PD-1 or anti-CTLA-4 therapy, including but not limited to; tumor resident (CD39+/-) CD103+ CD8 T cells[23–25], TCF7+ CD8 T cells [13], Tbet/ICOS+ CD4 T cells[11], T regulatory cells[26–28] , Macrophages (CD68+)[26], CD16 expressing cells[26], and tumor MHC class I expression[29,30]. Representative staining for each of the panels and markers can be found in Figure 1A. All patient tumors at baseline (timepoint 1) had tumor HLA-ABC expression (mean: 84.5 ±3.7, range: 15-100), T cells (mean: 864.2 ±172, range: 40-3365), and tumor resident CD8 T cells (mean: 31.3 ±10.7, range: 9-291) (Figure S2A), although the resident phenotype comprised a relatively low proportion of the total CD8 population (mean: 11.4 ±1.9, range: 2.9-51) (Figure S2A) compared to what has been described in other studies (~30%), suggesting a sub-optimal anti-tumoral response in these tumors before treatment. Differences in immune phenotypes between responders and non-responders to second line anti-PD-1 + anti-CTLA-4 were examined at all timepoints. No significant changes were observed in total T cell densities, CD8 T cell densities or CD4 T cell densities between responders or non-responders (Figure 1B-C). However, a higher proportion of CD4 T cells comprising the total T cell population in patient tumors at baseline was strongly associated with response to anti-PD-1 + anti-CTLA-4 immunotherapy after anti-PD-1 monotherapy failure (73.8 ±3.6 (responders) vs. 56.5 ±3.9 (non-responders), p=0.006) (Figure 1B-C). Conversely, a higher proportion of CD8 T cells comprising the total T cell population in tumors at baseline was strongly associated with non-responders (26.3 ±3.6 (responders) vs. 43.5 ±3.9 (non-responders), p=0.005). TCF7+ CD8 T cells, which have previously been described as an undifferentiated stem cell-like CD8 T cell critical to immunotherapy response [13], especially to anti-PD-1 monotherapy, were most strongly associated with responders (68.1 ±5.2) compared to non-responders (32.4 ±6.2) (p=0.0004) when analysed as a proportion of the total CD8 T cell population in tumors (Figure 1B-C). The proportion of these cells also appeared higher in this cohort of patients than what has previously been reported for anti-PD-1 responders in other cohorts[13] (Figure S2B). Additionally, the proportion of Tbet+ CD8+ T cells, which have been described as a well-differentiated CD8 T cell population with opposing characteristics to TCF7+ CD8+ T cells [31], trended lower in responders and higher in non-responders at baseline (Figure 1B and Figure S2C). Other immune phenotypes including regulatory T cells, ICOS+ CD4 T cells, CD68+ macrophages and CD16 expressing cells were no different between responders and non-responders (Figure 1B), although the proportion of Tbet+ CD4 T cells was significantly higher in non-responders (37 ±4.9) compared to responders (21 ±4.3) at timepoint 2 (p=0.03) (Figure S2C). The proportion of CD39+ CD103+ tumor resident CD8 T cells post combination therapy also trended higher in responders (23.1 ±5.8) compared to non-responders (7.8 ±1.8) (p=0.06), indicating a restoration of anti-tumoral immunity in responding patients at this timepoint (Figure S2C).

Immunological differences observed between responders and non-responders to second-line anti-PD-1+anti-CTLA-4 therapy are independent of clinical factors.

To examine whether any underlying clinical factors might be responsible for the strong immunological associations observed between responding and non-responding patients, we performed univariate analyses on key clinical factors that could potentially impact outcome. No differences in gender (Female vs Male, p=0.159), age at start of anti-PD-1+ anti-CTLA-4 treatment (p=0.948), ECOG status (0 vs >=1, p=0.4633), metastatic stage (III/M1a/M1b vs. M1c/M1d, p=0.314), LDH levels (normal vs elevated, p=0.710), number of metastases (<3 vs. >=3, p=0.516), or site of disease (Brain: p=0.803; Lung: p=0.968; Liver: p=0.642; Bone: p=0.956, and others) were seen between responding and non-responding patients (see Table 2 for details). This suggested that the higher proportion of CD4 T cells (of CD3) and TCF7+ CD8 T cells (of CD8) in responding patient tumors at baseline were independent of key clinical factors and were truly biological observations that correlated with response outcome.

Responding patient tumors to second-line combination anti-PD-1 + anti-CTLA-4 show biological changes in response to anti-PD-1 monotherapy, despite clinically failing anti-PD-1 therapy

TCF7+ CD8 T cells are thought to be a sub-population of T cells that provide the proliferative burst during anti-PD-1 treatment [13,32]. Given the strong association between TCF7+ CD8 T cells and response to second-line anti-PD-1 + anti-CTLA-4 treatment, we hypothesised that anti-PD-1 might play a significant role in determining patient outcome to combination treatment, despite previous failure to anti-PD-1 monotherapy. 2 We explored changes in the CD8 T cell compartment of patient-matched tumors between baseline (timepoint 1) and post-anti-PD-1 monotherapy (timepoint) in second-line combination responders (n=5) and non-responders (n=6). The purpose was to understand whether responding tumors showed any signs of biological sensitivity to anti-PD-1 that might suggest a role for anti-CTLA-4 in sensitizing tumors to anti-PD-1, rather than the alternative hypothesis that these tumors lack the biological mechanisms needed for any kind of response to anti-PD-1. Both the density and proportion of CD8 T cells (of total T cells) in responding patient tumors trended higher (4/5 patient tumors) after anti-PD-1 monotherapy (Figure 2A and B), consistent with the overall mechanism of action for anti-PD-1 and with what has previously been described for tumors responding to anti-PD-1 [12,33]. This trend was not apparent in non-responders (Figure 2A and B). CD8 phenotypic changes were also observed in responders post-anti-PD-1 therapy compared to baseline. Indeed, overall, the proportion of TCF7+ CD8 T cells decreased after anti-PD-1 monotherapy (p=0.03), with the change mostly seen in responders (p=0.06), and with greater magnitude (p=0.03), compared to non-responding tumors (p=0.44) (Figure 2C), demonstrating a clear biological effect of anti-PD-1 in responding patient tumors. The proportional decrease in stem cell-like CD8 T cells also suggested that these cells might be differentiating in the presence of anti-PD-1. In line with this, the proportion of differentiated Tbet+ CD8 T cells was higher overall (p=0.04) at timepoint 2 compared to baseline, with the trend mostly observed in responders (p=0.06) compared to non-responders (p=0.44) (Figure 2D). Lastly, the proportion of CD39+ CD103+ resident CD8 T cells was also higher (p=0.02) after anti-PD-1 therapy overall, with the effect significant in non-responders (p=0.03) and only trending (4/5) in responders, given the presence of an outlier (1/5) (Figure 2E). Together, these data suggested a clear biological change in responding tumors that resulted from anti-PD-1, which was not apparent in non-responding tumors to the same extent, indicating that responding tumors had the mechanisms necessary to anti-PD-1 in place.

RNA sequencing reveals higher expression of multiple cancer testis antigens in tumors of responding patients compared to non-responding patients.

We next performed RNA sequencing analysis on a subgroup of patient tumors (n=12; n=6 responders and n=6 non-responders) at timepoint 1 (pre-anti-PD-1 monotherapy) to investigate broader factors (immune and non-immune) associated with response to combination treatment after anti-PD-1 failure. Among immune-related genes, *IL13RA2* was significantly higher in responders (fold change (FC) 2.9, padj = 5.85e-39) compared to non-responders, while *CXCL10*, *SPP1* and *IFIT3* were higher in non-responders (FC 3.1, 3.44, 2.48; padj = 0.004, 0.006, 0.05, respectively (Table 3). Among non-immune related genes, multiple cancer testis antigens (CTAs) from the MAGE, GAGE and XAGE family were differentially expressed (Table 3). Indeed, responding patient tumors expressed higher levels of 1. MAGE (*MAGEA1*(FC 0.59, padj =1.46e-9); *MAGEA3*(FC 4.68, padj=3.2e-9); and *MAGEB2*(FC 0.9, padj= 3.4e-14)), 2. GAGE (*GAGE1*(FC 3.54, padj=1.09e-8); *GAGE12J*(FC 3.79, padj=4.01e-7); and *GAGE12C*(FC 3.88, padj= 1.71e-6)) and 3. XAGE (*XAGE1A*(FC 3.62, padj=3.64e-7) and *XAGE1B*(FC 3.81, padj=9.88e-7)) compared to non-responders (Table 3). Because the MAGE family is a well-known therapeutic target in melanoma, most notably MAGE-A3, we sought to validate these findings at the protein level using multi-fluorescence-IHC in all patient tumors at baseline. We quantified the expression of pan-MAGE as a percentage of total SOX10+ tumor cells (Figure 3A). No statistical differences were observed between responding (34 ±9.5) and non-responding patients (25.3 ±6.6)(P=0.91)(Figure 3B). However, responding patient tumors did comprise a higher percentage of tumors (7/10) with higher MAGE expression (>45%) compared to tumors with lower (<45%) MAGE expression (9/21) (Figure 3C).

TCF7+ CD8 T cells strongly correlate with the proportion of CD4 T cells in the tumor microenvironment, are proximally closer to CD4 T cells when compared to TCF7- CD8 T cells, and directly interact with Treg T cells.

Given that the proportion of TCF7+ CD8 T cells (of CD8) and CD4 T cells (of CD3) in the tumor microenvironment were the strongest factors predicting clinical response to combination anti-PD-1+ anti-CTLA-4 after anti-PD-1 failure, we tested whether combining both biological variables together would improve their association with clinical outcome. Patient tumors at baseline were separated according to high or low TCF7+ CD8 T cells (of CD8) and CD4 T cells (of CD3), with the median of each variable used to define high or low for that variable. 30% of patient tumors low in both variables were responders, while 0% and 50% of tumors low in just TCF7+ CD8 T cells (of CD8) or CD4 T cells (of CD3) respectively, were responders (Figure 4A). In contrast, 90% of patient tumors high in both TCF7+ CD8 T cells (of CD8) and CD4 T cells (of CD3) were comprised of responding tumors, pointing to a clear additive effect in their association with response outcome (Figure 4A). Interestingly however, patient tumors that were high or low in one variable tended to mirror high or low in the other (n=20/28, 71%)(Figure 4A), pointing to an association between TCF7+ CD8 T cell and CD4 T cells. Indeed, the proportion of CD4 T cells (of CD3) strongly correlated with the proportion of TCF7+ CD8 T cells (of CD8) in patient tumors at baseline ($r=0.71$, $p<0.0001$) (Figure 4B left), while the proportion of CD8 T cells (of CD3) negatively correlated with the proportion of TCF7+ CD8 T cells (of CD3) ($r=-0.71$, $p<0.0001$) (Figure 4B centre). A higher ratio of Tregs to CD8 T cells was also correlated with a higher proportion of TCF7+ CD8 T cells (of CD8) ($r=0.53$, $p<0.002$) (Figure 4B, right), however, because Treg numbers in tumors also correlated with CD4 numbers ($r=0.76$, $p<0.0001$) (Figure S3A), it is possible that this relationship is an effect of CD4 T cells rather than Treg cell themselves. Given the strong correlations between TCF7+ T cells and CD4 T cells, we performed spatial analysis on multifluorescence-IHC images to test the likelihood of cellular interactions between these phenotypes in patient tumors.

Indeed, TCF7+ CD8 T cells were closer to CD4 T cells when compared to their TCF7- CD8 T cell counterparts ($p<0.0001$)(Figure 4C, top left) and also clustered with CD4 T cells (Figure 4C, bottom left - R1). TCF7+ CD8 T cells were also closer to Treg T cells (Figure 3C centre) compared to TCF7- CD8 T cells ($p<0.0001$) and were seen to directly interact with T-regulatory cells through close contact (Figure 4C, centre (white arrows)) in patient tumors. Lastly, TCF7+ CD8 T cells were spatially located further away from tumor cells (Figure 4C, right (R1 and R2)) compared to TCF7- CD8 T cells (Figure 4C, right (R3))($p<0.0001$).

Discussion

A proportion of melanoma patients will not respond to anti-PD-1 monotherapy but will respond to combination anti-PD-1+ anti-CTLA-4 immunotherapy [5]. Our study is the first to examine the biological characteristics of these patient tumors using a unique and retrospective cohort of metastatic melanoma patients treated with combination anti-PD-1+ anti-CTLA-4 immunotherapy after failure to anti-PD-1 monotherapy. We characterised patient tumors at multiple timepoints in the treatment algorithm and correlated with objective response to second-line combination therapy to provide a foundation for the discovery of suitable biomarkers to help limit the toxicity of combination therapy to patients that actually require and are likely to benefit from it, and secondly, to provide rationale for their failure to anti-PD-1 and response to combination treatment. One of the obvious strengths to this study is the nature of the patient cohort itself, which allowed any biological finding to be directly linked to the addition of anti-CTLA-4 and not with innate response to anti-PD-1 alone (given all patients failed anti-PD-1 therapy). However, it is important to recognise that these patients do not simply represent anti-CTLA-4 responders either, given that combination anti-PD-1 + anti-CTLA-4 therapy yields higher objective response rates (33%) than anti-CTLA-4 alone (13%) in patients who have failed previous anti-PD-1 monotherapy [5], clearly pointing to a role for anti-PD-1 in anti-PD-1 failures. Therefore, in the current cohort it is likely that there is a combination of patients who would respond to anti-CTLA-4 alone and others who *require* combination anti-PD-1 + anti- CTLA-4 treatment, with the latter group comprising the majority of patients based on retrospective clinical studies (cited above). Still, no single biomarker is likely to predict response in all patients, and this represents a potential limitation of the study.

One of the key findings of this study is that the proportion of TCF7+ CD8 T cells in patient tumors at baseline strongly correlated with response to second-line combination anti-PD-1 + anti-CTLA-4 treatment, independent of clinical characteristics. TCF7+ CD8 T cells represent an undifferentiated stem-cell-like T cell population with greater proliferative capacity and potential to differentiate into all other CD8 T cell phenotypes [34]. Although an important biomarker of response to anti-PD-1 monotherapy [13], our data suggests that stemness within the CD8 T cell population is still an important feature in the tumors of patients responding to second-line combination therapy after anti-PD-1 failure. In line with this, non-responding tumors at baseline tended to have a higher proportion of differentiated Tbet+ CD8 T cells, a CD8 T cell population that has been associated with less proliferative capacity and stemness [31], compared to responding tumors. While “stemness” may be an important feature of response to anti-CTLA-4, an alternative explanation for the presence of this biomarker may be that anti-CTLA-4 appropriately sensitizes responding tumors to anti-PD-1. Supporting this view, responding patient tumors (to second-line combination anti-PD-1 + anti-CTLA-4) showed expected CD8 T cell phenotypic changes in response to anti-PD-1 monotherapy, despite clinically failing anti-PD-1 therapy, suggesting that these tumors were not devoid of all the necessary mechanisms required for anti-PD-1 response. Indeed, the proportional decrease in TCF7+ CD8 T cells and proportional increase in Tbet+ CD8 T cells following anti-PD-1

therapy in responding but not non-responding tumors, is in line with previous studies demonstrating a push towards cellular differentiation upon anti-PD-1 blockade and with what has been described as the prime mechanism of action for anti-PD-1 [31,32,35,36]. These results, taken in context, demonstrate that responding tumors clearly have an innate sensitivity to anti-PD-1 monotherapy, despite failing anti-PD-1 monotherapy, which may be further sensitised with the addition of anti-CTLA-4. While non-responding tumors may not be completely desensitized to anti-PD-1 given their proportional increase in CD39+ CD103+ resident CD8 T cells post anti-PD-1 monotherapy, they lack other important phenotypic patterns associated with response to anti-PD-1, which demonstrate a comparative inability to appropriately respond to anti-PD-1 even with the addition of anti-CTLA-4. If in the current treatment algorithm, anti-CLTA-4 works to overcome mechanisms of resistance associated with anti-PD-1 non-response, then the next question becomes what are these mechanisms of resistance and how does anti-CTLA-4 overcome them?

In the current study, our data points to a number of possible mechanisms by which anti-CTLA-4 could sensitize tumors to anti-PD-1 immunotherapy and contribute to response. Firstly, studies have shown that intratumoral CD8 T cells that are specific for melanoma associated antigens (MAAs) have a lower TCR avidity compared to neoantigen-specific intratumoral CD8 T cells [37]. CD8 T cells with lower TCR avidity are known to be less effective in recognising and reacting to MHC-bound cognate peptides and may also be a characteristic of CD8 T cells in anti-PD-1 non-responders [38]. We showed that responding tumors to second-line combination therapy had significantly higher expression levels of several melanoma associated antigens at baseline, including MAGE-A3, GAGE, and XAGE. While this was not replicated at the protein level using IHC, responding tumors did comprise a higher percentage of tumors with higher MAGE expression. It is possible therefore, that responding tumors have a high proportion of low avidity MAA- specific CD8 T cells that become effective after the addition of anti-CTLA-4, and therefore this warrants further investigation. Indeed, anti-CTLA-4 is known to lower the TCR threshold required for T cell activation [39] and epigenetic studies suggest TCF7 CD8+ T cells are likely to express high levels of CTLA-4 in addition to ICOS and CD28 [40]. PD-1 on cytotoxic T cells increases the TCR activation threshold via suppression of CD28 and thus anti-PD-1 is thought to work by restoring CD28 functionality to provide the proliferative burst in cancer immunotherapy [41]. In this way, anti-CTLA-4 could either directly lower the TCR threshold for T cells with low TCR avidity or could prevent competition between CD28 and CTLA-4 molecules and CD80/CD86 ligands on antigen presenting cells, thereby enhancing the effect of anti-PD-1.

Besides directly acting on CD8 T cells, anti-CTLA-4 may also work indirectly to boost the functionality of TCF7+ CD8 populations by having an effect on CD4 T cells. We showed that the proportion of CD4 T cells (of total T cells) at baseline significantly correlated with response to second-line combination therapy, such that lower proportions of CD4 T cells was associated with negative outcome. CD4 T cells are well known for supporting the effector function and differentiation of CD8+ T cells and have been shown to be critical in sustaining anti-tumoral CD8 T cell responses [42]. Anti-CTLA-4 is also known to enhance CD4 functionality [43] and the absence of CTLA-4 in murine models expands CD4 phenotypic boundaries [44]. Thus, it is plausible that the addition of anti-CTLA-4 enhances the functionality of CD8+ T cells via its direct effect on CD4 T cells. Supporting this notion, we found that CD4 T cells clustered closely together with TCF7+ CD8+ T cells and were on average closer to TCF7+ CD8 T cells compared to TCF7- CD8 T cells, pointing to a functional interaction between CD4 T cells and TCF7+ CD8 T cells. Interestingly, no specific CD4 phenotype was associated with response. Previous studies have shown that Th1-like ICOS+ CD4 T cells are the prime targets of anti-

CTLA-4 therapy on CD4 conventional T cells [11], however this phenotype did not correlate with response in our study. Within the CD4 compartment, T regulatory cells are also believed to be a target of anti-CTLA-4 therapy because of their high expression of anti-CTLA-4 [45] and because previous studies have shown that Tregs are depleted in an Fc-dependent manner in murine models administered with anti-CTLA-4 [26]. While Treg cells were not predictive of response to second-line combination therapy, they were closer to TCF7+ CD8 T cells in tumors compared to TCF7- CD8 T cells and were seen to directly interact with TCF7+ CD8 T cells, suggesting an immunosuppressive role for Tregs on this CD8 T cell population. If the addition of anti-CTLA-4 does indeed deplete intratumoral Tregs, this could also provide another mechanism by which TCF7+ CD8 T cells become more sensitized to the effects of anti-PD-1. Indeed, a recent study in mice showed that sequential administration of anti-CTLA-4 followed by anti-PD-1 increased the effectiveness of anti-PD-1 monotherapy [46]. The mechanism for this increased efficacy was due to anti-CTLA-4 depleting intratumoral follicular Tregs, which are known to reside in tertiary lymphoid structures (TLS) and have enhanced suppressive function compared to conventional Tregs [46]. Given TCF7+ CD8 T cells also have a bias for residing in TLS [35], this warrants further investigation to understand whether follicular Tregs have a role in supressing TCF7+ CD8 T cells in responding tumors to second-line combination therapy.

Finally, one of the remaining questions for our cohort of patients is why they failed anti-PD-1 monotherapy in the first instance. We showed that all patient tumors at baseline expressed HLA-ABC, and thus the absence of MHC-I presented peptides is unlikely to be the reason for anti-PD-1 failure, though it has been described as a primary mechanism of resistance to anti-PD-1 therapy [47]. Furthermore, all patient tumors contained tumor infiltrating lymphocytes (TILs), with a CD8 T cell density that was not outside the range of what has previously been described for anti-PD-1 responders [12]. This is important because higher CD8 T cell densities have been associated with anti-PD1 response [33]. However, the proportion of resident CD8 T cell phenotypes was lower (around 10%) in all tumors at baseline compared to what has previously been described in melanoma tumors (~30%)[23], and likely represents an inadequate anti-tumoral immune response in both responding and non-responding tumors, given that resident CD8 T cells have been described as tumour-specific T cells and are associated with protective immunity in many cancers [48]. Anti-PD-1 blockade, whose primary mechanism of action is via the expansion and differentiation of stem-cell-like CD8 T cells, is also likely to reinvigorate other PD-1+ CD8 T cell populations, including resident CD8 T cells, as others have shown [23,36,49]. Non-responding tumors, which lack both these important CD8 T cell populations, are therefore likely to be completely devoid of the immune populations required for response to anti-PD-1. There are many factors that may contribute to poor anti-tumoral responses in non-responding patient tumors. We showed that non-responding tumors had higher scores of peri-tumoral fibrosis compared to responding tumors. Indeed, cancer associated fibroblasts (CAFs) are known to express immunosuppressive cytokines such as TGF-beta , and this is in turn reduces the potency and the quality of the anti-tumoral response [50,51]. Other mechanisms of resistance are likely to be present, and this represents an area of research that warrants further investigation. In responding tumors, which have high stemness but low proportions of resident CD8 T cells, this suggests an anti-tumoral response with high capacity but limited anti-tumoral function. Indeed, responding tumors in our cohort had a significantly higher proportion of TCF7+ CD8 T cells than what has previously been described for anti-PD-1 monotherapy responders[13], and we demonstrated that TCF7+ CD8 T cells were located further away from melanoma cells than their TCF7- CD8 T cell counterparts. As discussed, CD4 T cells and T regulatory cells may have an important role in regulating these cells (which anti-CTLA-4 may enhance or disrupt) but it is also possible that they contribute and help maintain the stem-cell phenotype by limiting differentiation into resident-like CD8 T

cells. In support of this, we found that the proportion of CD4 T cells highly correlated with the proportion of TCF7+ CD8 T cells in tumors. While stemness may be an important feature of response to checkpoint blockade, perhaps too much stemness may also be indicative of mechanisms that restrain CD8 T cell differentiation too effectively, such that there is a lack of response even in the presence of anti-PD-1.

In conclusion, we have characterized the tumors of patients who are unresponsive to single agent anti-PD-1 but responsive to combination anti-CTLA-4 + anti-PD-1 therapy. We have identified multiple biomarkers of response, the strongest being the presence of stem-cell like TCF7+ CD8 T cells. We have also identified multiple possible reasons for why the addition of anti-CTLA-4 is necessary in these responding patients, including the lowering of TCR threshold activity on MAA-specific T cells, and enhanced CD4 and/or disruption of regulatory T cell interactions on TCF7+ CD8 T cells. These represent important findings that increase our understanding of the biology of these patient tumors and provide important implications on which patients are likely to benefit from the addition of anti-CTLA-4 in their treatment algorithm.

Figure 1

Immune characterization of patient tumors pre-anti-PD-1, post anti-PD-1 failure, and post second-line anti-PD-1 + anti-CTLA-4 combination therapy. A) Representative immunofluorescence-IHC staining from three panels consisting of 1) CD8 (green), CD103 (magenta), CD39 (cyan), TCF7 (red), FOXP3 (yellow) and SOX10 (orange) (Panel 1), 2) CD8 (green), Tbet (magenta), ICOS (cyan), TCF7 (red), CD3 (yellow) and SOX10 (orange) (Panel 2), and 3) CD16 (green), CD68 (red), HLA-ABC (yellow) and SOX10 (orange) (Panel 3). Composite images shown at the top for each panel, and individual markers from the same region shown below. B) Heatmap summarizing the statistical differences for various immune phenotypes between non-responders and responders to second-line combination therapy in baseline (pre- anti-PD-1, n=31; responders =16, non-responders = 15), post anti-PD-1 (n=14; responders =5, non-responders = 9), and post anti-PD-1 + anti-CTLA-4 (n=9, responders =2, non-responders = 7) patient tumors. Strong red indicates statistical significance ($p \leq 0.01$, min $p = 0.0004$), while blue indicates non-statistical significance (max p=0.99). C) Density and proportion of select immune phenotypes that display strong statistical differences between non-responders and responders to second-line combination therapy at baseline (pre-anti-PD-1). All error bars displayed represent the SEM. Non-parametric Mann-Whitney tests were used to determine statistical differences between non-responders and responders.

Figure 2

Patient-matched differences between pre-anti-PD-1 and post-anti-PD-1 responding and non-responding tumors to second-line combination anti-PD-1 + anti-CTLA-4 therapy. A) Patient matched differences between pre-anti-PD-1 and post anti-PD-1 tumors (n=13) for CD8 T cell densities in all tumors (left), non-responders (red, n=6) and responders (blue, n=5). B) Patient matched differences between pre-anti-PD-1 and post anti-PD-1 tumors (n=13) for the proportion of CD8 T cells (of CD3) in non-responding (red, n=6) and responding (blue, n=5) tumors. C) Patient matched differences between pre-anti-PD-1 and post anti-PD-1 tumors (n=13) for the proportion of TCF7+ T cells (of CD8) in all tumors (left), non-responders (red, n=6) (centre, left) and responders (blue, n=5) (centre, right). Change in the proportion of TCF7+ (of CD8) between pre-anti-PD-1 and post-anti-PD-1 for non-responders (red, n=6) and

responders (blue, n=5) (right). D) Patient matched differences between pre-anti-PD-1 and post anti-PD-1 tumors (n=13) for the proportion of Tbet+ T cells (of CD8) in all tumors (left), non-responders (red, n=6) and responders (blue, n=5). E) Patient matched differences between pre-anti-PD-1 and post anti-PD-1 tumors (n=13) for the proportion of CD39+ CD103+ T cells (of CD8) in all tumors (left), non-responders (red, n=6) and responders (blue, n=5). Non-parametric Wilcoxon matched-pairs signed rank tests were used to determine statistical differences between pre-anti-PD-1 and post-anti-PD-1.

Figure 3

Differential gene expression analysis and MAGE-A expression differences in responding and non-responding patient tumors to second-line combination therapy at baseline. A) Representative immunofluorescence staining of MAGE (cyan) expression on SOX10+ tumor cells (orange) (left). Composite image at the top and individual marker staining from the same region below. B) The proportion of MAGE staining (of total tumor) in non-responding (n=14) and responding tumors (n=16) to second-line combination therapy at baseline (pre-anti-PD-1) (centre). C) Number of responding (blue) and non-responding (red) patient tumors with <45% or >45% MAGE expression comprising tumors (right). All error bars displayed represent the SEM. Non-parametric Mann-Whitney tests were used to determine statistical differences between non-responders and responders for MAGE expression in tumors.

Figure 4

Relationship and characterization of TCF7+ CD8 T cells and CD4 T cell populations in the tumor microenvironment. A) Number of responding (blue) and non-responding (red) patient tumors at baseline (pre-anti-PD-1) in TCF7 high/low (% of TCF7+ CD8 T cells (of CD8), cutoff =58 (median)) and CD4 high/low (% of CD4 T cells (of CD3), cutoff =66 (median)) groups. B) Correlation between the proportion of TCF7+ T cells (of CD8) and CD4 T cells (of CD3) (left), CD8 T cells (of CD3) (centre), and Treg/CD8 T cell ratio (right) in all patient tumors (n=31) at baseline (pre-anti-PD-1). C) Average distance (μm) between TCF7+/TCF7- CD8 T cells and CD4 T cells (left), Treg cells (centre), and tumor cells (right) in all patient tumors (n=32) at baseline (pre-anti-PD-1). Representative images below depicting proximity of TCF7+ CD8 T cells (TCF7, red; CD8, green) cells and CD4 T cells (CD3, yellow; CD8 negative) (left, Region 1 (R1)), Tregs (FOXP3, cyan; CD8 negative) (centre, Region 1 and 2 (R1, R2)), and Tumor cells (SOX10, violet) (right, Region 1,2, and 3 (R1, R2, R3). Non-parametric spearman rho tests were performed for correlation analyses. Non-parametric Wilcoxon matched-pairs signed rank tests were used to determine statistical differences between TCF7+ and TCF7- T cell average distances in tumors.

Supplementary figure 1

Histological characterization of patient tumors pre-anti-PD-1, post anti-PD-1 failure, and post second-line anti-PD-1 + anti-CTLA-4 combination therapy. A) Schematic of patient cohort, treatment algorithm, and biopsy timepoints. B) Heatmap summarizing the statistical differences for various histological parameters between non-responders and responders to second-line combination therapy in baseline (pre- anti-PD-1, n=31; responders =16, non-responders = 15), post anti-PD-1 (n=14; responders =5, non-responders = 9), and post anti-PD-1 + anti-CTLA-4 (n=9, responders =2, non-responders = 7) patient tumors. C) Percentage of tumor involved and scoring of select histological parameters that display modest statistical differences between non-responders and responders to second-line combination therapy at

baseline (pre-anti-PD-1) or post anti-PD-1 + anti-CTLA-4. All error bars displayed represent the SEM. Non-parametric Mann-Whitney tests were used to determine statistical differences between non-responders and responders.

Supplementary figure 2

Broad characterization of patient tumors failing anti-PD-1. A) The proportion and density of HLA-ABC and T cell phenotypes in all patient tumors (baseline) failing anti-PD-1 (n=31). B) The proportion of TCF7+ CD8 T cells (of CD8) in responders to anti-PD-1 monotherapy (n=15) (Sade-Feldman et al., 2018) and responders to second-line combination anti-PD-1 + anti-CTLA-4 after anti-PD-1 failure in the current study. C) The proportion of select immune phenotypes in responders (blue) and non-responders (red) pre-anti-PD-1 (timepoint 1), post anti-PD-1 (timepoint 2), or post anti-PD-1 + anti-CTLA-4 (timepoint 3) immunotherapy. All error bars displayed represent the SEM. Non-parametric Mann-Whitney tests were used to determine statistical differences between non-responders and responders.

Supplementary figure 3

Correlation between Tregs and CD4 conventional T cells. A) Correlation between the density of Tregs and conventional CD4 T cells (non-Tregs) in all patient tumors (n=31) at baseline (pre-anti-PD-1).

Citations

1. Wolchok, J. D. *et al.* Safety and clinical activity of combined PD-1 (nivolumab) and CTLA-4 (ipilimumab) blockade in advanced melanoma patients Jedd. *Physiol. Behav.* **169**, 122–133 (2017).
2. Robert, C. *et al.* Nivolumab in Previously Untreated Melanoma without BRAF Mutation . *N. Engl. J. Med.* **372**, 320–330 (2015).
3. Ribas, A. *et al.* Pembrolizumab versus investigator-choice chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): A randomised, controlled, phase 2 trial. *Lancet Oncol.* **16**, 908–918 (2015).
4. Larkin, J. *et al.* Combined Nivolumab and Ipilimumab or Monotherapy in Previously Untreated Melanoma Corresponding authors. *N Engl J Med* **373**, 23–34 (2015).
5. Pires da Silva, I. *et al.* Ipilimumab alone or ipilimumab plus anti-PD-1 therapy in patients with metastatic melanoma resistant to anti-PD-(L)1 monotherapy: a multicentre, retrospective, cohort study. *Lancet Oncol.* **22**, 836–847 (2021).
6. Eroglu, Z. *et al.* High response rate to PD-1 blockade in desmoplastic melanomas. *Nature* **553**, 347–350 (2018).
7. Klemen, N. D. *et al.* Survival after checkpoint inhibitors for metastatic acral, mucosal and uveal melanoma. *J. Immunother. Cancer* **8**, 1–6 (2020).
8. Rossi, E. *et al.* Efficacy of immune checkpoint inhibitors in different types of melanoma. *Hum. Vaccin. Immunother.* **17**, 4–13 (2021).
9. Pires da Silva, I. *et al.* Site-specific response patterns, pseudoprogression, and acquired

resistance in patients with melanoma treated with ipilimumab combined with anti–PD-1 therapy. *Cancer* **126**, 86–97 (2020).
10. Florou, V., Puri, S., Garrido-Laguna, I. & Wilky, B. A. Considerations for immunotherapy in patients with cancer and comorbid immune dysfunction. *Ann. Transl. Med.* **9**, 1035–1035 (2021).
11. Wei, S. C. *et al.* Distinct Cellular Mechanisms Underlie Anti-CTLA-4 and Anti-PD-1 Checkpoint Blockade. LID - S0092-8674(17)30831-0 [pii] LID - 10.1016/j.cell.2017.07.024 [doi]. *Cell* **170**, 1120–1133 (2017).
12. Gide, T. N. *et al.* Distinct Immune Cell Populations Define Response to Anti-PD-1 Monotherapy and Anti-PD-1/Anti-CTLA-4 Combined Therapy. *Cancer Cell* **35**, 238-255.e6 (2019).
13. Sade-Feldman, M. *et al.* Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell* **175**, 998-1013.e20 (2018).
14. Zimmer, L. *et al.* Ipilimumab alone or in combination with nivolumab after progression on anti-PD-1 therapy in advanced melanoma. *Eur. J. Cancer* **75**, 47–55 (2017).
15. Eisenhauer, E. A. *et al.* New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur. J. Cancer* **45**, 228–247 (2009).
16. Edwards, J. *et al.* Tumor mutation burden and structural chromosomal aberrations are not associated with T-cell density or patient survival in acral, mucosal, and cutaneous melanomas. *Cancer Immunol. Res.* **8**, (2020).
17. Fridman, W. H. *et al.* Prognostic and predictive impact of intra- and peritumoral immune infiltrates. *Cancer Res.* **71**, 5601–5605 (2011).
18. Maibach, F., Sadozai, H., Seyed Jafari, S. M., Hunger, R. E. & Schenk, M. Tumor-Infiltrating Lymphocytes and Their Prognostic Value in Cutaneous Melanoma. *Front. Immunol.* **11**, 1–20 (2020).
19. Schnell, A., Schmidl, C., Herr, W. & Siska, P. J. The peripheral and intratumoral immune cell landscape in cancer patients: A proxy for tumor biology and a tool for outcome prediction. *Biomedicines* **6**, (2018).
20. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
21. Anders, S., Pyl, P. T. & Huber, W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).
22. Rawson, R. V. *et al.* Pathological response and tumour bed histopathological features correlate with survival following neoadjuvant immunotherapy in stage III melanoma. *Ann. Oncol.* **32**, 766–777 (2021).
23. Edwards, J. *et al.* $CD103^+$ tumor-resident $CD8^+$ T cells are associated with improved survival in immunotherapy-naïve melanoma patients and expand significantly during anti-PD-1 treatment. *Clin. Cancer Res.* **24**, (2018).
24. Duhen, T. *et al.* Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. *Nat. Commun.* **9**, (2018).
25. Simoni, Y. *et al.* Bystander CD8+ T cells are abundant and phenotypically distinct in human tumour infiltrates. *Nature* **557**, 575–579 (2018).
26. Simpson, T. R. *et al.* Fc-dependent depletion of tumor-infiltrating regulatory t cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J. Exp. Med.* **210**, 1695–1710 (2013).
27. Curran, M. A., Montalvo, W., Yagita, H. & Allison, J. P. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 4275–4280 (2010).

28. Liakou, C. I. *et al.* CD4 ⁄- ICOS hi cells to shift the ratio of effector to regulatory T cells in cancer patients. *Sci. York* 1–6 (2008).
29. Zaretsky, J. M. *et al.* Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. *N. Engl. J. Med.* **375**, 819–829 (2016).
30. Restifo, N. P. *et al.* Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. *J. Natl. Cancer Inst.* **88**, 100–108 (1996).
31. Chen, Z. *et al.* TCF-1-Centered Transcriptional Network Drives an Effector versus Exhausted CD8 T Cell-Fate Decision. *Immunity* **51**, 840-855.e5 (2019).
32. Siddiqui, I. *et al.* Intratumoral Tcf1 + PD-1 + CD8 + T Cells with Stem-like Properties Promote Tumor Control in Response to Vaccination and Checkpoint Blockade Immunotherapy. *Immunity* **50**, 195-211.e10 (2019).
33. Tumeh, P. C. *et al.* PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* **515**, 568–571 (2014).
34. van der Leun, A. M., Thommen, D. S. & Schumacher, T. N. CD8+ T cell states in human cancer: insights from single-cell analysis. *Nat. Rev. Cancer* **20**, 218–232 (2020).
35. Eberhardt, C. S. *et al.* Functional HPV-specific PD-1+ stem-like CD8 T cells in head and neck cancer. *Nature* **597**, 279–284 (2021).
36. Kurtulus, S. *et al.* Checkpoint Blockade Immunotherapy Induces Dynamic Changes in PD-1 − CD8 + Tumor-Infiltrating T Cells. *Immunity* **50**, 181-194.e6 (2019).
37. Oliveira, G. *et al.* Phenotype, specificity and avidity of antitumour CD8+ T cells in melanoma. *Nature* **596**, 119–125 (2021).
38. Caushi, J. X. *et al.* Transcriptional programs of neoantigen-specific TIL in anti-PD-1-treated lung cancers. *Nature* **596**, 126–132 (2021).
39. Gajewski, T. F., Fallarino, F., Fields, P. E., Rivas, F. & Alegre, M.-L. Absence of CTLA-4 Lowers the Activation Threshold of Primed CD8 + TCR-Transgenic T Cells: Lack of Correlation with Src Homology Domain 2-Containing Protein Tyrosine Phosphatase . *J. Immunol.* **166**, 3900–3907 (2001).
40. Jadhav, R. R. *et al.* Epigenetic signature of PD-1+ TCF1+ CD8 T cells that act as resource cells during chronic viral infection and respond to PD-1 blockade. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 14113–14118 (2019).
41. Hui, E. *et al.* T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* **355**, 1428–1433 (2017).
42. Tay, R. E., Richardson, E. K. & Toh, H. C. Revisiting the role of CD4+ T cells in cancer immunotherapy—new insights into old paradigms. *Cancer Gene Ther.* **28**, 5–17 (2021).
43. Binnewies, M. *et al.* Unleashing Type-2 Dendritic Cells to Drive Protective Antitumor CD4+ T Cell Immunity. *Cell* **177**, 556-571.e16 (2019).
44. Wei, S. C. *et al.* Negative Co-stimulation Constrains T Cell Differentiation by Imposing Boundaries on Possible Cell States. *Immunity* **50**, 1084-1098.e10 (2019).
45. Edwards, J. *et al.* Prevalence and cellular distribution of novel immune checkpoint targets across longitudinal specimens in treatment-naïve melanoma patients: Implications for clinical trials. *Clin. Cancer Res.* **25**, (2019).
46. Eschweiler, S. *et al.* Intratumoral follicular regulatory T cells curtail anti-PD-1 treatment efficacy. *Nat. Immunol.* **22**, 1052–1063 (2021).
47. Dhatchinamoorthy, K., Colbert, J. D. & Rock, K. L. Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation. *Front. Immunol.* **12**, (2021).
48. Okla, K., Farber, D. L. & Zou, W. Tissue-resident memory T cells in tumor immunity and immunotherapy. *J. Exp. Med.* **218**, 1–14 (2021).

49. Han, L. *et al.* Characterization of CD103+ CD8+ tissue-resident T cells in esophageal squamous cell carcinoma: may be tumor reactive and resurrected by anti-PD-1 blockade. *Cancer Immunol. Immunother.* **69**, 1493–1504 (2020).
50. Mariathasan, S. *et al.* TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature* **554**, 544–548 (2018).
51. Tauriello, D. V. F. *et al.* TGFβ drives immune evasion in genetically reconstituted colon cancer metastasis. *Nature* **554**, 538–543 (2018).

Table 1. Demographic and clinical characteristics at start of PD1 and IPI + PD1.

	Patients n=34
Sex – no. (%)	
Male	19 (56%)
Female	15 (44%)
Mutational status – no. (%)[1]	
BRAF mutant	7 (21%)
NRAS mutant	11 (33%)
BRAF & NRAS WT	15 (46%)
Age at start PD1 - yr	
Median	70
Range	36, 86
ECOG Performance Status at start PD1 – no. (%)	
0	29 (85%)
≥ 1	5 (15%)
Staging at start PD1	
III/M1A/M1B	23 (68%)
M1C/M1D	11 (32%)
LDH at start PD1 – no. (%)[2]	
Normal	26 (84%)
>UNL	5 (16%)
PD1 treatment setting – no. (%)	
Adjuvant	11 (32%)
Metastatic	23 (68%)
Resistance to PD1 – no. (%)[3]	
Innate	16 (70%)
Acquired	7 (30%)
Median Time to progression - mo	
Median	2.7
Range	1.2, 23.1
Age at start IPI+PD1 - yr	
Median	71.0
Range	37, 86

[1] Missing values: n=1.
[2] Missing values: n=3.
[3] Resistance to PD1, included patients treated with PD1 in the metastatic setting only: n=23.

ECOG Performance Status at start IPI+PD1 – no. (%)	
0	28 (82%)
≥ 1	6 (18%)
Staging at start IPI+PD1	
III/M1A/M1B	16 (47%)
M1C/M1D	18 (53%)
LDH at start IPI+PD1 – no. (%)[4]	
Normal	22 (69%)
>UNL	10 (31%)

[4] Missing values: n=2.

Table 2: Univariate Logistic Regression of Objective Response

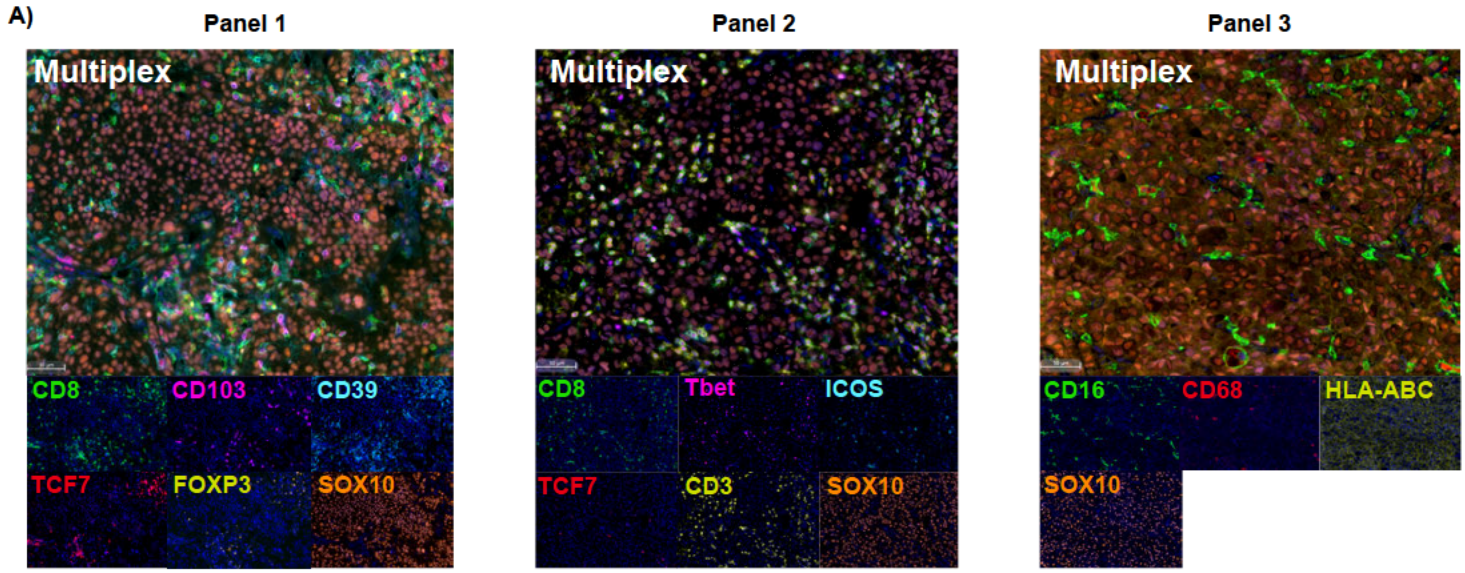
	Univariable	
Covariates	**OR**	**P-value**
Gender		
Female	1	0.1591
Male	2.75 (0.67, 11.24)	
Age at start of IPI+PD1	1.00 (0.94, 1.06)	0.9482
ECOG		
0	1	0.4633
>=1	0.50 (0.08, 3.19)	
Stage		
III/M1a/M1b	1	0.3139
M1c/M1d	0.49 (0.13, 1.95)	
LDH		
Normal	1	0.7100
Elevated	0.76 (0.18, 3.27)	
Brain metastasis		
No	1	0.8028
Yes	0.81 (0.15, 4.32)	
Lung metastasis		
No	1	0.9675
Yes	1.03 (0.26, 3.99)	
Liver metastasis		
No	1	0.6423
Yes	0.71 (0.17, 2.95)	
GI metastasis		
No	1	0.8547
Yes	0.87 (0.19, 4.01)	
Bone metastasis		
No	1	0.9560
Yes	NA	
Adrenal metastasis		
No	1	0.9784
Yes	NA	
Soft tissue metastasis		
No	1	0.1306
Yes	0.17 (0.02, 1.68)	
Lymph nodes metastasis		
No	1	0.3414
Yes	2.17 (0.44, 10.65)	
Spleen metastasis		
No	1	0.9314
Yes	1.13 (0.07, 19.74)	
Subcutaneous metastasis		
No	1	0.9675
Yes	0.97 (0.25, 3.77)	
Spleen metastasis		
No	1	0.9314
Yes	1.13 (0.07, 19.74)	
GU metastasis		
No	1	0.9784
Yes	NA	
Number of sites of met		

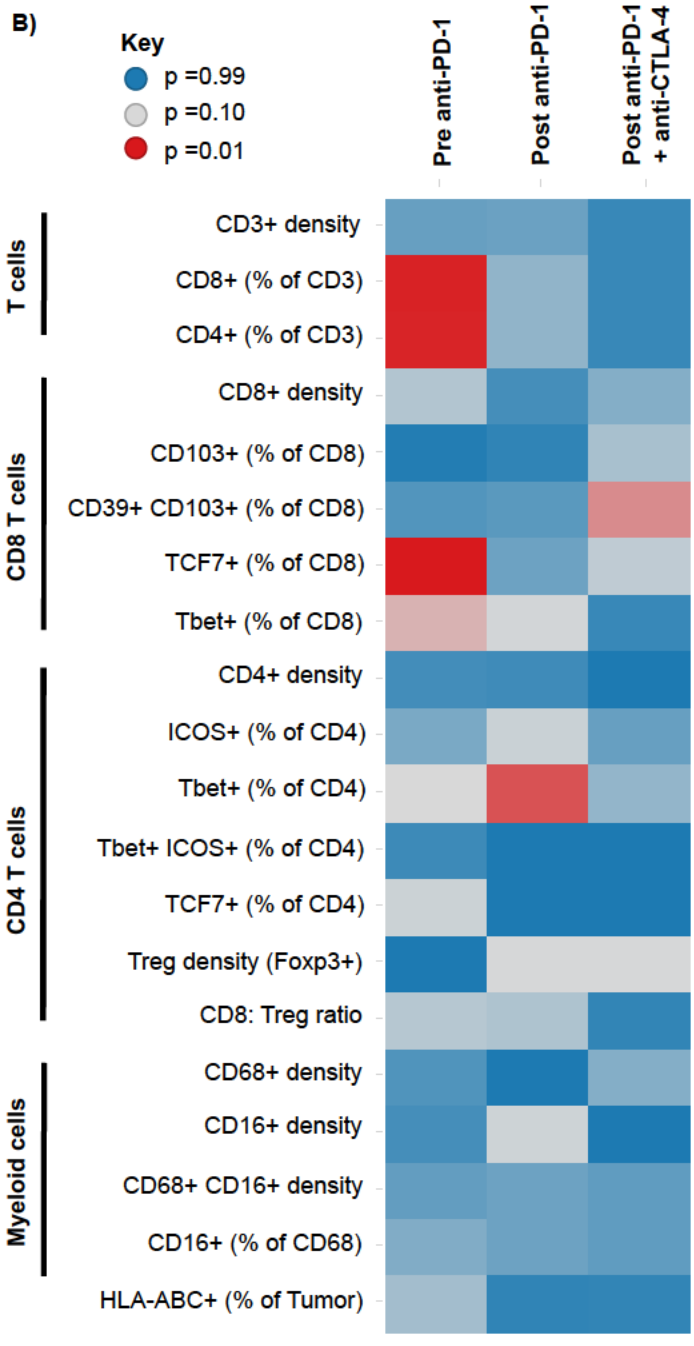
<3	1	0.5158
>=3	0.64 (0.16, 2.49)	
TCF7+ (% of CD8) at baseline	1.06 (1.02, 1.10)	**0.0055**

Table 3. List of differentially expressed genes in baseline tumors between responders and non-responders to second-line combination therapy

Gene	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
IL13RA2	169.8663236	2.903072888	0.973846996	13.7774406	3.48E-43	5.85E-39
SPINK1	312.9415217	1.152113211	0.950950267	9.93852788	2.83E-23	2.38E-19
CABCOCO1	103.8703116	-1.105325121	0.959190106	-9.8559786	6.46E-23	3.61E-19
OR5H6	9.734662476	0.97721109	0.803030507	9.50564224	1.99E-21	8.34E-18
REXO1L1P	541.7781185	-1 3297148	0.807112947	-9.0619037	1.28E-19	4.30E-16
SFTPA2	4676.241476	0.001933151	0.807202112	8.96391689	3.13E-19	7.51E-16
PGC	349.7820741	0.020447302	0.80669105	8.97655307	2.79E-19	7.51E-16
OR52E8	82.80378226	1.250243307	0.821696603	8.68407951	3.82E-18	8.01E-15
OR52E6	96.19159902	1.879890419	0.899052887	8.61133746	7.22E-18	1.35E-14
MAGEB2	172.6410441	0.908497242	0.806568315	8.49336549	2.01E-17	3.37E-14
ISL1	58.44900387	0.695866007	0.805531721	8.2887489	1.14E-16	1.75E-13
PADI3	47.86575556	-0.851646655	0.921238129	-8.2751895	1.28E-16	1.79E-13
LIN28B	166.2787722	0.615532936	0.916975128	8.19557381	2.49E-16	3.22E-13
ERVV-2	1335.514648	-1.178015987	0.807195083	-7.982236	1.44E-15	1.72E-12
CCDC190	16.18339151	-0.085202172	0.802828814	-7.9671725	1.62E-15	1.82E-12
NAA11	58.00880679	0.938485089	0.805265479	7.95712878	1.76E-15	1.85E-12
VSTM5	11.75538016	-0.764274868	0.803101181	-7.8932486	2.94E-15	2.91E-12
KRTAP9-2	34.22853005	-0.973295242	0.894056546	-7.8158974	5.46E-15	5.09E-12
SSX1	460.3998747	1.067876979	0.833628616	7.75657525	8.73E-15	7.71E-12
GKN2	89.79346718	-0.021083857	0.80517067	-7.7498742	9.20E-15	7.72E-12
FAM71E1	10.0456817	-0.659978113	0.803022362	-7.742999	9.71E-15	7.76E-12
CMTM5	15.45735719	1.012173419	0.910041592	7.73086388	1.07E-14	8.15E-12
HTN1	97.34344935	-0.774700196	0.905382666	-7.6455367	2.08E-14	1.52E-11
KRT83	71.40446284	-1.17917361	0.853971838	-7.5919798	3.15E-14	2.20E-11
WDR87	203.0960865	-1.324033559	0.806893172	-7.535822	4.85E-14	3.26E-11
SLC9A4	22.44698076	-0.516519739	0.804046742	-7.485494	7.13E-14	4.48E-11
MYLK2	9.354546578	-0.735546444	0.802983287	-7.4840141	7.21E-14	4.48E-11
TMEM139	141.6314724	-0.670728092	0.848361403	-7.4046783	1.31E-13	7.88E-11
FRG2	42.28883168	0.55341826	0.804545359	7.28255483	3.28E-13	1.90E-10
HOXD13	89.43457338	0.927681427	0.85264876	7.25528775	4.01E-13	2.24E-10
BPI	84.71329198	-0.796290615	0.840703147	-7.2362385	4.61E-13	2.50E-10
LCN8	8.146996985	0.952423957	0.802920446	7.22131985	5.15E-13	2.70E-10
MSMB	41.65680149	-0.583469568	0.871905008	-7.1720849	7.39E-13	3.76E-10
CNBD1	68.52782666	0.851809735	0.855388795	7.08327762	1.41E-12	6.95E-10
OR52A5	14.86187105	0.810231957	0.803270402	7.01454121	2.31E-12	1.11E-09
BEST3	7.649618711	-1.24132707	0.802588061	-6.9919186	2.71E-12	1.26E-09
SMR3A	235.2267475	-0.740054091	0.806960138	-6.9641788	3.30E-12	1.46E-09
MAGEA1	303.6286439	0.594426622	0.806904407	6.96713648	3.23E-12	1.46E-09
MAGEA3	1098.887753	4.675341214	0.889318724	6.84927206	7.42E-12	3.20E-09
NKAIN4	5.258459402	-0.517193862	0.802394162	-6.8357195	8.16E-12	3.42E-09
PRR9	637.6531044	-0.112671228	0.807024605	-6.5725145	4.95E-11	2.01E-08
HAND2	39.33740011	0.456665565	0.845214166	6.57027923	5.02E-11	2.01E-08
PIWIL1	12.62466727	-0.869661397	0.803135776	-6.4056217	1.50E-10	5.85E-08
G6PC2	26.3594816	0.531535858	0.803618534	6.38642483	1.70E-10	6.48E-08
GAGE1	4759.632187	3.541710748	0.957320012	6.30296361	2.92E-10	1.09E-07
DRD2	20.47675763	-0.693067132	0.804321084	-6.2446405	4.25E-10	1.55E-07
HOXB9	25.46578208	0.644299421	0.803426937	6.14991344	7.75E-10	2.77E-07
GBA3	16.1658383	0.815814196	0.805880332	6.136626	8.43E-10	2.95E-07
XAGE1A	4793.299111	3.621599804	0.949468596	6.09992994	1.06E-09	3.64E-07
GAGE12J	3841.085675	3.794196931	0.942395971	6.08110442	1.19E-09	4.01E-07
SFTPA1	1252.07917	0.003917136	0.80708534	5.98789125	2.13E-09	7.00E-07
RNF17	1187.311005	3.681350646	0.97547366	5.96339059	2.47E-09	7.98E-07
XAGE1B	2780.354869	3.813167723	0.944829628	5.92525274	3.12E-09	9.88E-07
GAGE12C	6177.392635	3.875532346	0.955466052	5.83156302	5.49E-09	1.71E-06
LCE3C	30.75834	-0.013243664	0.803190897	5.67972076	1.35E-08	4.12E-06
PNRC2	418.9436772	-5.010139779	0.889926509	-5.4898323	4.02E-08	1.21E-05
ERVV-1	725.5492496	-2.645815946	0.967910348	-5.3325174	9.69E-08	2.85E-05
ZNF322	1574.985371	-4.141625238	0.934478787	-5.1082233	3.25E-07	9.41E-05
PAGE5	2308.832	2.603151993	0.979079693	5.05718897	4.25E-07	0.00012107
STIMATE-MU	163.5375089	-2.389717363	0.932657938	-4.4510535	8.55E-06	0.00238492
MUC7	1243.758482	-3.985143822	0.946163525	-4.4480409	8.67E-06	0.00238492
CXCL10	3896.74408	-3.104496404	0.721524553	-4.3407246	1.42E-05	0.00384536
SPP1	51626.9732	-3.441221273	0.831425146	-4.2367147	2.27E-05	0.00604405
NELL1	2314.311115	2.887431767	0.970247519	4.16504473	3.11E-05	0.00816557
GABRG2	318.6941795	1.482289087	0.945454221	4.12224711	3.75E-05	0.00969041
TMIGD3	1172.981695	-3.702372963	0.899077689	-4.0382296	5.39E-05	0.01369904
FABP3	1272.802318	-1.908426735	0.501976411	-3.8058824	0.0001413	0.03540502
IFIT3	2689.128272	-2.480988706	0.664204155	-3.7279599	0.00019304	0.04765721
POU6F2	94.65016526	2.193619335	0.973926927	3.71506327	0.00020315	0.04942797

Figure 1


A)
Panel 1
Panel 2
Panel 3
Multiplex
Multiplex
Multiplex
CD8
CD103
CD39
TCF7
FOXP3
SOX10
CD8
Tbet
ICOS
TCF7
CD3
SOX10
CD16
CD68
HLA-ABC
SOX10


B)
Key
p =0.99
p =0.10
p =0.01
Pre anti-PD-1
Post anti-PD-1
Post anti-PD-1 + anti-CTLA-4
T cells
CD3+ density
CD8+ (% of CD3)
CD4+ (% of CD3)
CD8 T cells
CD8+ density
CD103+ (% of CD8)
CD39+ CD103+ (% of CD8)
TCF7+ (% of CD8)
Tbet+ (% of CD8)
CD4 T cells
CD4+ density
ICOS+ (% of CD4)
Tbet+ (% of CD4)
Tbet+ ICOS+ (% of CD4)
TCF7+ (% of CD4)
Treg density (Foxp3+)
CD8: Treg ratio
Myeloid cells
CD68+ density
CD16+ density
CD68+ CD16+ density
CD16+ (% of CD68)
HLA-ABC+ (% of Tumor)

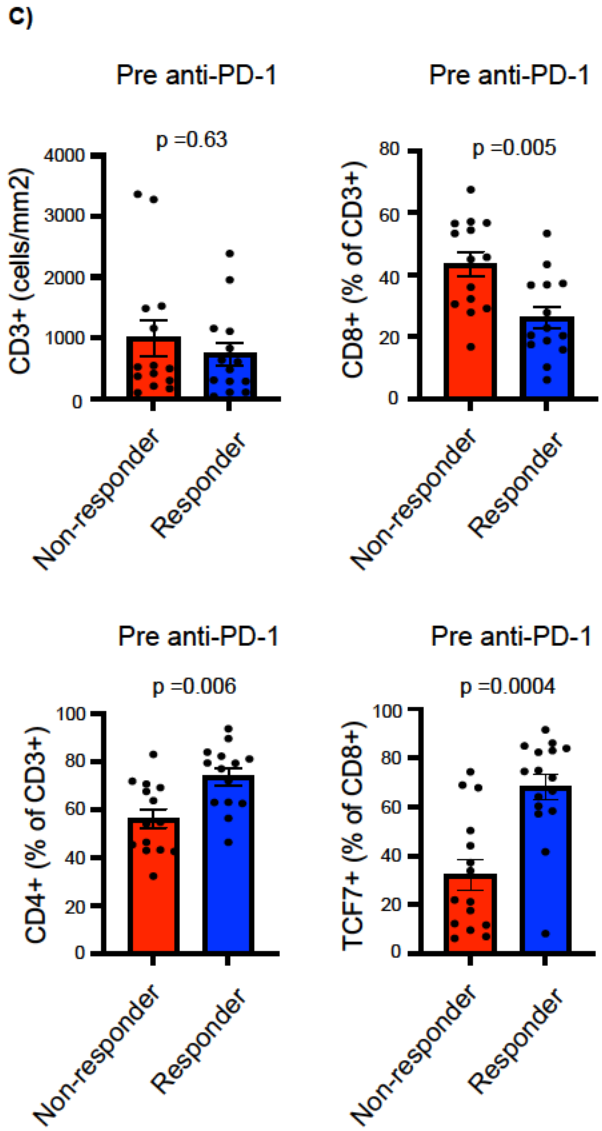

C)
Pre anti-PD-1
p =0.63
CD3+ (cells/mm2)
Non-responder
Responder
Pre anti-PD-1
p =0.005
CD8+ (% of CD3+)
Non-responder
Responder
Pre anti-PD-1
p =0.006
CD4+ (% of CD3+)
Non-responder
Responder
Pre anti-PD-1
p =0.0004
TCF7+ (% of CD8+)
Non-responder
Responder

Figure 2

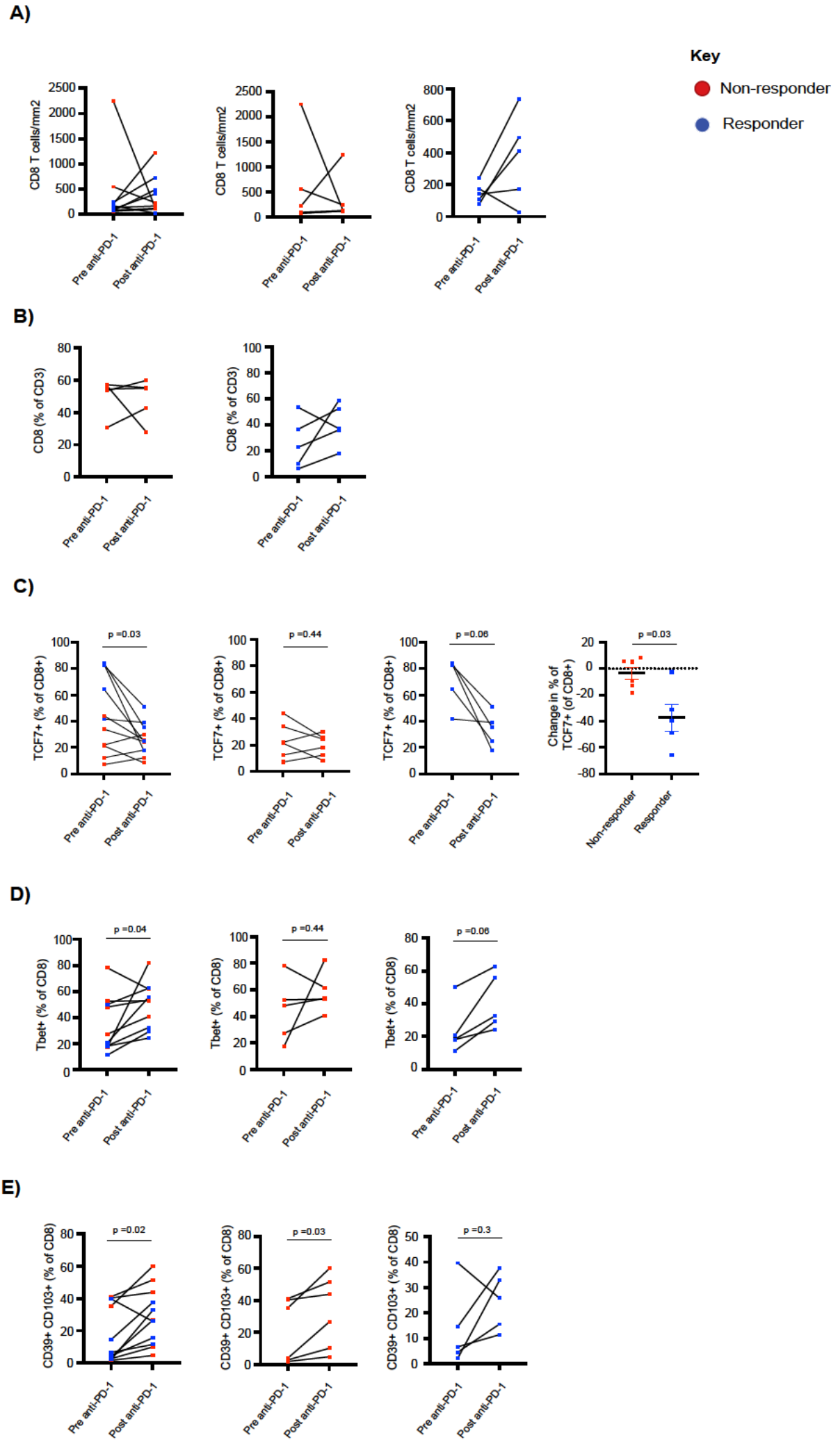
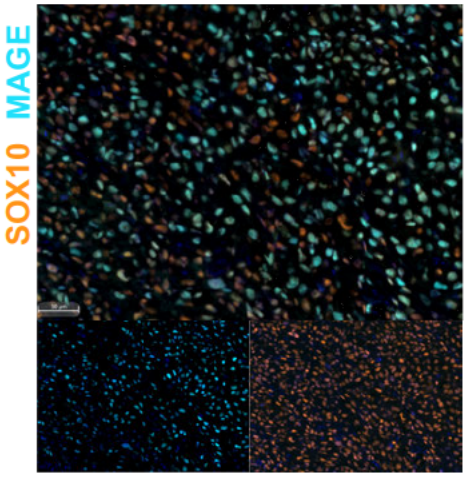
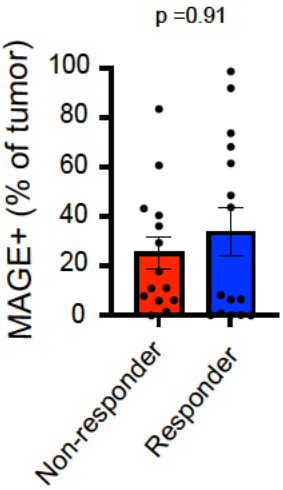

A)
Key
Non-responder
Responder
CD8 T cells/mm2
Pre anti-PD-1
Post anti-PD-1
B)
CD8 (% of CD3)
C)
TCF7+ (% of CD8+)
p =0.03
p =0.44
p =0.06
Change in % of TCF7+ (of CD8+)
Non-responder
Responder
D)
Tbet+ (% of CD8)
p =0.04
p =0.44
p =0.06
E)
CD39+ CD103+ (% of CD8)
p =0.02
p =0.03
p =0.3

Figure 3

A)


SOX10
MAGE

B)


p =0.91
MAGE+ (% of tumor)
100
80
60
40
20
0
Non-responder
Responder

C)

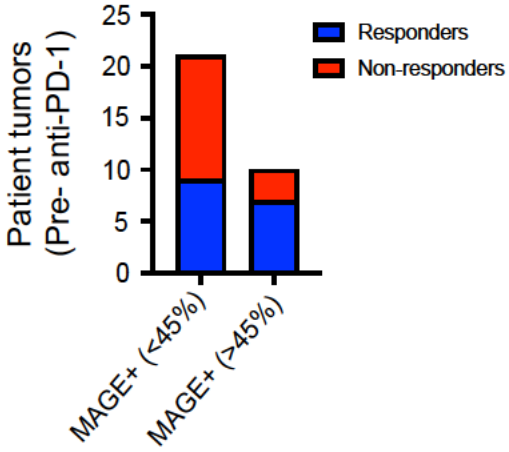
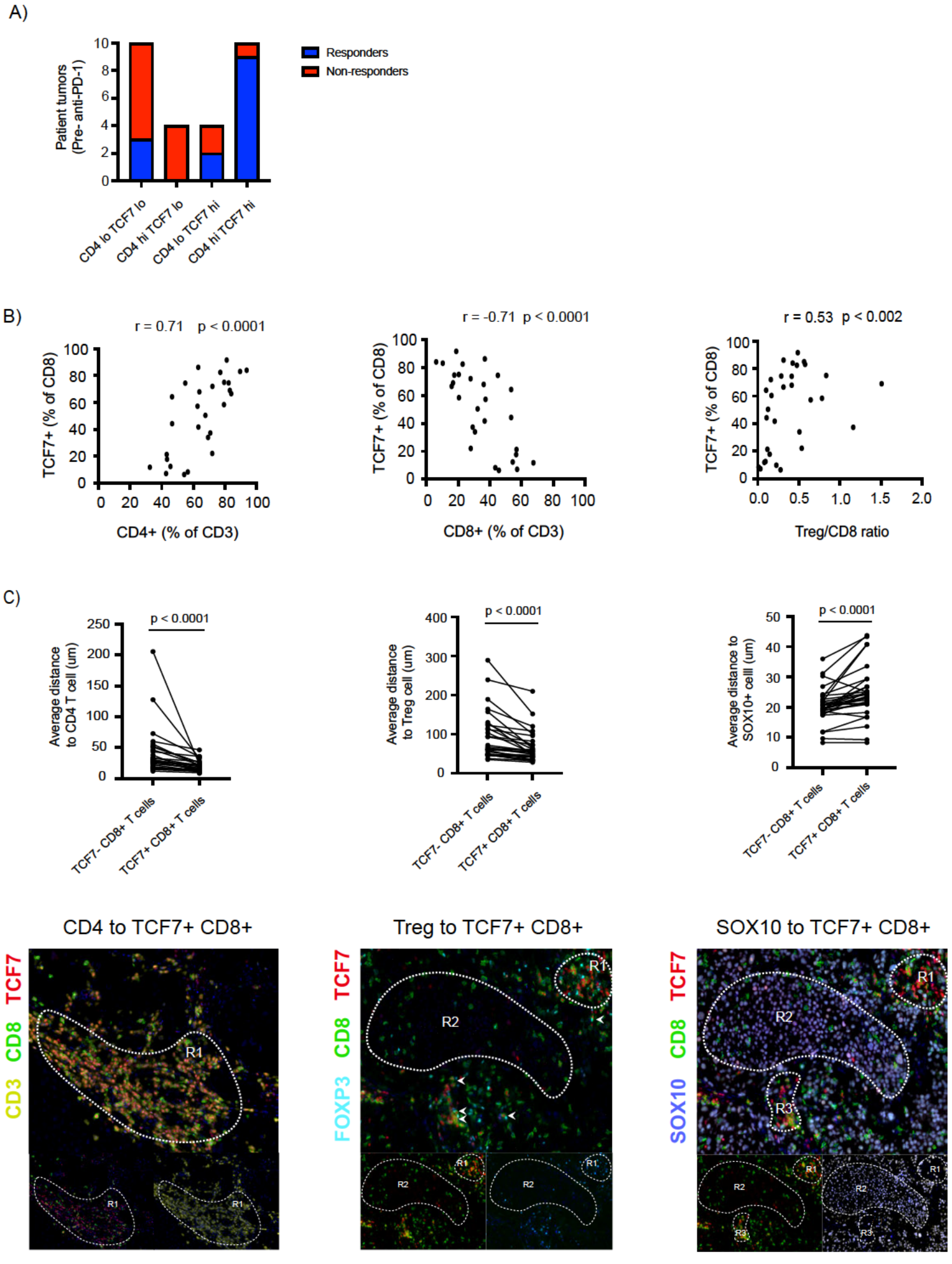
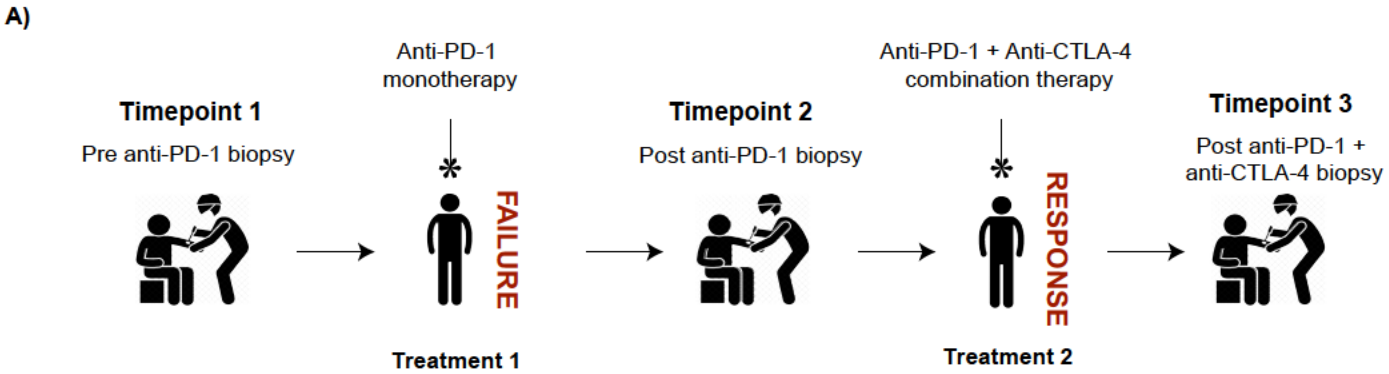
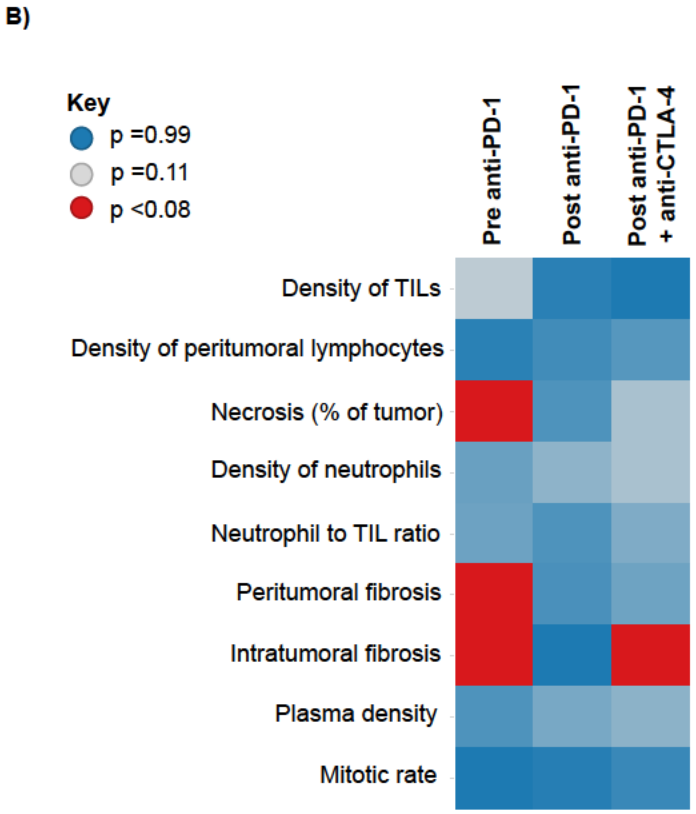

Patient tumors
(Pre- anti-PD-1)
25
20
15
10
5
0
Responders
Non-responders
MAGE+ (<45%)
MAGE+ (>45%)

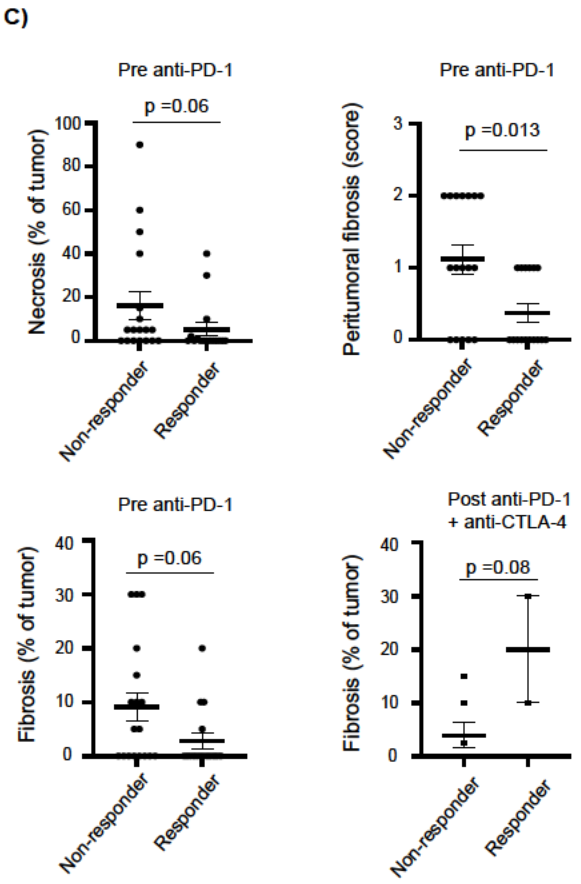
Figure 4


A)
Patient tumors (Pre- anti-PD-1)
Responders
Non-responders
CD4 lo TCF7 lo
CD4 hi TCF7 lo
CD4 lo TCF7 hi
CD4 hi TCF7 hi
B)
r = 0.71 p < 0.0001
TCF7+ (% of CD8)
CD4+ (% of CD3)
r = -0.71 p < 0.0001
CD8+ (% of CD3)
r = 0.53 p < 0.002
Treg/CD8 ratio
C)
p < 0.0001
Average distance to CD4 T cell (um)
TCF7- CD8+ T cells
TCF7+ CD8+ T cells
Average distance to Treg cell (um)
Average distance to SOX10+ cell (um)
CD4 to TCF7+ CD8+
Treg to TCF7+ CD8+
SOX10 to TCF7+ CD8+
CD3 CD8 TCF7
FOXP3 CD8 TCF7
SOX10 CD8 TCF7
R1
R2
R3

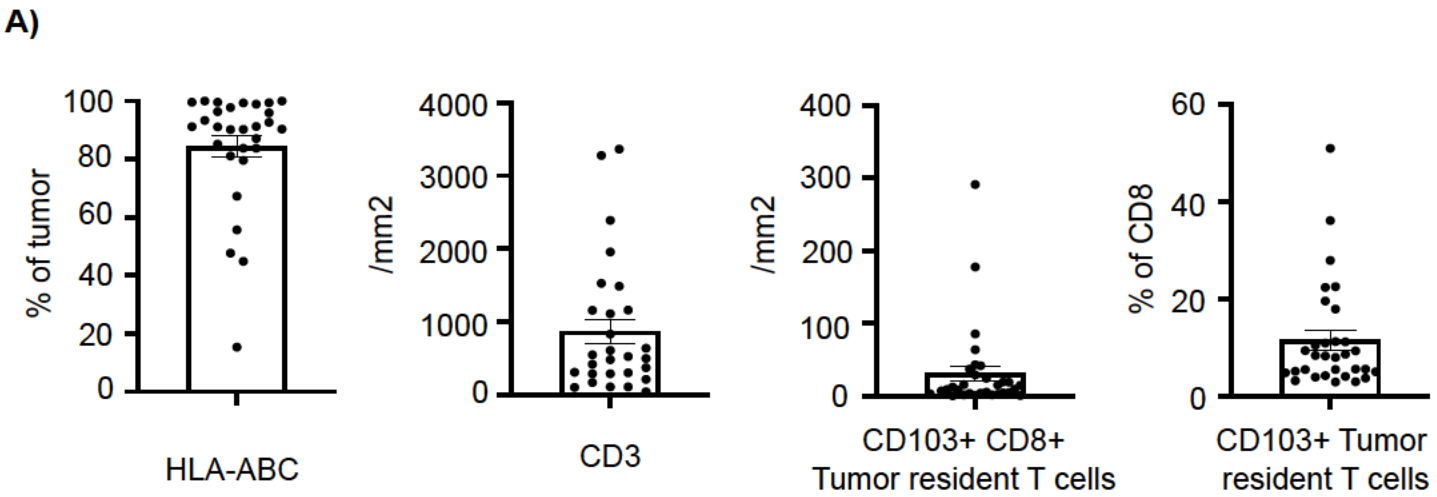
Supplementary Figure 1

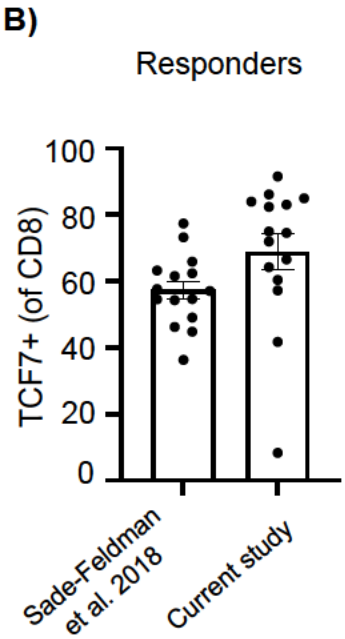


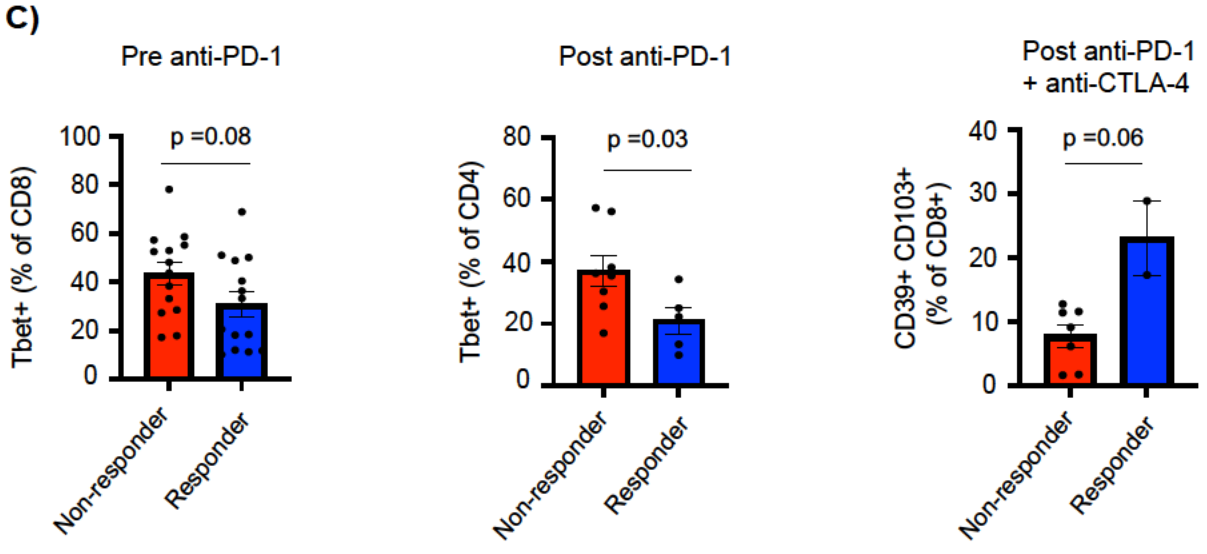




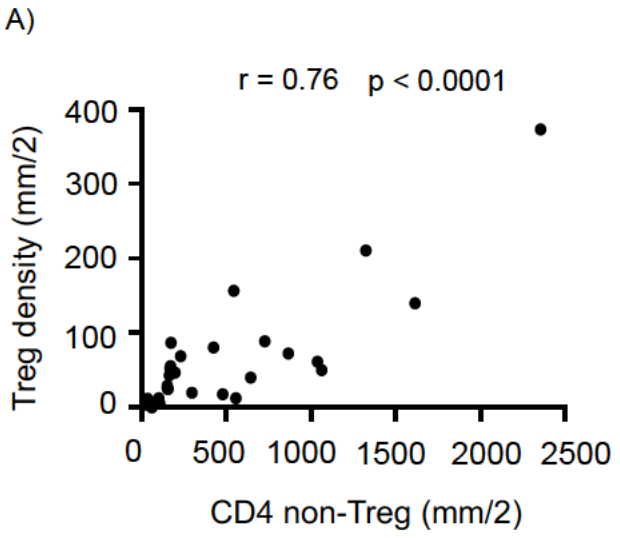
Supplementary Figure 2







Supplementary Figure 3



Chapter 4 - Concluding discussion

To conclude this chapter, we have examined and explored the tumor characteristics of melanoma patients who have failed anti-PD-1 monotherapy but responded to subsequent combination anti-PD-1 + anti-CTLA-4 therapy. The work of this chapter fits into the overall theme of advancing opportunities to overcome anti-PD-1 resistance. The cohort of patients that we have examined here represents a very real and clinically practiced method of salvaging anti-PD-1 refractory patients. However, its implementation is limited because of the absence of predictive biomarkers that can be used to assist clinicians in their clinical decision-making process. Specifically, the absence of biomarkers prevents clinicians from limiting the use of combination therapy to those that require it and are likely to benefit from it. To address this unmet need, we focused our work on phenotyping immune cells that had previously been associated with response to anti-PD-1 and anti-CTLA-4 therapies, including resident CD8 T cells, CD4 T cells, Tregs, and other special CD8 T cell populations, including TCF7+ CD8 T cells. In fulfillment of our first two aims for this chapter, we found several differences between non-responding and responding tumors to second-line combination therapy, including the proportion of CD8 and CD4 T cells, but most strikingly the proportion of TCF7+ CD8+ T cells, which was strongly associated with response. As has been discussed, given that this is a shared biomarker for anti-PD-1 immunotherapy, this likely indicates that the addition of anti-CTLA-4 sensitizes responding tumors to the effects of anti-PD-1 therapy. This is supported by the fact that anti-PD-1 resistant patients derive higher objective response rates with subsequent combination anti-PD-1 + anti-CTLA-4 (30%) compared to subsequent single agent anti-CTLA-4 therapy (13%) (Pires da Silva et al., 2021), clearly demonstrating the importance of anti-PD-1 even in patients resistant to single agent anti-PD-1. The issue with a shared biomarker is the fact that it cannot be used in the treatment-naïve setting to predict whether a patient would require combination anti-CTLA-4 + anti-PD-1 over single agent anti-PD-1 immunotherapy. Yet, such a biomarker could still be used in patients that have already failed anti-PD-1 therapy. In this way, understanding the proportion of TCF7+ CD8 T cells might be able to help limit the exposure of anti-CTLA-4 to those that are likely to benefit from it, and secondly from patient populations that may be particularly vulnerable to the added risks of toxicity associated with combination treatment. Given that very little research has been conducted in this area, our data represents a significant step forward in the advancement of this therapeutic strategy. One of the intriguing questions that remains is why tumor resident CD8 T cells were not also predictive at baseline, especially if anti-CTLA-4 does indeed sensitize patient tumors to the effects of anti-PD-1? Certainly, tumor resident CD8 T cells have a role to play in anti-PD-1 immunotherapy response. They are tumor antigen specific TILs, express PD-1, and we have shown that they expand early during treatment with anti-PD-1 therapy. One reason might be due to the fact that at baseline, tumor resident CD8 T cells were lacking and substantially lower in both responding and non-responding patient tumors when compared to average levels seen in melanoma tumors. Thus, the contribution of these cells (at baseline) to immunotherapy responses may be minimal due to their underrepresentation in the tumor. However, post combination therapy, responding tumors did have higher proportions of tumor resident CD8 T cells compared to non-responding tumors, reaffirming the importance of this population in immunotherapy responses. How then is this to be reconciled? A possible explanation for this apparent discordance is that TCF7+ CD8 T cells and tumor resident CD8 T cells may very well represent the same T cell clonotypes at different time points in their differentiation pathway. As others have shown, TCF7+ CD8 T cells are undifferentiated stem-cell like CD8 T cells that are tumor specific and are thought to be the main source of the proliferative burst during anti-PD-1 treatment (Im et al., 2016; Sade-Feldman et al., 2018; Siddiqui et al., 2019). Upon anti-PD-1 blockade, they proliferate and differentiate into memory

and effector subsets (Chen et al., 2019; Kurtulus et al., 2019). TCF7+ CD8 T cells themselves likely do not engage with tumor cells or perform cytotoxic functions as we and others have shown that they are located distantly from tumors compared to TCF7- CD8 T cells (Eberhardt et al., 2021). However, upon differentiation into memory (tumor resident memory?) and effector populations, they then become essential for tumor clearance, performing effector functions, and establishing tumor-immune equilibriums (Eberhardt et al., 2021; S.L. Park et al., 2019). Indeed, Eberhardt and colleagues showed that 3 distinct CD8 T cell phenotypes (TCF7+ CD8 T cells, a transitory cell population, and a terminally differentiated-like population) existed in head and neck tumors and which were comprised of the same T cell clonotypes. Thus, it is possible that the high capacity of TCF7+ CD8 T cells in responding patients at baseline provides significant anti-tumor potential that can be unlocked by checkpoint blockade, and which can lead to the differentiation of protective CD8 immune cell subsets, including resident CD8 T cell subsets. In line with this, we showed that proportions of TCF7+ CD8 T cells decreased post immunotherapy, while resident CD8 T cells constituted a higher proportion of CD8 T cells in responding patients compared to non-responding patients post combination therapy. Thus, higher proportions of TCF7+ CD8 T cells are predictive pre-immunotherapy, while higher proportions of resident CD8 T cells post therapy (expansion) are associated with response potentially because that they demonstrate the successful proliferation and differentiation of TCF7+ CD8 T cells into protective resident T cell populations. Therefore, both TCF7+ CD8 T cells and resident CD8 T cells represent important populations, each playing a role in immunotherapy response. In our patient cohort however, their significance might be more separated in space and time. Another key question, as it pertains to our third aim for this chapter, is why the addition of anti-CTLA-4 is necessary to unlock the potential of TCF7+ CD8 T cells with anti-PD-1 therapy? While our study has not fully detailed the mechanism of action for this, our data does provide potential explanations. For example, we found that high proportions of CD4 T cells (of CD3) were higher at baseline in responding tumors compared to non-responding tumors. These CD4 T cells were also proximally closer to TCF7+ CD8 T cells compared to non-TCF7+ CD8 T cells. This was also true for the proximity of T-regulatory cells and TCF7+ CD8+ T cells. Given that anti-CTLA-4 is expressed highly on CD4 and T-regulatory cells and is known to activate or deplete these cells respectively (Binnewies et al., 2019; Buchan et al., 2018; Simpson et al., 2013), it is very possible that anti-CTLA-4 acts to provide CD4 “help” to TCF7+ CD8 populations or dampen Treg suppression on TCF7+ CD8 T cells. Perhaps this is necessary in tumors in which the vast CD8 T cell pool is undifferentiated (too much stemness/capacity?). Alternatively, anti-CTLA-4 may also be working on TCF7+ CD8 T cells directly, as these cells themselves can express CTLA-4 (Eberhardt et al., 2021; Jadhav et al., 2019). In this sense, the alternative checkpoint hypothesis might be relevant where the addition of another checkpoint inhibitor is necessary to overcome the threshold necessary for TCR activation. Lastly, it is also possible that cancer testis antigen (CTA) specific T cells might also play a role in this cohort of patients, given that a number of CTAs were differentially expressed between responding and non-responding tumors. The addition of anti-CTLA-4 has been shown to lower the TCR threshold necessary to activate antigen specific T cell populations (Gajewski, Fallarino, Fields, Rivas, & Alegre, 2001), and it has also been shown that CTA specific T cells have lower TCR avidity for antigen compared to neoantigen-specific T cells (Oliveira et al., 2021). Thus, it is possible that anti-CTLA-4 has an important role in tumors that contain a high proportion of CTA specific and not neoantigen specific T cells. Each of these hypotheses are worthy of further investigation, however our study provides a significant step-forward in better understanding the mechanism of action for response to combination anti-CTLA-4 + anti-PD1 after single agent PD-1 failure.

Thesis Concluding Discussion

The immune system is now appreciated as an integral component in the rejection and clearance of tumors. Immunotherapies that target T cells have proven efficacious, especially checkpoint inhibitors that target the T cell co-inhibitory receptors PD-1 and CTLA-4. In metastatic melanoma, this has revolutionised treatment and patient management. A decade earlier, metastatic melanoma was considered a fatal disease with a survival rate from diagnosis of under 2 years (Luke et al., 2017). Now, melanoma patients on anti-PD-1 immunotherapy can expect an objective response rate of 40%, and up to 60% with combination anti-PD-1 + anti-CTLA-4 immunotherapy (Larkin et al., 2015). Most of these responders will experience durable progression-free survival that will last years, if not for the rest of their lives. Some, however, will acquire resistance (P. Sharma et al., 2017). Despite these advances, innate and acquired resistance to checkpoint inhibitors represent the treatment experience of most patients, and therefore there is a need to develop strategies to improve current immunotherapies and overcome resistance mechanisms. The purpose of this thesis, broadly speaking, was to approach this problem in two ways. Firstly, we sought to better understand the exact T cell phenotypes associated and likely critical for immunotherapy responses. We reasoned that the absence of these T cell targets in tumors would explain patient non-response and provide opportunities to understand and develop strategies for increasing target populations in patients lacking them. Secondly, we sought to understand the tumor microenvironment of anti-PD-1 non-responding melanoma patients as well as the expression profile of alternative checkpoint receptors, especially in the context of current clinical strategies to overcome anti-PD-1 resistance (addition of anti-CTLA-4 and other checkpoint inhibitors in clinical trials). We reasoned that this approach would provide a better understanding of immunotherapy non-response and provide insights into patients that are likely to benefit from the addition of anti-CTLA-4 and other emerging checkpoint inhibitors.

Our investigation into T cell phenotypes and anti-PD-1 response began with our exploration of CD103+ tumor resident CD8 T cells in melanoma because previous studies had shown the protective function of CD103+ CD8 T cells at epithelial sites (Djenidi et al., 2015; Thomas Gebhardt et al., 2009; L. K. Mackay et al., 2012; John R. Webb et al., 2014), and other reports suggested that anti-PD-1 response was independent of circulating T cell subsets (Spranger et al., 2014). We showed that this phenotype was strongly associated with patient overall survival in the treatment-naïve setting and that this association was stronger than total CD8 T cells in the tumor (Figure 1). CD8 T cell densities in the TME had already been associated with overall survival and anti-PD-1 response (Tumeh et al., 2014), however our data supported the notion that particular CD8 T cell phenotypes might be more protective and relevant as targets in anti-PD-1 therapy. Since then, a plethora of studies have explored the prognostic potential of CD103+ CD69+ (+/- CD49a) Trm in breast cancer (Egelston et al., 2019; Savas et al., 2018; Z. Q. Wang et al., 2016), bladder cancer (Hartana et al., 2018; B. Wang et al., 2015), cervical cancer and endometrial cancer (Komdeur et al., 2017; Workel et al., 2016), head and neck (Duhen et al., 2018), oropharyngeal squamous cell carcinoma (Hewavisenti et al., 2020), and others, each highlighting the superior prognostic and functional capacity of this subset and their importance in tumor control. There are a few potential explanations for why CD103+ CD8 T cells are a superior subset over total CD8 T cells. Firstly, CD103+ CD8 T cells likely encompass a higher proportion of tumor-antigen specific clones compared to other CD8 subsets in the tumor microenvironment. Using tetramer staining with the MART-1 antigen in melanoma, we showed that all MART-1 specific T cell clones had a resident phenotype. In lung and urothelial/glioma cancers, similar techniques revealed higher proportions of CD103+

T cells comprising antigen specific clones (Djenidi et al., 2015; B. Wang et al., 2015) compared to CD103- CD8 T cell populations. This is relevant because it is now understood that significant bystander CD8 T cell populations exist in tumors that are specific for non-tumor antigens and are therefore unlikely to provide any functional benefit for tumor control (Simoni et al., 2018). PD-1+ CD8+ T cells are thought to represent clonally expanded tumor reactive populations (Gros et al., 2014), and we, along with others (Djenidi et al., 2015; Savas et al., 2018; J. R. Webb, Milne, & Nelson, 2015), have shown that CD103+ CD8 T cells express the highest levels of PD-1, providing further evidence that these cells are enriched for clones specific to tumor antigen. CD39 has been implicated as a marker that phenotypes tumor specific T cell clones (Duhen et al., 2018), and resident CD103+ CD8 T cells are enriched for this marker (Duhen et al., 2018; Lin et al., 2020). Tumor resident CD8 T cells might also be superior because of their location within tumors. Tumor resident CD8 T cells have been found in various cancers to have a bias towards residing intratumorally rather than within the stroma surrounding tumors (Egelston et al., 2019; B. Wang et al., 2015; Z. Q. Wang et al., 2016; John R. Webb et al., 2014; Workel et al., 2016). This is significant given that CD8 T cell infiltration into tumoral regions is more prognostic than CD8 T cells located in stromal regions (Egelston et al., 2019; Workel et al., 2016). Some have postulated that the superior prognostic potential of tumor resident CD8 T cell is solely based on location rather than function (Egelston et al., 2019), however there is also evidence to suggest that these cells might possess unique functional capabilities that help them outperform their non-resident counterparts in the tumor microenvironment. For example, single cell sequencing of PD-1 expressing tumor resident CD8+ T cells in lung cancer revealed that they were enriched for transcripts linked to cytotoxicity compared to PD-1 expressing non-tumor resident T cells (Clarke et al., 2019). Other studies corroborate these findings (Ganesan et al., 2017; Komdeur et al., 2017), and we along with others have shown that CD103+ resident CD8 T cells have high expression of Granzyme B (Djenidi et al., 2015; Savas et al., 2018) and low DNA methylation of the gene locus involved in perforin expression (Hartana et al., 2018). Besides antigen specificity, the molecular expression of CD103 on T lymphocytes may be functionally important for the killing of tumour cells, as binding of CD103 to E-cadherin on tumour cells was shown to enhance TCR-mediated tumour cell lysis by triggering lytic granule polarisation and exocytosis (Le Floc'h et al., 2011, 2007). E- cadherin is expressed on melanoma tumours (Tang et al., 1994) and it is possible that CD103+ CD8+ tumour resident populations employ such mechanisms to facilitate better tumour killing and tumour control compared to CD103- counterparts. Although we did not explore E-cadherin expression and correlate with tumor-resident T cell densities or patient outcome, others have since shown that loss of E-cadherin in melanoma leads to reduced CD103 anti-tumor activity and increased tumor growth (Shields et al., 2019). Other studies, demonstrate that CD103+ tumor resident CD8 T cells may also possess a unique metabolic program that allows them to persist and withstand the harsh tumor microenvironment, which may also partially explain their enhanced anti-tumor functionality. For example, in skin, resident CD8 T cells were shown to preferentially utilize exogenous lipids via Fabp4/Fabp5 for FAO and cell survival over glucose (Pan et al., 2017). More recently, this was also confirmed to be the case in gastric adenocarcinoma (Lin et al., 2020). Many cancers are deprived of glucose, and therefore this would be an environment conducive to the tumor resident phenotype. Further work is warranted to unravel the unique metabolic features of tumor resident CD8 T cells to better understand metabolic programs that provide them a competitive edge in the TME. Lastly, there is also evidence to suggest that tumor resident CD8+ T cells can amplify CD8 anti-tumor immunity through crosstalk with dendritic cells and improve survival (Menares et al., 2019).

Another significant finding from this thesis is that CD103+ tumor resident CD8 T cells were found to expand early during treatment with anti-PD-1 monotherapy in metastatic melanoma patient tumors. This expansion occurred in both responding and non-responding patients, and though not statistically significant (probably due to sample size), the magnitude of this expansion trended higher in responding patients to anti-PD-1 therapy compared to non-responders. Since our finding, other studies have confirmed these results and provide further evidence that resident T cells are a likely target of PD-1/PD-L1 inhibition and expand during immunotherapy. Indeed, in esophageal cancer resident CD8 T cell populations were seen to expand more significantly than total CD8 T cell populations during PD-L1 blockade (Han et al., 2020). In gastric cancer non-responding patient tumors to anti-PD-1 blockade had very low percentages of CD8 T cells expressing a resident phenotype when compared to responding patient tumors (Lin et al., 2020). Single cell sequencing in lung cancer models showed that the PD-1+ TIM-3+ tumor resident CD8 T cell subset was enriched in responders to anti-PD-1 compared to non-responders (Clarke et al., 2019). We also showed that the PD-1+ TIM-3+ subset was enriched in CD103+ tumor resident CD8 T cells. Again, there are several reasons why tumor resident CD8 T cells are a target of anti-PD-1 monotherapy. The most obvious one is that they are enriched for the target receptor PD-1. Anti-PD-1 reinvigorates T cells through the disruption of PD-1/PD-L1 ligand interactions, and therefore it is very likely that reinvigorated and expanded T cell phenotypes are those that express PD-1. Initially, PD-1+ TILs were labelled exhausted based on findings that they were unable to produce cytokines or perform effector functions (Ahmadzadeh et al., 2009). However, it is now clear that tumor resident CD8 T cells are not exhausted in many respects, and therefore have the capacity to be functionally enhanced during anti-PD-1 blockade. We showed that CD103+ tumor resident CD8 T cells expressed an effector memory phenotype (CD45RA-CCR7-) over a terminally differentiated phenotype (CD45RA+ CCR7-), suggesting proliferative and functional capacity. Djennidi and colleagues demonstrated that CD103+ CD69+ CD8+ T cells stimulated with rIL-2 in the presence of autologous tumor expressed higher levels of granzyme B and CD107, markers of cytotoxic potential, compared to their CD103- CD8+ counterparts (Djenidi et al., 2015). Others showed that CD103+ PD-1+ T cells were quiescent when assessed ex vivo with autologous tumour cells, but upon stimulation, produced robust production of TNF-alpha and IFN-gamma, demonstrating that these cells were still capable of effector function (J. R. Webb et al., 2015). More recently, single cell sequencing in lung cancer revealed that tumor resident CD8 T cells were enriched for effector cytokines (IFN-gamma, TNF-alpha, and IL-2), proliferation markers (Ki67) and cytotoxic granules (Granzyme B) compared to CD103- non-resident phenotypes (Clarke et al., 2019). Other studies corroborate many aspects of these findings (Ganesan et al., 2017; Komdeur et al., 2017; Savas et al., 2018), while others show no dysfunctional impairment in tumor resident CD8 T cells compared to other CD8 T cell subsets in the TME (Egelston et al., 2019). While PD-1+ CD8 T cells appear to retain function, there is less evidence to support TIM-3+ PD-1+ subsets as a non-dysfunctional population. Indeed, PD-1+ TIM-3+ subsets have been shown in multiple settings to correspond more closely with the "exhausted" phenotype (Fourcade et al., 2010; Jin et al., 2010; Sade-Feldman et al., 2018). However, we and others have shown that PD-1+TIM-3+ tumor resident CD8 T cells comprise a relatively small proportion of PD-1+ tumor resident CD8 T cells, indicating that the vast majority are probably not exhausted and can regain function. Lastly, because tumor resident CD8 T cells utilize exogenous lipids and FAO for their homeostasis and survival, it is possible that anti-PD-1 therapy (which alters the nutrient availability in the TME) selectively enhances resident cells. Indeed, a recent study showed that PD-L1 blockade reduced Fabp4/Fabp5 transporters on tumor cells, decreasing tumor exogenous lipid uptake, while simultaneously increasing tumor resident Fabp4/Fabp5 transporter expression and uptake, providing another

potential mechanism of response for resident T cells during anti-PD-1 immunotherapy (Lin et al., 2020).

It is important to recognise that tumor resident CD8 T cells are unlikely to be the only target of anti-PD-1 immunotherapy. Within this thesis we have also shown that TCF7+ CD8 T cells in tumors at baseline strongly predict response to combination anti-CTLA-4 + anti-PD-1 therapy (Figure 1). While this was in the context of failure to anti-PD-1 monotherapy, previous studies have shown that this cellular phenotype corresponds to response to anti-PD-1 monotherapy. Indeed, CXCR5+ T cells, which were marked by PD-1 and TCF7 expression, were shown to provide the proliferative burst to anti-PD-1 therapy during chronic viral infection (Im et al., 2016). These cells expressed co-stimulatory markers (ICOS and CD28) and were shown to have self-renewal capacity. Later, single cell sequencing and multiplex IHC in melanoma revealed that responding tumors to both anti-PD-1 monotherapy had higher proportions of CD8 T cells comprising the TCF7+ phenotype compared to non-responders (Sade-Feldman et al., 2018). Once again this cellular phenotype had properties of self-renewal, high expression of the IL-7 receptor, and low expression of co-inhibitory receptors (Sade-Feldman et al., 2018). Combined with our findings, this clearly demonstrates that this is a key phenotype targeted and associated with response to immunotherapy. TCF7+ T cells also express the target receptor PD-1, but more importantly they represent one of the most undifferentiated memory T cell phenotypes (Gattinoni et al., 2011), making it an ideal target population to provide a burst of CD8 T cell phenotypes in the TME. One potential conundrum with having identified both TCF7+ CD8 T cells and dysfunctional-like CD103+ resident CD8 T cell phenotypes as targets to anti-PD-1 therapy is the fact that the two phenotypes are often considered mutually exclusive and opposing. Indeed, resident CD103+ CD8 T cells have lower expression of TCF7 as a subset and TCF7 negatively regulates tissue resdient memory T cell development (J. Wu et al., 2020), while TCF7+ CD8 T cells tend to have lower expression of co-inhibitory receptors and the marker CD39 (enriched in the resident phenotype) (Sade-Feldman et al., 2018). Thus, the two phenotypes are likely distinct. Which then, is the true target of anti-PD-1 immunotherapy? Is it the stem-cell like TCF7+ CD8 T cell population, or the tumor resident CD8 T cell population? Is one more important than the other for response? This thesis has not directly addressed these questions, however, inferences can be made based on our data as well as emerging data from other studies. It is very likely that TCF7+ CD8 T cells and tumor resident CD103+ CD8 T cells represent the same tumor reactive T cell clonotype but at different points in space and time. Thus it is likely that they are both critical in immunotherapy response but for different reasons and points in time. TCF7+ CD8 T cells (being the most undifferentiated memory phenotype) probably predict response at baseline because they have the greatest proliferative potential as well as the greatest capacity to drive the differentiation of other ciritical immune phenotypes (Eberhardt et al., 2021; Im et al., 2016; Kurtulus et al., 2019; Siddiqui et al., 2019). Indeed, TCF7+ CD8 T cells were shown to proliferate during anti-PD-1 immunotherapy into TCF7+ and TCF7- CD8 T cells (Siddiqui et al., 2019), suggesting that anti-PD-1 initiated response through the proliferation and differentiation of stem-cell like subsets rather than the reversal of exhausted-like subsets. We have also shown that TCF7+ CD8 T cells during anti-PD-1 therapy decrease as a proportion of total CD8 T cells, while other phenotypes, particulalrly the resident phenotyope, expand. Thus it is possible that the expansion of resident CD103+ CD8 T cells in responding patient tumors, early during or post therapy, is largely a consequence of the proliferation and differentation of stem-cell like cells. This might explain why the association of CD103+ resident CD8 T cells with anti-PD-1 response has come from the observation of their expansion in treatment samples rather than their use as a prediction tool at baseline. However, tumor resident CD8 T cells, as a whole, are not terminally differentiated and do possess proliferative potential (although not

as much as stem cell -like cells), and therefore they are likely to also respond de-novo to anti-PD-1. Thus it is possible that both resident self-renewal and stem cell-like differentiation contribute to the expansion of resident CD8 T cells, and future studies will need to dilineate the exact contribution of each. What is more clear, is that TCF7+ CD8 T cells are unlikley to provide direct anti-tumor functions. We showed that TCF7+ CD8 T cells are located further away from tumor cells than their TCF7- CD8 T cell counterparts. Others have shown that they reside preferentially in tertiary lymphoid structures and stromal regions rather than in tumor crests (Eberhardt et al., 2021). Furthermore, TCF7+ CD8 T cells, as yet, have not been associated with patient overall survival in the treatment-naïve setting, only as a predictor of immunotherapy response. Resident CD8 T cells, on the other hand, express cytotoxic markers, are located closer to tumor cells, are strongly associated with natural immunity and survival, and express molecular markers that may assist their anti-tumor functions (CD103); strongly suggesting their importance in the direct control and elimination of tumors. Indeed, one study showed that TNF-alpha production by tumor resident CD8 T cells was essential in maintaining a melanoma-immune equilibrium (S.L. Park et al., 2019). Together, these results suggest that while TCF7+ CD8 T cells are an important phenotype for initiating response to immunotherapy, CD103+ tumor resident CD8 T cells are critical for directing and carrying out anti-tumor functions that ultimately lead to tumor regression and long-term remission (through maintenance of anti-tumor immunity). Thus both immune cell phenotypes are critical for immunotherapy response and future studies will need to assess each as potential biomarkers to various clinical outcomes before, during, and post-immunotherapy.

As part of our aim to understand the factors responsible for the presence and absence of cellular targets in patient tumors, we explored a number of tumor intrinsic and extrinsic properties and correlated these with the densities of various T cell phenotypes in the tumor. We found that IL-15 levels in melanoma tumors was associated with the presence of CD103+ tumor resident CD8 T cells (Figure 1). Indeed, this was also true for total CD8 T cells, such that tumors with higher IL-15 expression contained higher densities of tumor resident and total CD8 T cells. This is an important finding because it suggests that one way to improve anti-tumor immunity and immunotherapy responses in patient non-responders is to utilise therapeutic strategies that bolster growth and self-renewal signals important for resident CD8 T cells. IL-15 is a critical cytokine for the generation and maintenance of tissue resident memory T cells in skin (L. Mackay et al., 2013) and other organs (Takamura, 2018; Zheng & Wakim, 2021). However we are the first to show that this is likely the case in tumors as well. We and others have also shown that IL-15 itself, is a prognostic feature in melanoma (Figure 1) and colorectal cancer (Mlecnik et al., 2014), and it is probable that its prognostic effect is due to its association with critical anti-tumor phenotypes, such as the resident CD8 T cell phenotype. IL-15 is thought to be derived mainly from myeloid/dendtric cell populations in the TME (Santana Carrero et al., 2019) and STING agonists have been shown to increase IL-15 expression in these populations (Santana Carrero et al., 2019). STING agonists are currently being combined with anti-PD1 in clinical trials (Le Naour, Zitvogel, Galluzzi, Vacchelli, & Kroemer, 2020) and it will be interesting to see whether this improves responses in patients resistant to anti-PD-1 monotherapy. Within this thesis we also explored the role of tumor mutation burden (TMB) on the presence of tumor-resident CD8 T cells in melanoma. Interestingly, relatively high TMB in cutaneous melanoma tumors was not correlated with higher CD103+ tumor resident CD8 T cells. We also showed that the prognostic effect of CD103+ resident CD8 T cells on overall patient survival (treatment-naïve) was independent of TMB. This suggests that TMB is not a driving factor for protective anti-tumor immunity in cutaneous melanoma. While TMB has been associated with immunotherapy response, it has also recently been shown to be independent of CD8 T cell densities and independent of a prognostic IFN-gamma signature

(Newell et al., 2021), suggesting that the prognostic value of TMB may not be a result of generating important immunotherapy targets in pre-treatment samples. There are many reasons why TMB may not correlate with protective anti-tumor immunity. For example, other sources of antigen including cancer associated antigens (cancer testes antigens) and viral/bacterial antigen expression by tumor cells may be important but unaccounted for in models to predict neoantigens. It may also simply reflect the fact that there are many more important driving factors that predict robust anti-tumoral responses in tumors. Interestingly, we did find that acral and mucosal melanoma subtypes (which have low TMB and are less responsive to checkpoint blockade) had lower densities of CD8 T cells, including the tumor-resident CD8 T cell phenotype. However, the densities of these cell types did not correlate with TMB or other genomic factors (chromosomal structural abberations), suggesting that other immunospressive mechanisms are probbaly responsbile for the reduced T cell infiltrate in these tumors as a whole compared to cutaneous tumors. Lastly, with regards to TCF7+ CD8 T cell populations, we have shown that their presence is strongly correlated with CD4 T cells as a proportion of total CD3+ T cells. Indeed, in pre-treatmemt patient melanoma biopsies higher percentages of CD4 T cells (of CD3) and lower percentages of CD8 T cells (of CD3) correlated with a higher proportion of TCF7+ CD8+ T cells (of total CD8) (Figure 1). Since TCF7+ CD8 T cells are strongly predictive of anti-PD-1 immunotherapy response, this suggests that CD4 T cells have a role in regulating CD8 phenotypes necessary for immunotherapy response. Future studies are warranted to understand the exact mechansims responsible for the generation of TCF7+ CD8 T cells.

The most utilised and clinically tested methods for overcoming resistance to anti-PD-1 immunotherapy is the addition of anti-CTLA-4 or alternative checkpoint inhibitors/ co-stimulatory agnosists. As part of this thesis, we aimed to understand the tumor microenvironment of anti-PD-1 non-responders as well as provide a foundation for the use of biomarkers in such patient cohorts. In a particularly rare cohort of melanoma patients who failed first-line anti-PD-1 therapy and went on to receive second-line combination therapy, we profiled melanoma patient tumors at pre-treatment, post anti-PD-1 treatment, and post anti-CTLA-4 + anti-PD-1 therapy. We found that these patient tumors did contain CD8 TILs (known progostic feature in anti-PD-1 immunotherapy) and robust expression of MHC-class 1 (often associated with immunotherapy resistance), indicating the existence of an anti-tumor response capable of recognising and interacting with tumors, pointing to other resistance mechanisms. Upon investigation of specific CD8 TIL types, we discovered low proportions of CD103+ tumor resident CD8 T cells and high proportions of TCF7+ CD8 TILs (Figure 1). This is relevant for two reasons. Firstly, the absence of protective CD103+ tumor resident CD8 T cells likely indicates that the quality of the anti-tumor response is poor in these patients pre-therapy, which although broad, partially may explain non-response to anti-PD-1 monotherapy. This is reinforced by our observation that post-combination therapy, resident CD8 T cells percentages normalised in responding patients. Secondly, TCF7+ CD8 TILs have been proposed as a precise marker for determining response to anti-PD-1 therapy (Sade-Feldman et al., 2018). All non-responding tumors to ant-PD-1 had low percentages of TCF7+ CD8 TILs, and any discordant patient tumors were explained by the absence of MHC-class 1 expression or mutations in the IFN-gamma pathway (Sade-Feldman et al., 2018). Our results clearly show however, that despite high proportions of TCF7+ CD8 T cells and intact antigen presentation pathways, other mechanisms of resistance exist, which may make TCF7+ CD8 TILs an imperfect marker for prediciting anti-PD-1 reponse in a subset of patients. Upon further examination of the TME of these patient tumors, we observed that Tregs and CD4 T cells were located closer to TCF7+ CD8 TILs than their TCF7- CD8 TIL counterparts, with many direct interactions observed. It is tempting to speculate that the addition of anti-CTLA-4 enhances

CD4 T cell helper activity on this subset or dampens regulatory T cell supressive functions on the TCF7+ CD8 T cell phenotype, consistent with the mechanism of action for anti-CTLA-4 (Binnewies et al., 2019; Wei et al., 2017). Indeed, a recent study showed that sequential treatment with anti-CTLA-4 and then anti-PD-1 was more effective than anti-PD-1 alone in murine models (Eschweiler et al., 2021). The mechansim for this improved efficacy towards anti-PD-1 was found to be through the depletion of intratumoral follicular-like regulatory T cells with potent supressive functions (Eschweiler et al., 2021). Given we and others have shown that TCF7+ CD8 T cells are located further away from tumors and within tertiary lymphoid structures, it is possible that the regulatory T cells with close proximity to TCF7+ CD8 T cells represent the same follicular-like regulatory T cells described. Future studies will need to examine the functional relevance of CD4 and Treg cells on TCF7+ CD8 T cell subsets. Despite these data gaps, however, we have shown that the TCF7+ CD8 T cell subset may be a useful biomarker of response to patients receiving combination anti-CTLA-4 + anti-PD-1 after anti-PD-1 monotherapy failure, providing a significant step forward in helping clinicians limit the toxicity of combination therapy to patients that are likely to derive benefit from it.

Lastly, this thesis explored the expression profile of alternative checkpoint receptors in primary, regional, and distant metastatic melanoma tumors, providing the first comprehensive evaluation of these markers in melanoma. One interesting finding as it pertains to immunotherapy response and resistance is the fact that PD-1 negative melanoma tumors had significantly lower expression of alternative checkpoint receptors in the TME compared to PD-1 positive tumors. While PD-L1 and PD-1 expression are imperfect biomarkers of anti-PD-1 response, higher PD-1 expression at baseline has been associated with positive clinical outcomes (Gide et al., 2019; Tumeh et al., 2014), suggesting that patients with low anti-PD-1 expression and which exhibit non-response to anti-PD-1 therapy may be less responsive to alternative checkpoint inhibitors compared to patient tumors with PD-1 expression. We also showed that co-inhibitory receptors were more abundant in the TME compared to co-stimulatory receptors at all stages of disease, except for the co-stimualtory receptor ICOS. Indeed the co-stimulatory receptors GITR and OX-40 were minimally expressed in the TME and were largely restricted to regulatory T cells. If marker expression does correlate with responsiveness (as is the case for some drug targets) this may suggest that the target populations are too small to cause significant clinical benefit. Anti-CTLA-4 is thought to partially work through the depletion of T-regs (Simpson et al., 2013), and therefore it remains to be determined what added benefit would be derived from targeting receptors largely restricted to this immune subset. Interestingly, our data also suggests that the use of some targets may be better suited at earlier stages of disease (GITR expression higher in primary compared to distant metatstatic disease). Indeed, preclinical models with co-stimulatory agonists have shown that timing and sequence of therapies may be ciritical for efficacy (Messenheimer et al., 2017). In terms of the expression profile of targets on immune cell subsets, we found that TIGIT had a very similar expression profile to PD-1, with enrichment on CD103+ tumor resident CD8 T cells and regulatory T cells. Recently the importance of the CD155/TIGIT axis has been highlighted in models of pancreatic cancer and show that CD155/TIGIT is sufficient to cause immune evasion (Freed-Pastor et al., 2021). Indeed, combination anti-PD-1+anti-TIGIT+ CD40 agonisism initiated complete responses in a subset of pre-clinical models, whereas monotherapies did not (Freed-Pastor et al., 2021). One hypothesis as it relates to the targeting of additional checkpoint receptors is that these additional targets are likley to be redundant. Certainly this would seem more probable if the receptors are enriched on similar anti-PD-1 target cells. However, the above study suggests otherwise. Indeed, an alternative hypothesis termed the “alternative checkpoint theory” states that the inhibtion of anti-PD-1 alone may be not be sufficient to rescue T cell functionality and that the inhibition of alternative checkpoint

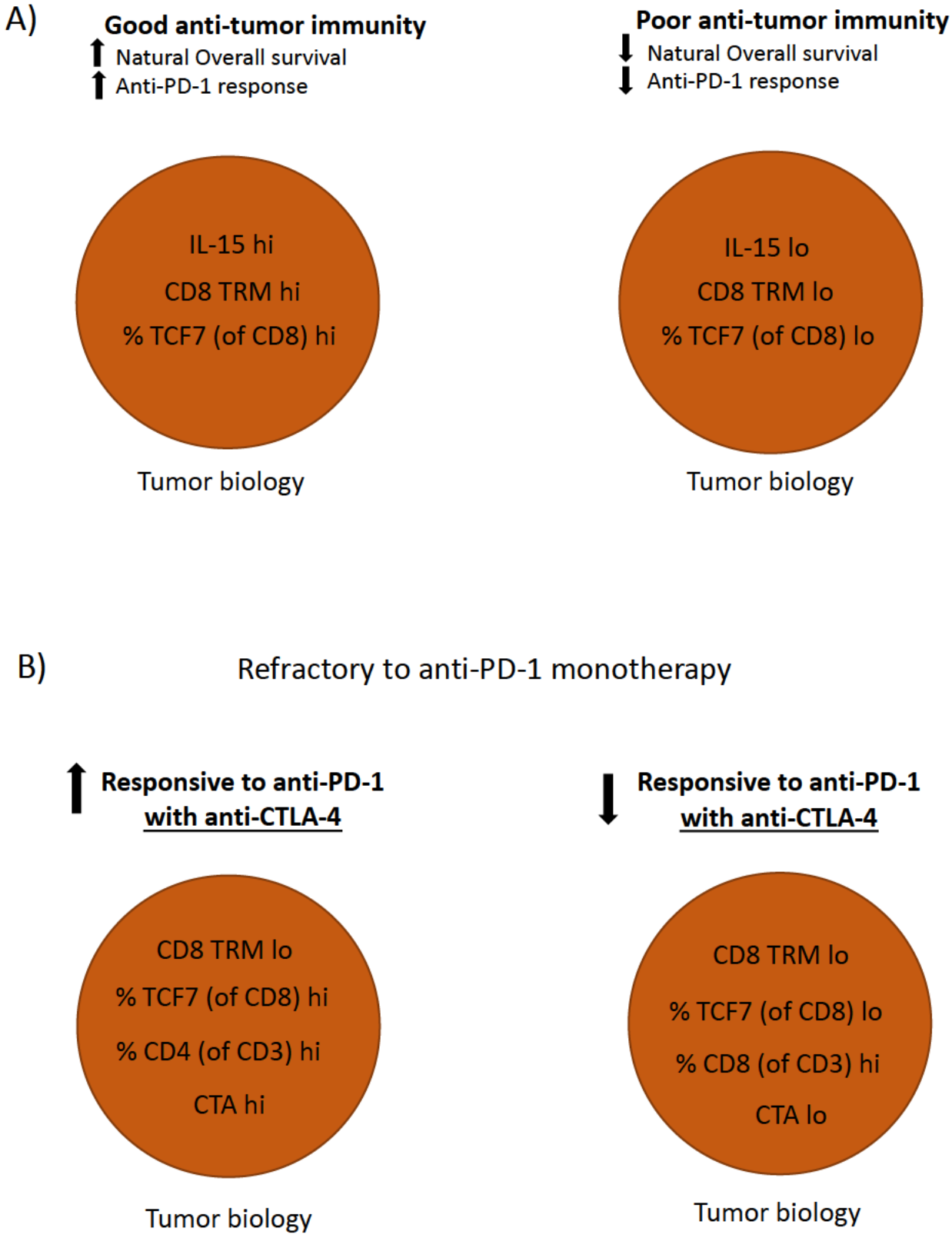
receptors may be required. We have shown that LAG-3 is also enriched on the tumor-resident CD8 T cell phenotype, and recent phase 3 trails show that combination anti-PD-1+ anti-LAG-3 provides superior PFS in melanoma compared to anti-PD-1 monotherapy (Lipson et al., 2021). However, the differences appear to be modest, and this may suggest that inhibition of alternative checkpoint receptors only rescues a small subset of PD-1 non-responders. Our data would suggest that it is likely to be PD-1 non-responders with T cell infiltration, and perhaps T cells expressing alternative markers, such as the resident phenotype. It is important to recognise that not all targets are expressed predominantly by T cells. We have shown that TIM-3 and VISTA are expressed largely on dendritic and myeloid cells, respectively, and therefore there is scope for these alternative checkpoint inhibitors to modulate innate immune phenotypes, including MDSCs, which are emerging as a significant mechanism of resistance to anti-PD-1. Nevertheless, our data will serve as an important foundation for the optimal selection of targets for clinical trials, interpretation of early phase data, and development of predictive biomarkers.

In conclusion, this thesis has contributed to the field of cancer immunotherapy by identifying T cell subsets likely targeted by anti-PD-1 immunotherapy. We have phenotyped these cell subsets in melanoma, have explored their changes early during treatment, and correlated these with clinical response. We have also identified factors likley to be important and not important for the generation and maintenance of these phenotypes. This thesis has provided the first comprehensive study of the expression profile of novel checkpoint targets in melanoma and explored the tumor microenvironment of anti-PD-1 non-responders as well as biomarkers of response to subsequent combination anti-PD-1 + anti-CTLA-4 therapy.

Figure 1

Patient tumor characteristics associated with overall survival and response to single agent anti-PD-1 and combination anti-PD-1 + anti-CTLA-4 immunotherapies. A) Tumor biology of patient tumors associated with higher (left) and lower (right) overall survival in treatment-naïve setting and responsiveness to anti-PD-1 immunotherapy. B) Tumor biology of patients responsive (left) or unresponsive (right) to combination anti-PD-1+anti-CTLA-4 after failure on anti-PD-1 immunotherapy.

Figure 1


A)
Good anti-tumor immunity
Natural Overall survival
Anti-PD-1 response
IL-15 hi
CD8 TRM hi
% TCF7 (of CD8) hi
Tumor biology
Poor anti-tumor immunity
Natural Overall survival
Anti-PD-1 response
IL-15 lo
CD8 TRM lo
% TCF7 (of CD8) lo
Tumor biology
B)
Refractory to anti-PD-1 monotherapy
Responsive to anti-PD-1
with anti-CTLA-4
CD8 TRM lo
% TCF7 (of CD8) hi
% CD4 (of CD3) hi
CTA hi
Tumor biology
Responsive to anti-PD-1
with anti-CTLA-4
CD8 TRM lo
% TCF7 (of CD8) lo
% CD8 (of CD3) hi
CTA lo
Tumor biology

References

Ahmadzadeh, M., Johnson, L. a, Heemskerk, B., Wunderlich, J. R., Dudley, M. E., White, D. E., … Dc, W. (2009). Tumor antigen − specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired Tumor antigen – specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood*, *114*(8), 1537–1544. https://doi.org/10.1182/blood-2008-12-195792

Ahmadzadeh, M., & Rosenberg, S. A. (2006). IL-2 administration increases CD4+ CD25(hi) Foxp3+ regulatory T cells in cancer patients. *Blood*, *107*(6), 2409–2414. https://doi.org/10.1182/blood-2005-06-2399

Akbani, R., Akdemir, K. C., Aksoy, B. A., Albert, M., Ally, A., Amin, S. B., … Zou, L. (2015). Genomic Classification of Cutaneous Melanoma. *Cell*, *161*(7), 1681–1696. https://doi.org/10.1016/j.cell.2015.05.044

Alexandrov, L. B., Nik-zainal, S., Wedge, D. C., & Aparicio, S. A. J. R. (2013). Signatures of mutational processes in human cancer. *Nature*, *500*(7463), 415–421. https://doi.org/10.1038/nature12477.Signatures

Angelin, A., Gil-de-Gómez, L., Dahiya, S., Jiao, J., Guo, L., Levine, M. H., … Beier, U. H. (2017). Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metabolism*, *25*(6), 1282-1293.e7. https://doi.org/10.1016/j.cmet.2016.12.018

Antony, R., Emery, C. M., Sawyer, A. M., & Garraway, L. A. (2013). C-RAF mutations confer resistance to RAF inhibitors. *Cancer Research*, *73*(15), 4840–4851. https://doi.org/10.1158/0008-5472.CAN-12-4089

Ariotti, S. (2014). Skin-resident memory CD8+ T cells trigger a stat of tissue-wide pathogen alert. *Science (New York, N.Y.)*, *101*(6205), 101. https://doi.org/10.1126/science.1254803

Arlauckas, S. P., Garris, C. S., Kohler, R. H., Kitaoka, M., Cuccarese, M. F., Yang, K. S., … Pittet, M. J. (2017). In vivo imaging reveals a tumor-associated macrophage-mediated resistance pathway in anti-PD-1 therapy. *Science Translational Medicine*, *9*(389). https://doi.org/10.1126/scitranslmed.aal3604

Atkins, M. B., Lotze, M. T., Dutcher, J. P., Fisher, R. I., Weiss, G., Margolin, K., … Rosenberg, S. A. (1999). High-Dose Recombinant Interleukin 2 Therapy for Patients With Metastatic Melanoma: Analysis of 270 Patients Treated Between 1985 and 1993. *Journal of Clinical Oncology*, *17*(7), 2105. https://doi.org/10.1200/JCO.1999.17.7.2105

Australian Institute of Health and Welfare. (2021). *Cancer in Australia 2021* (Cancer ser). Canberra: AIHW.

Ayers, M., Lunceford, J., Nebozhyn, M., Murphy, E., Loboda, A., Kaufman, D. R., … McClanahan, T. K. (2017). IFN-γ-related mRNA profile predicts clinical response to PD-1 blockade. *Journal of Clinical Investigation*, *127*(8), 2930–2940. https://doi.org/10.1172/JCI91190

Azimi, F., Scolyer, R. A., Rumcheva, P., Moncrieff, M., Murali, R., McCarthy, S. W., … Thompson, J. F. (2012). Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. *Journal of Clinical Oncology*, *30*(21), 2678–2683. https://doi.org/10.1200/JCO.2011.37.8539

Bai, A., Hu, H., Yeung, M., & Chen, J. (2007). Kruppel-like factor 2 controls T cell trafficking by activating L-selectin (CD62L) and sphingosine-1-phosphate receptor 1 transcription. *Journal of Immunology (Baltimore, Md. : 1950)*, *178*(12), 7632–7639.

Balatoni, T., Mohos, A., Papp, E., Sebestyén, T., Liszkay, G., Oláh, J., … Ladányi, A. (2018). Tumor-infiltrating immune cells as potential biomarkers predicting response to treatment and survival in patients with metastatic melanoma receiving ipilimumab therapy. *Cancer Immunology, Immunotherapy*, *67*(1), 141–151. https://doi.org/10.1007/s00262-017-2072-1

Ballbach, M., Dannert, A., Singh, A., Siegmund, D. M., Handgretinger, R., Piali, L., … Hartl, D. (2017). Expression of checkpoint molecules on myeloid-derived suppressor cells. *Immunology Letters*, *192*, 1–6. https://doi.org/10.1016/j.imlet.2017.10.001

Bankovich, A. J., Shiow, L. R., & Cyster, J. G. (2010). CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4. *Journal of Biological Chemistry*, *285*(29), 22328–22337. https://doi.org/10.1074/jbc.M110.123299

Barsoum, I. B., Smallwood, C. A., Siemens, D. R., & Graham, C. H. (2014). A mechanism of hypoxia-mediated escape from adaptive immunity in cancer cells. *Cancer Research*, *74*(3), 665–674. https://doi.org/10.1158/0008-5472.CAN-13-0992

Bartolomé-Casado, R., Landsverk, O. J. B., Chauhan, S. K., Richter, L., Phung, D., Greiff, V., … Jahnsen, F. L. (2019). Resident memory CD8 T cells persist for years in human small intestine. *Journal of Experimental Medicine*, *216*(10), 2412–2426. https://doi.org/10.1084/jem.20190414

Berele, B. A., Cai, Y., & Yang, G. (2021). Prognostic Value of Tumor Infiltrating Lymphocytes in Nasopharyngeal Carcinoma Patients: Meta-Analysis. *Technology in Cancer Research & Treatment*, *20*, 1–13. https://doi.org/10.1177/15330338211034265

Binnewies, M., Mujal, A. M., Pollack, J. L., Combes, A. J., Hardison, E. A., Barry, K. C., … Krummel, M. F. (2019). Unleashing Type-2 Dendritic Cells to Drive Protective Antitumor CD4+ T Cell Immunity. *Cell*, *177*(3), 556-571.e16. https://doi.org/10.1016/j.cell.2019.02.005

Blank, C. U., Reijers, I. L. M., Pennington, T., Versluis, J. M., Saw, R. P. M., Rozeman, E. A., … Long, G. V. (2020). First safety and efficacy results of PRADO: A phase II study of personalized response-driven surgery and adjuvant therapy after neoadjuvant ipilimumab (IPI) and nivolumab (NIVO) in resectable stage III melanoma. *Journal of Clinical Oncology*, *38*(15_suppl), 10002. https://doi.org/10.1200/JCO.2020.38.15_suppl.10002

Blattman, J. N., Antia, R., Sourdive, D. J. D., Wang, X., Kaech, S. M., Murali-Krishna, K., … Ahmed, R. (2002). Estimating the precursor frequency of naive antigen-specific CD8 T cells. *The Journal of Experimental Medicine*, *195*(5), 657–664. https://doi.org/10.1084/jem.20001021

Bohn, T., Rapp, S., Luther, N., Klein, M., Bruehl, T. J., Kojima, N., … Bopp, T. (2018). Tumor immunoevasion via acidosis-dependent induction of regulatory tumor-associated macrophages. *Nature Immunology*, *19*(12), 1319–1329. https://doi.org/10.1038/s41590-018-0226-8

Boon, T., Coulie, P. G., & Van Den Eynde, B. (1997). Tumor antigens recognized by T cells. *Immunology Today*, *18*(6), 267–268. https://doi.org/10.1016/S0167-5699(97)80020-5

Boutet, M., Gauthier, L., Leclerc, M., Gros, G., De Montpreville, V., Theret, N., … Mami-Chouaib, F. (2016). TGFβ signaling intersects with CD103 integrin signaling to promote T-Lymphocyte accumulation and antitumor activity in the lung tumor microenvironment. *Cancer Research*, *76*(7), 1757–1769. https://doi.org/10.1158/0008-5472.CAN-15-1545

Brand, A., Singer, K., Koehl, G. E., Kolitzus, M., Schoenhammer, G., Thiel, A., … Kreutz, M. (2016). LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells. *Cell Metabolism*, *24*(5), 657–671.

https://doi.org/10.1016/j.cmet.2016.08.011
Brandenburg, S., Takahashi, T., de la Rosa, M., Janke, M., Karsten, G., Muzzulini, T., … Scheffold, A. (2008). IL-2 induces in vivo suppression by CD4(+)CD25(+)Foxp3(+) regulatory T cells. *European Journal of Immunology*, *38*(6), 1643–1653. https://doi.org/10.1002/eji.200737791
Buchan, S. L., Dou, L., Remer, M., Booth, S. G., Dunn, S. N., Lai, C., … Beers, S. A. (2018). Antibodies to Costimulatory Receptor 4-1BB Enhance Anti-tumor Immunity via T Regulatory Cell Depletion and Promotion of CD8 T Cell Effector Function. *Immunity*, *49*(5), 958-970.e7. https://doi.org/10.1016/j.immuni.2018.09.014
Budhu, S., Schaer, D. A., Li, Y., Toledo-Crow, R., Panageas, K., Yang, X., … Wolchok, J. D. (2017). Blockade of surface-bound TGF-β on regulatory T cells abrogates suppression of effector T cell function in the tumor microenvironment. *Science Signaling*, *10*(494), eaak9702. https://doi.org/10.1126/scisignal.aak9702
Cahill, B. Y. R. N. P., Poskitt, D. C., Frost, H., & Trnka, Z. (1977). TWO DISTINCT POOLS OF R E C I R C U L A T I N G T LYMPHOCYTES : MIGRATORY CHARACTERISTICS OF NODAL AND I N T E S T I N A L T L Y M P H O C Y T E S Thymus-derived (T) lymphocytes display considerable functional heterogene- ity , but although it has been. *The Journal of Experimental Medicinehe Journal of Experimental Medicine*, *145*, 420–428.
Carlson, C. M., Endrizzi, B. T., Wu, J., Ding, X., Weinreich, M. a, Walsh, E. R., … Jameson, S. C. (2006). Kruppel-like factor 2 regulates thymocyte and T-cell migration. *Nature*, *442*(7100), 299–302. https://doi.org/10.1038/nature04882
Casey, K. A., Fraser, K. A., Schenkel, J. M., Moran, A., Abt, M. C., Beura, L. K., … Masopust, D. (2012). Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. *Journal of Immunology (Baltimore, Md. : 1950)*, *188*(10), 4866–4875. https://doi.org/10.4049/jimmunol.1200402
Caudana, P., Nuñez, N. G., De La Rochere, P., Pinto, A., Denizeau, J., Alonso, R., … Piaggio, E. (2019). IL2/Anti-IL2 complex combined with CTLA-4, But Not PD-1, blockade rescues antitumor NK cell function by regulatory T-cell Modulation. *Cancer Immunology Research*, *7*(3), 443–457. https://doi.org/10.1158/2326-6066.CIR-18-0697
Cepek, K. L., Parker, C. M., Madara, J. L., & Brenner, M. B. (1993). Integrin aEb7 mediates adhesion of T lymphocytes to epithelial cells. *J.Immunol.*, *150*, 3450–3470.
Cepek, K., Shaw, S., Parker, C., Russel, G., Morrow, J., Rimm, D., & Brenner, M. (1994). © 19 9 4 Nature Publishing Group. *Nature*, *372*(10), 190–193.
Chang, C. H., Curtis, J. D., Maggi, L. B., Faubert, B., Villarino, A. V., O'Sullivan, D., … Pearce, E. L. (2013). Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell*, *153*(6), 1239. https://doi.org/10.1016/j.cell.2013.05.016
Chapman, P. B., Hauschild, A., Robert, C., Haanen, J. B., Ascierto, P., Larkin, J., … McArthur, G. A. (2011). Improved Survival with Vemurafenib in Melanoma with BRAF V600E Mutation. *New England Journal of Medicine*, *364*(26), 2507–2516. https://doi.org/10.1056/nejmoa1103782
Chen, Z., Ji, Z., Ngiow, S. F., Manne, S., Cai, Z., Huang, A. C., … Wherry, E. J. (2019). TCF-1-Centered Transcriptional Network Drives an Effector versus Exhausted CD8 T Cell-Fate Decision. *Immunity*, *51*(5), 840-855.e5. https://doi.org/10.1016/j.immuni.2019.09.013
Cheuk, S., Schlums, H., Gallais Sérézal, I., Martini, E., Chiang, S. C., Marquardt, N., … Eidsmo, L. (2017). CD49a Expression Defines Tissue-Resident CD8+ T Cells Poised for Cytotoxic Function in Human Skin. *Immunity*, *46*(2), 287–300. https://doi.org/10.1016/j.immuni.2017.01.009
Chiche, J., Ilc, K., Laferrière, J., Trottier, E., Dayan, F., Mazure, N. M., … Pouysségur, J.

(2009). Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. *Cancer Research*, *69*(1), 358–368. https://doi.org/10.1158/0008-5472.CAN-08-2470

Christian, L. S., Wang, L., Lim, B., Deng, D., Wu, H., Wang, X.-F., & Li, Q.-J. (2021). Resident memory T cells in tumor-distant tissues fortify against metastasis formation. *Cell Reports*, *35*(6), 109118. https://doi.org/10.1016/j.celrep.2021.109118

Christo, S. N., Evrard, M., Park, S. L., Gandolfo, L. C., Burn, T. N., Fonseca, R., … Mackay, L. K. (2021). Discrete tissue microenvironments instruct diversity in resident memory T cell function and plasticity. *Nature Immunology*, *22*(9), 1140–1151. https://doi.org/10.1038/s41590-021-01004-1

Clambey, E. T., McNamee, E. N., Westrich, J. A., Glover, L. E., Campbell, E. L., Jedlicka, P., … Eltzschig, H. K. (2012). Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. *Proceedings of the National Academy of Sciences*, *109*(41), E2784–E2793. https://doi.org/10.1073/pnas.1202366109

Clark, R. A., Watanabe, R., Teague, J. E., Schlapbach, C., Tawa, M. C., Adams, N., … Kupper, T. S. (2012). *Skin efefctor memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients*. *4*(117), 1–10. https://doi.org/10.1126/scitranslmed.3003008.Skin

Clarke, J., Panwar, B., Madrigal, A., Singh, D., Gujar, R., Wood, O., … Vijayanand, P. (2019). Single-cell transcriptomic analysis of tissue-resident memory T cells in human lung cancer. *Journal of Experimental Medicine*, *216*(9), 2128–2149. https://doi.org/10.1084/jem.20190249

Colegio, O. R., Chu, N. Q., Szabo, A. L., Chu, T., Rhebergen, A. M., Jairam, V., … Medzhitov, R. (2014). Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*, *513*(7519), 559–563. https://doi.org/10.1038/nature13490

Corzo, C. A., Condamine, T., Lu, L., Cotter, M. J., Youn, J.-I., Cheng, P., … Gabrilovich, D. I. (2010). HIF-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *The Journal of Experimental Medicine*, *207*(11), 2439–2453. https://doi.org/10.1084/jem.20100587

Curran, M. A., Montalvo, W., Yagita, H., & Allison, J. P. (2010). PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(9), 4275–4280. https://doi.org/10.1073/pnas.0915174107

Curti, A., Pandolfi, S., Valzasina, B., Aluigi, M., Isidori, A., Ferri, E., … Lemoli, R. M. (2007). Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25- into CD25+ T regulatory cells. *Blood*, *109*(7), 2871–2877. https://doi.org/10.1182/blood-2006-07-036863

Curti, B., & Faries, M. (2021). Recent advances in the treatment of melanoma. *The New England Journal of Medicine*, *384*(23), 2229–2240. https://doi.org/10.1056/nejmra2034861

D'Urso, C. M., Wang, Z., Cao, Y., Tatake, R., Zeff, R. A., & Ferrone, S. (1991). Lack of HLA class I antigen expression by cultured melanoma cells FO-1 due to a defect in B2m gene expression. *Journal of Clinical Investigation*, *87*(1), 284–292. https://doi.org/10.1172/JCI114984

Dagenborg, V. J., Marshall, S. E., Grzyb, K., Fretland, Å. A., Lund-Iversen, M., Mælandsmo, G. M., … Flatmark, K. (2021). Low Concordance Between T-Cell Densities in Matched Primary Tumors and Liver Metastases in Microsatellite Stable Colorectal Cancer.

Frontiers in Oncology, *11*(June), 1–9. https://doi.org/10.3389/fonc.2021.671629
Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., … Futreal, P. A. (2002). Mutations of the BRAF gene in human cancer. *Nature*, *417*(6892), 949–954. https://doi.org/10.1038/nature00766
Dennis, K. L., Blatner, N. R., Gounari, F., & Khazaie, K. (2013). Current status of interleukin-10 and regulatory T-cells in cancer. *Current Opinion in Oncology*, *25*(6), 637–645. https://doi.org/10.1097/CCO.0000000000000006
Desbois, M., Le Vu, P., Coutzac, C., Marcheteau, E., Béal, C., Terme, M., … Chaput, N. (2016). IL-15 Trans -Signaling with the Superagonist RLI Promotes Effector/Memory CD8 + T Cell Responses and Enhances Antitumor Activity of PD-1 Antagonists . *The Journal of Immunology*, *197*(1), 168–178. https://doi.org/10.4049/jimmunol.1600019
Dhatchinamoorthy, K., Colbert, J. D., & Rock, K. L. (2021). Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation. *Frontiers in Immunology*, *12*(March). https://doi.org/10.3389/fimmu.2021.636568
Dietl, K., Renner, K., Dettmer, K., Timischl, B., Eberhart, K., Dorn, C., … Kreutz, M. P. (2010). Lactic Acid and Acidification Inhibit TNF Secretion and Glycolysis of Human Monocytes. *The Journal of Immunology*, *184*(3), 1200–1209. https://doi.org/10.4049/jimmunol.0902584
Ding, L.-W., Sun, Q.-Y., Edwards, J. J., Fernández, L. T., Ran, X.-B., Zhou, S.-Q., … Koeffler, H. P. (2019). LNK suppresses interferon signaling in melanoma. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-09711-y
Djenidi, F., Adam, J., Goubar, A., Durgeau, A., Meurice, G., de Montpreville, V., … Mami-Chouaib, F. (2015). CD8+CD103+ Tumor-Infiltrating Lymphocytes Are Tumor-Specific Tissue-Resident Memory T Cells and a Prognostic Factor for Survival in Lung Cancer Patients. *J Immunol*, *194*(7), 3475–3486. https://doi.org/10.4049/jimmunol.1402711
Doedens, A. L., Rubinstein, M. P., Gross, E. T., Best, J. A., Craig, D. H., Baker, M. K., … Goldrath, A. W. (2016). Molecular programming of tumor-infiltrating CD8+ T cells and IL15 resistance. *Cancer Immunology Research*, 799–812. https://doi.org/10.1158/2326-6066.CIR-15-0178
Doedens, Andrew L., Phan, A. T., Stradner, M. H., Fujimoto, J. K., Nguyen, J. V., Yang, E., … Goldrath, A. W. (2013). Hypoxia-inducible factors enhance the effector responses of CD8 + T cells to persistent antigen. *Nature Immunology*, *14*(11), 1173–1182. https://doi.org/10.1038/ni.2714
Doedens, Andrew L., Stockmann, C., Rubinstein, M. P., Liao, D., Zhang, N., DeNardo, D. G., … Johnson, R. S. (2010). Macrophage expression of hypoxia-inducible factor-1α suppresses T-cell function and promotes tumor progression. *Cancer Research*, *70*(19), 7465–7475. https://doi.org/10.1158/0008-5472.CAN-10-1439
Doroshow, D. B., Bhalla, S., Beasley, M. B., Sholl, L. M., Kerr, K. M., Gnjatic, S., … Hirsch, F. R. (2021). PD-L1 as a biomarker of response to immune-checkpoint inhibitors. *Nature Reviews. Clinical Oncology*, *18*(6), 345–362. https://doi.org/10.1038/s41571-021-00473-5
Duhen, T., Duhen, R., Montler, R., Moses, J., Moudgil, T., De Miranda, N. F., … Weinberg, A. D. (2018). Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-05072-0
Dunn, G. P., Old, L. J., & Schreiber, R. D. (2004). The three Es of cancer immunoediting. *Annual Review of Immunology*, *22*(4), 329–360. https://doi.org/10.1146/annurev.immunol.22.012703.104803
Eberhardt, C. S., Kissick, H. T., Patel, M. R., Cardenas, M. A., Prokhnevska, N., Obeng, R.

C., … Ahmed, R. (2021). Functional HPV-specific PD-1+ stem-like CD8 T cells in head and neck cancer. *Nature*, *597*(7875), 279–284. https://doi.org/10.1038/s41586-021-03862-z

Egelston, C. A., Yuan, Y., & Lee, P. P. (2019). Resident memory CD8 + T cells within cancer islands mediate survival in breast cancer patients Graphical abstract Find the latest version : *Journal of Clinical Investigation*, *4*(19), 1–15.

El-Asady, R., Yuan, R., Liu, K., Wang, D., Gress, R. E., Lucas, P. J., … Hadley, G. A. (2005). TGF-β-dependent CD103 expression by CD8+ T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. *Journal of Experimental Medicine*, *201*(10), 1647–1657. https://doi.org/10.1084/jem.20041044

Elliot, T. A. E., Jennings, E. K., Lecky, D. A. J., Thawait, N., Flores-Langarica, A., Copland, A., … Bending, D. (2021). Antigen and checkpoint receptor engagement recalibrates T cell receptor signal strength. *Immunity*, *54*(11), 2481-2496.e6. https://doi.org/10.1016/j.immuni.2021.08.020

Emily, K., & Gershenwald, J. E. (2018). The eighth edition American Joint Committee on Cancer (AJCC) melanoma staging system: implications for melanoma treatment and care. *Expert Rev. Anticancer Therapy*, *18*(8), 775–784. https://doi.org/10.4049/jimmunol.1801473.The

Erdag, G., Schaefer, J. T., Smolkin, M. E., Deacon, D. H., Shea, S. M., Dengel, L. T., … Slingluff, C. L. J. (2012). Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Research*, *72*(5), 1070–1080. https://doi.org/10.1158/0008-5472.CAN-11-3218

Eschweiler, S., Clarke, J., Ramírez-Suástegui, C., Panwar, B., Madrigal, A., Chee, S. J., … Vijayanand, P. (2021). Intratumoral follicular regulatory T cells curtail anti-PD-1 treatment efficacy. *Nature Immunology*, *22*(8), 1052–1063. https://doi.org/10.1038/s41590-021-00958-6

Evrard, M., Wynne-Jones, E., Peng, C., Kato, Y., Christo, S. N., Fonseca, R., … Mackay, L. K. (2022). Sphingosine 1-phosphate receptor 5 (S1PR5) regulates the peripheral retention of tissue-resident lymphocytes. *Journal of Experimental Medicine*, *219*(1). https://doi.org/10.1084/jem.20210116

Faries, M. B., Thompson, J. F., Cochran, A. J., Andtbacka, R. H., Mozzillo, N., Zager, J. S., … Elashoff, R. M. (2017). Completion Dissection or Observation for Sentinel-Node Metastasis in Melanoma. *New England Journal of Medicine*, *376*(23), 2211–2222. https://doi.org/10.1056/nejmoa1613210

Feng, S., Cheng, X., Zhang, L., Lu, X., Chaudhary, S., Teng, R., … Lu, X. (2018). Myeloid-derived suppressor cells inhibit T cell activation through nitrating LCK in mouse cancers. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(40), 10094–10099. https://doi.org/10.1073/pnas.1800695115

Fischer, K., Voelkl, S., Meidenbauer, N., Ammer, J., Gottfried, E., Schwarz, S., … Kreutz, M. (2007). Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood*, *109*(9), 3812–3819. https://doi.org/10.1182/blood-2006-07-035972

Flaherty, K. T., Infante, J. R., Daud, A., Gonzalez, R., Kefford, R. F., Sosman, J., … Weber, J. (2012). Combined BRAF and MEK Inhibition in Melanoma with BRAF V600 Mutations. *New England Journal of Medicine*, *367*(18), 1694–1703. https://doi.org/10.1056/nejmoa1210093

Flaherty, K. T., Robert, C., Hersey, P., Nathan, P., Garbe, C., Milhem, M., … Schadendorf, D. (2012). Improved Survival with MEK Inhibition in BRAF-Mutated Melanoma. *New England Journal of Medicine*, *367*(2), 107–114. https://doi.org/10.1056/nejmoa1203421

Fletcher, M., Ramirez, M. E., Sierra, R. A., Raber, P., Thevenot, P., Al-Khami, A. A., … Rodriguez, P. C. (2015). L-Arginine depletion blunts antitumor T-cell responses by

inducing myeloid-derived suppressor cells. *Cancer Research*, *75*(2), 275–283. https://doi.org/10.1158/0008-5472.CAN-14-1491

Fourcade, J., Sun, Z., Benallaoua, M., Guillaume, P., Luescher, I. F., Sander, C., … Zarour, H. M. (2010). Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *The Journal of Experimental Medicine*, *207*(10), 2175–2186. https://doi.org/10.1084/jem.20100637

Freed-Pastor, W. A., Lambert, L. J., Ely, Z. A., Pattada, N. B., Bhutkar, A., Eng, G., … Jacks, T. (2021). The CD155/TIGIT axis promotes and maintains immune evasion in neoantigen-expressing pancreatic cancer. *Cancer Cell*, *39*(10), 1342-1360.e14. https://doi.org/10.1016/j.ccell.2021.07.007

Freeman, G. J., Long, A. J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., … Honjo, T. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *Journal of Experimental Medicine*, *192*(7), 1027–1034. https://doi.org/10.1084/jem.192.7.1027

Fu, Q., Chen, N., Ge, C., Li, R., Li, Z., Zeng, B., … Li, G. (2019). Prognostic value of tumor-infiltrating lymphocytes in melanoma: a systematic review and meta-analysis. *OncoImmunology*, *8*(7), 1–14. https://doi.org/10.1080/2162402X.2019.1593806

Furtado, G. C., De Curotto Lafaille, M. A., Kutchukhidze, N., & Lafaille, J. J. (2002). Interleukin 2 signaling is required for CD4+ regulatory T cell function. *Journal of Experimental Medicine*, *196*(6), 851–857. https://doi.org/10.1084/jem.20020190

Gajewski, T. F., Fallarino, F., Fields, P. E., Rivas, F., & Alegre, M.-L. (2001). Absence of CTLA-4 Lowers the Activation Threshold of Primed CD8 + TCR-Transgenic T Cells: Lack of Correlation with Src Homology Domain 2-Containing Protein Tyrosine Phosphatase . *The Journal of Immunology*, *166*(6), 3900–3907. https://doi.org/10.4049/jimmunol.166.6.3900

Galon, Jerome, Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pages, C., … Pages, F. (2006). Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. *Science*, *313*(5795), 1960–1964. https://doi.org/10.1126/science.1129139

Galon, Jérôme, Pagès, F., Marincola, F. M., Angell, H. K., Thurin, M., Lugli, A., … Fox, B. A. (2012). Cancer classification using the Immunoscore: a worldwide task force. *Journal of Translational Medicine*, *10*, 205. https://doi.org/10.1186/1479-5876-10-205

Ganesan, A.-P., Clarke, J., Wood, O., Garrido-Martin, E. M., Chee, S. J., Mellows, T., … Ottensmeier, C. H. (2017). Tissue-resident memory features are linked to the magnitude of cytotoxic T cell responses in human lung cancer. *Nature Immunology*, *18*(8), 940–950. https://doi.org/10.1038/ni.3775

Gao, G., Wang, Z., Qu, X., & Zhang, Z. (2019). *Prognostic value of tumor-infiltrating lymphocytes in patients with triple-negative breast cancer: a systematic review and meta-analysis*. https://doi.org/10.21203/rs.2.19214/v1

Gao, J., Shi, L. Z., Zhao, H., Chen, J., Xiong, L., He, Q., … Sharma, P. (2016). Loss of IFN-γ Pathway Genes in Tumor Cells as a Mechanism of Resistance to Anti-CTLA-4 Therapy. *Cell*, *167*(2), 397-404.e9. https://doi.org/10.1016/j.cell.2016.08.069

Gao, J., Ward, J. F., Pettaway, C. A., Shi, L. Z., Subudhi, S. K., Vence, L. M., … Sharma, P. (2017). VISTA is an inhibitory immune checkpoint that is increased after ipilimumab therapy in patients with prostate cancer. *Nature Medicine*, *23*(5), 551–555. https://doi.org/10.1038/nm.4308

Gattinoni, L., Lugli, E., Ji, Y., Pos, Z., Paulos, C. M., Quigley, M. F., … Restifo, N. P. (2011). A human memory T cell subset with stem cell-like properties. *Nature Medicine*, *17*(10), 1290–1297. https://doi.org/10.1038/nm.2446

Gebhardt, T, Whitney, P. G., Zaid, A., Mackay, L. K., Brooks, A. G., Heath, W. R., …

Mueller, S. N. (2011). Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature*, *477*(7363), 216–219. https://doi.org/10.1038/nature10339

Gebhardt, Thomas, Wakim, L. M., Eidsmo, L., Reading, P. C., Heath, W. R., & Carbone, F. R. (2009). Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nature Immunology*, *10*(5), 524–530. https://doi.org/10.1038/ni.1718

Geginat, J, Sallusto, F., & Lanzavecchia, A. (2001). Cytokine-driven proliferation and differentiation of human naive, central memory and effector memory CD4+ T cells. *Journal of Experimental Medicine*, *194*(12), 1711–1719. https://doi.org/S0369811403000981 [pii]

Geginat, Jens, Geginat, J., Lanzavecchia, A., Lanzavecchia, A., Sallusto, F., & Sallusto, F. (2003). Proliferation and differentiation potential of human CD8. *Blood*, *101*(June), 4260–4266. https://doi.org/10.1182/blood-2002-11-3577.Supported

Gide, T. N., Quek, C., Menzies, A. M., Tasker, A. T., Shang, P., Holst, J., … Wilmott, J. S. (2019). Distinct Immune Cell Populations Define Response to Anti-PD-1 Monotherapy and Anti-PD-1/Anti-CTLA-4 Combined Therapy. *Cancer Cell*, *35*(2), 238-255.e6. https://doi.org/10.1016/j.ccell.2019.01.003

Gilchrest, B. a, Eller, M. S., Geller, A., & Yaar, M. (1999). The pathogenesis of melanoma induced by ultraviolet radiation. *N Engl J Med*, *340*(17), 1341–1348. https://doi.org/10.1056/NEJMra0804595

Gooden, M. J. M., De Bock, G. H., Leffers, N., Daemen, T., & Nijman, H. W. (2011). The prognostic influence of tumour-infiltrating lymphocytes in cancer: A systematic review with meta-analysis. *British Journal of Cancer*, *105*(1), 93–103. https://doi.org/10.1038/bjc.2011.189

Gordon, S. R., Maute, R. L., Dulken, B. W., Hutter, G., George, B. M., McCracken, M. N., … Weissman, I. L. (2017). PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature*, *545*(7655), 495–499. https://doi.org/10.1038/nature22396

Grasso, C. S., Tsoi, J., Onyshchenko, M., Abril-Rodriguez, G., Ross-Macdonald, P., Wind-Rotolo, M., … Ribas, A. (2020). Conserved Interferon-γ Signaling Drives Clinical Response to Immune Checkpoint Blockade Therapy in Melanoma. *Cancer Cell*, *38*(4), 500-515.e3. https://doi.org/10.1016/j.ccell.2020.08.005

Gros, A., Robbins, P. F., Yao, X., Li, Y. F., Turcotte, S., Tran, E., … Rosenberg, S. a. (2014). PD-1 identi fi es the patient-speci fi c in fi ltrating human tumors. *The Journal of Clinical Investigation*, *124*(5), 2246–2259. https://doi.org/10.1172/JCI73639.2246

Han, L., Gao, Q. L., Zhou, X. M., Shi, C., Chen, G. Y., Song, Y. P., … Gao, Y. F. (2020). Characterization of CD103+ CD8+ tissue-resident T cells in esophageal squamous cell carcinoma: may be tumor reactive and resurrected by anti-PD-1 blockade. *Cancer Immunology, Immunotherapy*, *69*(8), 1493–1504. https://doi.org/10.1007/s00262-020-02562-3

Hartana, C. A., Ahlén Bergman, E., Broomé, A., Berglund, S., Johansson, M., Alamdari, F., … Winqvist, O. (2018). Tissue-resident memory T cells are epigenetically cytotoxic with signs of exhaustion in human urinary bladder cancer. *Clinical and Experimental Immunology*, *194*(1), 39–53. https://doi.org/10.1111/cei.13183

Hatfield, S. M., Kjaergaard, J., Lukashev, D., Schreiber, T. H., Belikoff, B., Abbott, R., … Sitkovsky, M. V. (2015). Immunological mechanisms of the antitumor effects of supplemental oxygenation. *Science Translational Medicine*, *7*(277), 277ra30. https://doi.org/10.1126/scitranslmed.aaa1260

Hauschild, A., Grob, J. J., Demidov, L. V., Jouary, T., Gutzmer, R., Millward, M., … Chapman, P. B. (2012). Dabrafenib in BRAF-mutated metastatic melanoma: A

multicentre, open-label, phase 3 randomised controlled trial. *The Lancet*, *380*(9839), 358–365. https://doi.org/10.1016/S0140-6736(12)60868-X

Hayward, N. K., Wilmott, J. S., Waddell, N., Johansson, P. A., Field, M. A., Nones, K., … Mann, G. J. (2017). Whole-genome landscapes of major melanoma subtypes. *Nature*, *545*(7653), 175–180. https://doi.org/10.1038/nature22071

Hewavisenti, R., Ferguson, A., Wang, K., Jones, D., Gebhardt, T., Edwards, J., … Palendira, U. (2020). CD103+ tumor-resident CD8+ T cell numbers underlie improved patient survival in oropharyngeal squamous cell carcinoma. *Journal for ImmunoTherapy of Cancer*, *8*(1), 1–8. https://doi.org/10.1136/jitc-2019-000452

Hodi, F. S., O'Day, S. J., McDermott, D. F., Weber, R. W., Sosman, J. A., Haanen, J. B., … Urba, W. J. (2010). new england journal. *New England Journal of Medicine*, *363*(8), 711–723.

Hombrink, P., Helbig, C., Backer, R. A., Piet, B., Oja, A. E., Stark, R., … Van Lier, R. A. W. (2016). Programs for the persistence, vigilance and control of human CD8 + lung-resident memory T cells. *Nature Immunology*, *17*(12), 1467–1478. https://doi.org/10.1038/ni.3589

Huang, B., Pan, P. Y., Li, Q., Sato, A. I., Levy, D. E., Bromberg, J., … Chen, S. H. (2006). Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Research*, *66*(2), 1123–1131. https://doi.org/10.1158/0008-5472.CAN-05-1299

Hui, E., Cheung, J., Zhu, J., Su, X., Taylor, M. J., Wallweber, H. A., … Vale, R. D. (2017). T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science (New York, N.Y.)*, *355*(6332), 1428–1433. https://doi.org/10.1126/science.aaf1292

Husain, Z., Huang, Y., Seth, P., & Sukhatme, V. P. (2013). Tumor-derived lactate modifies antitumor immune response: effect on myeloid-derived suppressor cells and NK cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *191*(3), 1486–1495. https://doi.org/10.4049/jimmunol.1202702

Idos, G. E., Kwok, J., Bonthala, N., Kysh, L., Gruber, S. B., & Qu, C. (2020). The Prognostic Implications of Tumor Infiltrating Lymphocytes in Colorectal Cancer: A Systematic Review and Meta-Analysis. *Scientific Reports*, *10*(1), 3360. https://doi.org/10.1038/s41598-020-60255-4

Iijima, N., Iwasaki, A., Sallusto, F., Lenig, D., Förster, R., Lipp, M., … Carlson, C. S. (2014). T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science (New York, N.Y.)*, *346*(6205), 93–98. https://doi.org/10.1126/science.1257530

Im, S. J., Hashimoto, M., Gerner, M. Y., Lee, J., Kissick, H. T., Burger, M. C., … Ahmed, R. (2016). Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature*, *537*(7620), 417–421. https://doi.org/10.1038/nature19330

Ishizuka, J. J., Manguso, R. T., Cheruiyot, C. K., Bi, K., Panda, A., Iracheta-Vellve, A., … Haining, W. N. (2019). Loss of ADAR1 in tumours overcomes resistance to immune checkpoint blockade. *Nature*, *565*(7737), 43–48. https://doi.org/10.1038/s41586-018-0768-9

Iwai, Y., Ishida, M., Tanaka, Y., Okazaki, T., Honjo, T., & Minato, N. (2002). Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(19), 12293–12297. https://doi.org/10.1073/pnas.192461099

Jadhav, R. R., Im, S. J., Hu, B., Hashimoto, M., Li, P., Lin, J. X., … Goronzy, J. J. (2019). Epigenetic signature of PD-1+ TCF1+ CD8 T cells that act as resource cells during

chronic viral infection and respond to PD-1 blockade. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(28), 14113–14118. https://doi.org/10.1073/pnas.1903520116

Jayaprakash, P., Ai, M., Liu, A., Budhani, P., Bartkowiak, T., Sheng, J., … Curran, M. A. (2018). Targeted hypoxia reduction restores T cell infiltration and sensitizes prostate cancer to immunotherapy. *The Journal of Clinical Investigation*, *128*(11), 5137–5149. https://doi.org/10.1172/JCI96268

Ji, R. R., Chasalow, S. D., Wang, L., Hamid, O., Schmidt, H., Cogswell, J., … Shahabi, V. (2012). An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunology, Immunotherapy*, *61*(7), 1019–1031. https://doi.org/10.1007/s00262-011-1172-6

Jiang, X., Clark, R. A., Liu, L., Wagers, A. J., Fuhlbrigge, R. C., & Kupper, T. S. (2012). Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature*, *483*(7388), 227–231. https://doi.org/10.1038/nature10851

Jin, H.-T., Anderson, A. C., Tan, W. G., West, E. E., Ha, S.-J., Araki, K., … Ahmed, R. (2010). Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(33), 14733–14738. https://doi.org/10.1073/pnas.1009731107

Jing, X., Yang, F., Shao, C., Wei, K., Xie, M., Shen, H., & Shu, Y. (2019). Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Molecular Cancer*, *18*(1), 157. https://doi.org/10.1186/s12943-019-1089-9

Johnston, R. J., Comps-Agrar, L., Hackney, J., Yu, X., Huseni, M., Yang, Y., … Grogan, J. L. (2014). The Immunoreceptor TIGIT Regulates Antitumor and Antiviral CD8+ T Cell Effector Function. *Cancer Cell*, *26*(6), 923–937. https://doi.org/10.1016/j.ccell.2014.10.018

Kaidbey, K. H., Agin, P. P., Sayre, R. M., & Kligman, A. M. (1979). Photoprotection by melanin—a comparison of black and Caucasian skin. *Journal of the American Academy of Dermatology*, *1*(3), 249–260. https://doi.org/10.1016/S0190-9622(79)70018-1

Kakavand, H., Jackett, L. A., Menzies, A. M., Gide, T. N., Carlino, M. S., Saw, R. P. M., … Scolyer, R. A. (2017). Negative immune checkpoint regulation by VISTA: A mechanism of acquired resistance to anti-PD-1 therapy in metastatic melanoma patients. *Modern Pathology*, *30*(12), 1666–1676. https://doi.org/10.1038/modpathol.2017.89

Karimkhani, C., Green, A. C., Nijsten, T., Weinstock, M. A., Dellavalle, R. P., Naghavi, M., & Fitzmaurice, C. (2017). The global burden of melanoma: results from the Global Burden of Disease Study 2015. *British Journal of Dermatology*, *177*(1), 134–140. https://doi.org/10.1111/bjd.15510

Khayat, D., Rixe, O., Martin, G., Soubrane, C., Banzet, M., Bazex, J. A., … Banzet, P. (2003). Surgical margins in cutaneous melanoma (2 cm versus 5 cm for lesions measuring less than 2.1-mm thick): Long-term results of a large European multicentric phase III study. *Cancer*, *97*(8), 1941–1946. https://doi.org/10.1002/cncr.11272

Kim, K., Skora, A. D., Li, Z., Liu, Q., Tam, A. J., Blosser, R. L., … Zhou, S. (2014). Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(32), 11774–11779. https://doi.org/10.1073/pnas.1410626111

Kirkwood, J. M., Strawderman, M. H., Ernstoff, M. S., Smith, T. J., Borden, E. C., & Blum, R. H. (1996). Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. *Journal of Clinical Oncology*, *14*(1), 7–17. https://doi.org/10.1200/JCO.1996.14.1.7

Kishton, R. J., Sukumar, M., & Restifo, N. P. (2017). Metabolic Regulation of T Cell

Longevity and Function in Tumor Immunotherapy. *Cell Metabolism*, *26*(1), 94–109. https://doi.org/10.1016/j.cmet.2017.06.016
Klonowski, K. D., Williams, K. J., Marzo, A. L., Blair, D. A., Lingenheld, E. G., & Lefrançois, L. (2004). Dynamics of blood-borne CD8 memory T cell migration in vivo. *Immunity*, *20*(5), 551–562. https://doi.org/10.1016/S1074-7613(04)00103-7
Kohlhapp, F. J., Broucek, J. R., Hughes, T., Huelsmann, E. J., Lusciks, J., Zayas, J. P., … Kaufman, H. L. (2015). NK cells and CD8+ T cells cooperate to improve therapeutic responses in melanoma treated with interleukin-2 (IL-2) and CTLA-4 blockade. *Journal for Immunotherapy of Cancer*, *3*(1), 18. https://doi.org/10.1186/s40425-015-0063-3
Komdeur, F. L., Prins, T. M., van de Wall, S., Plat, A., Wisman, G. B. A., Hollema, H., … Nijman, H. W. (2017). CD103+ tumor-infiltrating lymphocytes are tumor-reactive intraepithelial CD8+ T cells associated with prognostic benefit and therapy response in cervical cancer. *Oncoimmunology*, *6*(9), e1338230. https://doi.org/10.1080/2162402X.2017.1338230
Koyama, S., Akbay, E. A., Li, Y. Y., Herter-Sprie, G. S., Buczkowski, K. A., Richards, W. G., … Hammerman, P. S. (2016). Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nature Communications*, *7*, 10501. https://doi.org/10.1038/ncomms10501
Krieg, C., Nowicka, M., Guglietta, S., Schindler, S., Hartmann, F. J., Weber, L. M., … Becher, B. (2018). High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. *Nature Medicine*, *24*(2), 144–153. https://doi.org/10.1038/nm.4466
Krummel, M. F., & Allison, J. P. (1995). CD28 and CTLA-4 Have Opposing Effects on the Response of T ceils to Stimulation. *Journal of Experimental Medicine*, *182*, 459–465.
Kumar, B. V., Ma, W., Miron, M., Granot, T., Guyer, R. S., Carpenter, D. J., … Farber, D. L. (2017). Human tissue-resident memory T cells are defined by core transcriptional and functional signatures in lymphoid and mucosal sites Brahma. *Cell Reports*, *20*(12), 2921–2934. https://doi.org/10.1016/j.celrep.2017.08.078.Human
Kurd, N. S., He, Z., Louis, T. L., Milner, J. J., Omilusik, K. D., Jin, W., … Chang, J. T. (2020). Early precursors and molecular determinants of tissue-resident memory CD8+ T lymphocytes revealed by single-cell RNA sequencing. *Science Immunology*, *5*(47). https://doi.org/10.1126/sciimmunol.aaz6894
Kurtulus, S., Madi, A., Escobar, G., Klapholz, M., Nyman, J., Christian, E., … Anderson, A. C. (2019). Checkpoint Blockade Immunotherapy Induces Dynamic Changes in PD-1 − CD8 + Tumor-Infiltrating T Cells. *Immunity*, *50*(1), 181-194.e6. https://doi.org/10.1016/j.immuni.2018.11.014
Larkin, J., Chiarion-Sileni, V., Gonzalez, R., Jacques Grob, J., Lao, C. D., Schadendorf, D., … Jdw, D. (2015). Combined Nivolumab and Ipilimumab or Monotherapy in Previously Untreated Melanoma Corresponding authors. *N Engl J Med*, *373*(1), 23–34. https://doi.org/10.1056/NEJMoa1504030.Combined
Le Floc'h, A., Jalil, A., Franciszkiewicz, K., Validire, P., Vergnon, I., & Mami-Chouaib, F. (2011). Minimal engagement of CD103 on cytotoxic T lymphocytes with an E-cadherin-Fc molecule triggers lytic granule polarization via a phospholipase C??-dependent pathway. *Cancer Research*, *71*(2), 328–338. https://doi.org/10.1158/0008-5472.CAN-10-2457
Le Floc'h, A., Jalil, A., Vergnon, I., Le Maux Chansac, B., Lazar, V., Bismuth, G., … Mami-Chouaib, F. (2007). Alpha E beta 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. *The Journal of Experimental Medicine*, *204*(3), 559–570. https://doi.org/10.1084/jem.20061524
Le Naour, J., Zitvogel, L., Galluzzi, L., Vacchelli, E., & Kroemer, G. (2020). Trial watch:

STING agonists in cancer therapy. *Oncoimmunology*, *9*(1), 1777624. https://doi.org/10.1080/2162402X.2020.1777624
Leach, D., Matthew, K., & James, A. (1996). Enhancement of Antitumor Immunity by CTLA-4 Blockade. *Science*, *271*(5256), 1734–1736. https://doi.org/10.1126/science.271.5256.1734
Lee, G. K., Park, H. J., MacLeod, M., Chandler, P., Munn, D. H., & Mellor, A. L. (2002). Tryptophan deprivation sensitizes activated T cells to apoptosis prior to cell division. *Immunology*, *107*(4), 452–460. https://doi.org/10.1046/j.1365-2567.2002.01526.x
Lee, H., Quek, C., Silva, I., Tasker, A., Batten, M., Rizos, H., … Wilmott, J. S. (2019). Integrated molecular and immunophenotypic analysis of NK cells in anti-PD-1 treated metastatic melanoma patients. *OncoImmunology*, *8*(2). https://doi.org/10.1080/2162402X.2018.1537581
Lee, J. H., Shklovskaya, E., Lim, S. Y., Carlino, M. S., Menzies, A. M., Stewart, A., … Rizos, H. (2020). Transcriptional downregulation of MHC class I and melanoma de-differentiation in resistance to PD-1 inhibition. *Nature Communications*, *11*(1), 1–12. https://doi.org/10.1038/s41467-020-15726-7
Leiter, U., Stadler, R., Mauch, C., Hohenberger, W., Brockmeyer, N., Berking, C., … Garbe, C. (2016). Complete lymph node dissection versus no dissection in patients with sentinel lymph node biopsy positive melanoma (DeCOG-SLT): a multicentre, randomised, phase 3 trial. *The Lancet Oncology*, *17*(6), 757–767. https://doi.org/10.1016/S1470-2045(16)00141-8
Leonardi, G. C., Falzone, L., Salemi, R., Zanghì, A., Spandidos, D. A., Mccubrey, J. A., … Libra, M. (2018). Cutaneous melanoma: From pathogenesis to therapy (Review). *International Journal of Oncology*, *52*(4), 1071–1080. https://doi.org/10.3892/ijo.2018.4287
Li, C., Zhu, B., Son, Y. M., Wang, Z., Jiang, L., Xiang, M., … Sun, J. (2019). The Transcription Factor Bhlhe40 Programs Mitochondrial Regulation of Resident CD8(+) T Cell Fitness and Functionality. *Immunity*, *51*(3), 491-507.e7. https://doi.org/10.1016/j.immuni.2019.08.013
Li, T., Liu, T., Zhu, W., Xie, S., Zhao, Z., Feng, B., … Yang, R. (2021). Targeting MDSC for Immune-Checkpoint Blockade in Cancer Immunotherapy: Current Progress and New Prospects. *Clinical Medicine Insights: Oncology*, *15*. https://doi.org/10.1177/11795549211035540
Liakou, C. I., Kamat, A., Tang, D. N., Chen, H., Sun, J., Troncoso, P., … Sharma, P. (2008). CD4 ⁄ ICOS hi cells to shift the ratio of effector to regulatory T cells in cancer patients. *Sciences-New York*, 1–6.
Lian, C. G., Bueno, E. M., Granter, S. R., Laga, A. C., Saavedra, A. P., Lin, W. M., … Murphy, G. F. (2014). Biomarker evaluation of face transplant rejection: Association of donor T cells with target cell injury. *Modern Pathology*, *27*(6), 788–799. https://doi.org/10.1038/modpathol.2013.249
Lin, R., Zhang, H., Yuan, Y., He, Q., Zhou, J., Li, S., … He, W. (2020). Fatty acid oxidation controls CD8+Tissue-resident memory t-cell survival in gastric adenocarcinoma. *Cancer Immunology Research*, *8*(4), 479–492. https://doi.org/10.1158/2326-6066.CIR-19-0702
Lipson, E. J., Tawbi, H. A.-H., Schadendorf, D., Ascierto, P. A., Matamala, L., Gutiérrez, E. C., … Hodi, F. S. (2021). Relatlimab (RELA) plus nivolumab (NIVO) versus NIVO in first-line advanced melanoma: Primary phase III results from RELATIVITY-047 (CA224-047). *Journal of Clinical Oncology*, *39*(15_suppl), 9503. https://doi.org/10.1200/JCO.2021.39.15_suppl.9503
Litchfield, K., Reading, J. L., Puttick, C., Thakkar, K., Abbosh, C., Bentham, R., … Swanton, C. (2021). Meta-analysis of tumor- and T cell-intrinsic mechanisms of

sensitization to checkpoint inhibition. *Cell*, *184*(3), 596-614.e14. https://doi.org/10.1016/j.cell.2021.01.002

Long, G. V., Atkinson, V., Lo, S., Sandhu, S., Guminski, A. D., Brown, M. P., … McArthur, G. A. (2018). Combination nivolumab and ipilimumab or nivolumab alone in melanoma brain metastases: a multicentre randomised phase 2 study. *The Lancet Oncology*, *19*(5). https://doi.org/10.1016/S1470-2045(18)30139-6

Long, G. V., Stroyakovskiy, D., Gogas, H., Levchenko, E., De Braud, F., Larkin, J., … Flaherty, K. (2015). Dabrafenib and trametinib versus dabrafenib and placebo for Val600 BRAF-mutant melanoma: A multicentre, double-blind, phase 3 randomised controlled trial. *The Lancet*, *386*(9992), 444–451. https://doi.org/10.1016/S0140-6736(15)60898-4

Luke, J. J., Bao, R., Sweis, R. F., Spranger, S., & Gajewski, T. F. (2019). WNT/b-catenin pathway activation correlates with immune exclusion across human cancers. *Clinical Cancer Research*, *25*(10), 3074–3083. https://doi.org/10.1158/1078-0432.CCR-18-1942

Luke, J. J., Flaherty, K. T., Ribas, A., & Long, G. V. (2017). Targeted agents and immunotherapies: Optimizing outcomes in melanoma. *Nature Reviews Clinical Oncology*, *14*(8), 463–482. https://doi.org/10.1038/nrclinonc.2017.43

Luke, J. J., & Schwartz, G. (2013). Chemotherapy in the Management of Advanced Cutaneous Malignant Melanoma. *Clinical Dermatology*, *31*(3), 290–297. https://doi.org/10.1016/j.clindermatol.2012.08.016.Chemotherapy

Ma, C., Mishra, S., Demel, E. L., Liu, Y., & Zhang, N. (2017). TGF-β Controls the Formation of Kidney-Resident T Cells via Promoting Effector T Cell Extravasation. *The Journal of Immunology*, *198*(2), 749–756. https://doi.org/10.4049/jimmunol.1601500

Mackay, C., Wayne, K., Malcolm, B., & Ross, C. (1988). Lymphocyte subsets show marked differences in their distribution between blood and the afferent and efferent lymph or peripheral lymph nodes. *Journal of Experimental Medicine*, *167*, 1755–1765.

Mackay, C., Wendy, M., & Lisabeth, D. (1990). Naive and memory T cells show distinct pathways of lymphocyte recirculation. *Journal of Experimental Medicine*, *171*, 801–817.

Mackay, L., Gebhardt, T., Collins, F. N., Tebartz, C., Bedoui, S., Mackay, L. K., … Carbone, F. R. (2015). CD69 Interference with Sphingosine- 1-Phosphate Receptor Function Regulates Peripheral T Cell Retention. *Journal of Immunology (Baltimore, Md. : 1950)*, 1–5. https://doi.org/10.4049/jimmunol.1402256

Mackay, L. K., Minnich, M., Kragten, N. A. M., Liao, Y., Nota, B., Seillet, C., … van Gisbergen, K. P. J. M. (2016). Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science (New York, N.Y.)*, *352*(6284), 459–463. https://doi.org/10.1126/science.aad2035

Mackay, L. K., Stock, A. T., Ma, J. Z., Jones, C. M., Kent, S. J., Mueller, S. N., … Gebhardt, T. (2012). Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(18), 7037–7042. https://doi.org/10.1073/pnas.1202288109

Mackay, L. K., Wynne-Jones, E., Freestone, D., Pellicci, D. G., Mielke, L. A., Newman, D. M., … Carbone, F. R. (2015). T-box Transcription Factors Combine with the Cytokines TGF-β and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity*, *43*(6), 1101–1111. https://doi.org/10.1016/j.immuni.2015.11.008

Mackay, L., Rahimpour, A., Ma, J., Collins, N., Stock, a T., Hafon, M. L., … Gebhardt, T. (2013). The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin. *Nat Immunol*, *14*(12), 1294–1301. https://doi.org/10.1038/ni.2744

Mahmoud, S. M. A., Paish, E. C., Powe, D. G., Macmillan, R. D., Grainge, M. J., Lee, A. H.

S., … Green, A. R. (2011). Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *Journal of Clinical Oncology*, *29*(15), 1949–1955. https://doi.org/10.1200/JCO.2010.30.5037

Mao, L., Qi, Z., Zhang, L., Guo, J., & Si, L. (2021). Immunotherapy in Acral and Mucosal Melanoma: Current Status and Future Directions. *Frontiers in Immunology*, *12*(June), 1–13. https://doi.org/10.3389/fimmu.2021.680407

Mariathasan, S., Turley, S. J., Nickles, D., Castiglioni, A., Yuen, K., Wang, Y., … Powles, T. (2018). TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature*, *554*(7693), 544–548. https://doi.org/10.1038/nature25501

Matloubian, M., Lo, C. G., Cinamon, G., Lesneski, M. J., Xu, Y., Brinkmann, V., … Cyster, J. G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*, *427*(6972), 355–360. https://doi.org/10.1038/nature02284

McGrail, D. J., Pilié, P. G., Rashid, N. U., Voorwerk, L., Slagter, M., Kok, M., … Lin, S. Y. (2021). High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types. *Annals of Oncology*, *32*(5), 661–672. https://doi.org/10.1016/j.annonc.2021.02.006

Menares, E., Gálvez-Cancino, F., Cáceres-Morgado, P., Ghorani, E., López, E., Díaz, X., … Lladser, A. (2019). Tissue-resident memory CD8+ T cells amplify anti-tumor immunity by triggering antigen spreading through dendritic cells. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-12319-x

Messenheimer, D. J., Jensen, S. M., Afentoulis, M. E., Wegmann, K. W., Feng, Z., Friedman, D. J., … Fox, B. A. (2017). Timing of PD-1 Blockade Is Critical to Effective Combination Immunotherapy with Anti-OX40. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *23*(20), 6165–6177. https://doi.org/10.1158/1078-0432.CCR-16-2677

Meyer, C., Cagnon, L., Costa-Nunes, C. M., Baumgaertner, P., Montandon, N., Leyvraz, L., … Speiser, D. E. (2014). Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. *Cancer Immunology, Immunotherapy*, *63*(3), 247–257. https://doi.org/10.1007/s00262-013-1508-5

Mezrich, J. D., Fechner, J. H., Zhang, X., Johnson, B. P., Burlingham, W. J., & Bradfield, C. A. (2010). An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *185*(6), 3190–3198. https://doi.org/10.4049/jimmunol.0903670

Miller, A., & Mihim, M. (2006). Review Article. *The New England Journal of Medicine*, *355*(1), 51–65. https://doi.org/10.1111/head.12009

Milner, J. J., Toma, C., Yu, B., Zhang, K., Omilusik, K., Anthony, T., … Goldrath, A. W. (2017). Runx3 programs CD8+ T cell residency in non-lymphoid tissues and tumors. *Nature*, *552*(7684), 253–257. https://doi.org/10.1038/nature24993.Runx3

Miska, J., Lee-Chang, C., Rashidi, A., Muroski, M. E., Chang, A. L., Lopez-Rosas, A., … Lesniak, M. S. (2019). HIF-1α Is a Metabolic Switch between Glycolytic-Driven Migration and Oxidative Phosphorylation-Driven Immunosuppression of Tregs in Glioblastoma. *Cell Reports*, *27*(1), 226-237.e4. https://doi.org/10.1016/j.celrep.2019.03.029

Mlecnik, B., Bindea, G., Angell, H. K., Sasso, M. S., Obenauf, A. C., Fredriksen, T., … Galon, J. (2014). Functional network pipeline reveals genetic determinants associated with in situ lymphocyte proliferation and survival of cancer patients. *Sci Transl Med*, *6*(228), 228ra37. https://doi.org/10.1126/scitranslmed.3007240 6/228/228ra37 [pii]

Montagut, C., Sharma, S. V, Shioda, T., Mcdermott, U., Ulkus, L. E., Dias-santagata, D., … Settleman, J. (2008). Elevated CRAF as a potential mechanism of acquired resistance to

BRAF inhibition in melanoma. *Cancer Research*, *68*(12), 4853–4861. https://doi.org/10.1158/0008-5472.CAN-07-6787.Elevated
Moore, K. W., O'Garra, A., Malefyt, R. D. W., Vieira, P., & Mosmann, T. R. (1993). INTERLEUKIN-10. *Annu Rev Immunol*, *11*, 165–190.
Mueller, S. N., Gebhardt, T., Carbone, F. R., & Heath, W. R. (2013). Memory T Cell Subsets, Migration Patterns, and Tissue Residence. *Annual Review of Immunology*, *31*(1), 137–161. https://doi.org/doi:10.1146/annurev-immunol-032712-095954
Mumm, J. B., Emmerich, J., Zhang, X., Chan, I., Wu, L., Mauze, S., … Oft, M. (2011). IL-10 Elicits IFNγ-Dependent tumor immune surveillance. *Cancer Cell*, *20*(6), 781–796. https://doi.org/10.1016/j.ccr.2011.11.003
Munn, D. H., Shafizadeh, E., Attwood, J. T., Bondarev, I., Pashine, A., & Mellor, A. L. (1999). Inhibition of T cell proliferation by macrophage tryptophan catabolism. *Journal of Experimental Medicine*, *189*(9), 1363–1372. https://doi.org/10.1084/jem.189.9.1363
Muruganandah, V., Sathkumara, H. D., Navarro, S., & Kupz, A. (2018). A systematic review: The role of resident memory T cells in infectious diseases and their relevance for vaccine development. *Frontiers in Immunology*, *9*(JUL). https://doi.org/10.3389/fimmu.2018.01574
Newell, F., Pires da Silva, I., Johansson, P. A., Menzies, A. M., Wilmott, J. S., Addala, V., … Long, G. V. (2021). Multiomic profiling of checkpoint inhibitor-treated melanoma: Identifying predictors of response and resistance, and markers of biological discordance. *Cancer Cell*. https://doi.org/https://doi.org/10.1016/j.ccell.2021.11.012
Nishimura, H., Nose, M., Hiai, H., Minato, N., & Honjo, T. (1999). Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity*, *11*(2), 141–151. https://doi.org/10.1016/S1074-7613(00)80089-8
Noman, M. Z., Desantis, G., Janji, B., Hasmim, M., Karray, S., Dessen, P., … Chouaib, S. (2014). PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *The Journal of Experimental Medicine*, *211*(5), 781–790. https://doi.org/10.1084/jem.20131916
O'Hara, J. M., Redhu, N. S., Cheung, E., Robertson, N. G., Patik, I., Sayed, S. El, … Horwitz, B. H. (2020). Generation of protective pneumococcal-specific nasal resident memory CD4+ T cells via parenteral immunization. *Mucosal Immunology*, *13*(1), 172–182. https://doi.org/10.1038/s41385-019-0218-5
O'Neill, L. A. J., Kishton, R. J., & Rathmell, J. (2016). A guide to immunometabolism for immunologists. *Nature Reviews Immunology*, *16*(9), 553–565. https://doi.org/10.1038/nri.2016.70
Oberg, A., Samii, S., Stenling, R., & Lindmark, G. (2002). Different occurrence of CD8+, CD45R0+, and CD68+ immune cells in regional lymph node metastases from colorectal cancer as potential prognostic predictors. *International Journal of Colorectal Disease*, *17*(1), 25–29.
Okla, K., Farber, D. L., & Zou, W. (2021). Tissue-resident memory T cells in tumor immunity and immunotherapy. *Journal of Experimental Medicine*, *218*(4), 1–14. https://doi.org/10.1084/jem.20201605
Oliveira, G., Stromhaug, K., Klaeger, S., Kula, T., Frederick, D. T., Le, P. M., … Wu, C. J. (2021). Phenotype, specificity and avidity of antitumour CD8+ T cells in melanoma. *Nature*, *596*(7870), 119–125. https://doi.org/10.1038/s41586-021-03704-y
Opitz, C. A., Litzenburger, U. M., Sahm, F., Ott, M., Tritschler, I., Trump, S., … Platten, M. (2011). An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature*, *478*(7368), 197–203. https://doi.org/10.1038/nature10491
Oweida, A., Hararah, M. K., Phan, A., Binder, D., Bhatia, S., Lennon, S., … Karam, S. D.

(2018). Resistance to radiotherapy and PD-L1 blockade is mediated by TIM-3 upregulation and regulatory T-cell infiltration. *Clinical Cancer Research*, *24*(21), 5368–5380. https://doi.org/10.1158/1078-0432.CCR-18-1038

Pagès, F. (2007). Effector Memory T Cells, Early Metastasis, and Survival in Colorectal Cancer. *The New England Journal of Medicine*, *2007*, 307–308. https://doi.org/10.1016/S0090-3671(08)70229-5

Pal, S., Nandi, M., Dey, D., Chakraborty, B. C., Shil, A., Ghosh, S., … Datta, S. (2019). Myeloid-derived suppressor cells induce regulatory T cells in chronically HBV infected patients with high levels of hepatitis B surface antigen and persist after antiviral therapy. *Alimentary Pharmacology & Therapeutics*, *49*(10), 1346–1359. https://doi.org/10.1111/apt.15226

Palazon, A., Tyrakis, P. A., Macias, D., Veliça, P., Rundqvist, H., Fitzpatrick, S., … Johnson, R. S. (2017). An HIF-1α/VEGF-A Axis in Cytotoxic T Cells Regulates Tumor Progression. *Cancer Cell*, *32*(5), 669-683.e5. https://doi.org/10.1016/j.ccell.2017.10.003

Pallett, L. J., Davies, J., Colbeck, E. J., Robertson, F., Hansi, N., Easom, N. J. W., … Maini, M. K. (2017). IL-2high tissue-resident T cells in the human liver: Sentinels for hepatotropic infection. *Journal of Experimental Medicine*, *214*(6), 1567–1580. https://doi.org/10.1084/jem.20162115

Pan, Y., Tian, T., Park, C. O., Lofftus, S. Y., Mei, S., Liu, X., … Kupper, T. S. (2017). Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. *Nature*, *543*(7644), 252–256. https://doi.org/10.1038/nature21379

Papandreou, I., Cairns, R. A., Fontana, L., Lim, A. L., & Denko, N. C. (2006). HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metabolism*, *3*(3), 187–197. https://doi.org/10.1016/j.cmet.2006.01.012

Park, S.L., Buzzai, A., Rautela, J., Hor, J. L., Hochheiser, K., Effern, M., … Gebhardt, T. (2019). Tissue-resident memory $CD8^+$ T cells promote melanoma–immune equilibrium in skin. *Nature*, *565*(7739). https://doi.org/10.1038/s41586-018-0812-9

Park, Simone L., Zaid, A., Hor, J. L., Christo, S. N., Prier, J. E., Davies, B., … MacKay, L. K. (2018). Local proliferation maintains a stable pool of tissue-resident memory T cells after antiviral recall responses article. *Nature Immunology*, *19*(2), 183–191. https://doi.org/10.1038/s41590-017-0027-5

Paulson, K. G., Voillet, V., McAfee, M. S., Hunter, D. S., Wagener, F. D., Perdicchio, M., … Chapuis, A. G. (2018). Acquired cancer resistance to combination immunotherapy from transcriptional loss of class I HLA. *Nature Communications*, *9*(1), 3868. https://doi.org/10.1038/s41467-018-06300-3

Pilon-Thomas, S., Kodumudi, K. N., El-Kenawi, A. E., Russell, S., Weber, A. M., Luddy, K., … Gillies, R. J. (2016). Neutralization of tumor acidity improves antitumor responses to immunotherapy. *Cancer Research*, *76*(6), 1381–1390. https://doi.org/10.1158/0008-5472.CAN-15-1743

Pilotte, L., Larrieu, P., Stroobant, V., Colau, D., Dolusic, E., Frédérick, R., … Van den Eynde, B. J. (2012). Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(7), 2497–2502. https://doi.org/10.1073/pnas.1113873109

Pires da Silva, I., Ahmed, T., Reijers, I. L. M., Weppler, A. M., Betof Warner, A., Patrinely, J. R., … Long, G. V. (2021). Ipilimumab alone or ipilimumab plus anti-PD-1 therapy in patients with metastatic melanoma resistant to anti-PD-(L)1 monotherapy: a multicentre, retrospective, cohort study. *The Lancet Oncology*, *22*(6), 836–847. https://doi.org/10.1016/S1470-2045(21)00097-8

Pizzolla, A., Nguyen, T. H. O., Smith, J. M., Brooks, A. G., Kedzieska, K., Heath, W. R., …

Wakim, L. M. (2017). Resident memory CD8(+) T cells in the upper respiratory tract prevent pulmonary influenza virus infection. *Science Immunology*, *2*(12). https://doi.org/10.1126/sciimmunol.aam6970

Poynter, J. N., Elder, J. T., Fullen, D. R., Nair, R. P., Soengas, M. S., Johnson, T. M., … Gruber, S. B. (2006). BRAF and NRAS mutations in melanoma and melanocytic nevi. *Melanoma Research*, *16*(4), 267–273. https://doi.org/10.1097/01.cmr.0000222600.73179.f3

Purwar, R., Campbell, J., Murphy, G., Richards, W. G., Clark, R. A., & Kupper, T. S. (2011). Resident Memory T cells (TRM) are abundant in human lung: Diversity, function, and antigen specificity. *PLoS ONE*, *6*(1). https://doi.org/10.1371/journal.pone.0016245

Qureshi, O. S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E. M., … Sansom, D. M. (2011). Trans-endocytosis of CD80 and CD86: a molecular basis for the cell extrinsic function of CTLA-4. *Science*, *332*(6029), 600–603. https://doi.org/10.1126/science.1202947.Trans-endocytosis

Restifo, N. P., Marincola, F. M., Kawakami, Y., Taubenberger, J., Yannelli, J. R., & Rosenberg, S. A. (1996). Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. *Journal of the National Cancer Institute*, *88*(2), 100–108. https://doi.org/10.1093/jnci/88.2.100

Ribas, A., Puzanov, I., Dummer, R., Schadendorf, D., Hamid, O., Robert, C., … Daud, A. (2015). Pembrolizumab versus investigator-choice chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): A randomised, controlled, phase 2 trial. *The Lancet Oncology*, *16*(8), 908–918. https://doi.org/10.1016/S1470-2045(15)00083-2

Robert, C., Long, G. V., Brady, B., Dutriaux, C., Maio, M., Mortier, L., … Ascierto, P. A. (2015). Nivolumab in Previously Untreated Melanoma without BRAF Mutation . *New England Journal of Medicine*, *372*(4), 320–330. https://doi.org/10.1056/nejmoa1412082

Robert, C., Schachter, J., Long, G. V., Arance, A., Grob, J. J., Mortier, L., … Ribas, A. (2015). Pembrolizumab versus Ipilimumab in Advanced Melanoma. *New England Journal of Medicine*, *372*(26), 2521–2532. https://doi.org/10.1056/nejmoa1503093

Robert, C., Thomas, L., Bondarenko, I., O'Day, S., Weber, J., Garbe, C., … Wolchok, J. D. (2011). Ipilimumab plus Dacarbazine for Previously Untreated Metastatic Melanoma. *New England Journal of Medicine*, *364*(26), 2517–2526. https://doi.org/10.1056/nejmoa1104621

Rodig, S. J., Gusenleitner, D., Jackson, D. G., Gjini, E., Giobbie-Hurder, A., Jin, C., … Hodi, F. S. (2018). MHC proteins confer differential sensitivity to CTLA-4 and PD-1 blockade in untreated metastatic melanoma. *Science Translational Medicine*, *10*(450). https://doi.org/10.1126/scitranslmed.aar3342

Rodriguez, P. C., Quiceno, D. G., & Ochoa, A. C. (2007). L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood*, *109*(4), 1568–1573. https://doi.org/10.1182/blood-2006-06-031856

Rodriguez, P. C., Quiceno, D. G., Zabaleta, J., Ortiz, B., Zea, A. H., Piazuelo, M. B., … Ochoa, A. C. (2004). Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Research*, *64*(16), 5839–5849. https://doi.org/10.1158/0008-5472.CAN-04-0465

Rooney, M. S., Shukla, S. A., Wu, C. J., Getz, G., & Hacohen, N. (2015). Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell*, *160*(1–2), 48–61. https://doi.org/10.1016/j.cell.2014.12.033

Rusakiewicz, S., Semeraro, M., Sarabi, M., Desbois, M., Locher, C., Mendez, R., … Zitvogel, L. (2013). Immune Infiltrates Are Prognostic Factors in Localized Gastrointestinal Stromal Tumors. *Cancer Research*, *73*(12), 3499–3510. https://doi.org/10.1158/0008-5472.CAN-13-0371

Sabbagh, L., Kaech, S. M., Bourbonnière, M., Woo, M., Cohen, L. Y., Haddad, E. K., … Sékaly, R.-P. (2004). The Selective Increase in Caspase-3 Expression in Effector but Not Memory T Cells Allows Susceptibility to Apoptosis. *The Journal of Immunology*, *173*(9), 5425–5433. https://doi.org/10.4049/jimmunol.173.9.5425

Sade-Feldman, M., Yizhak, K., Bjorgaard, S. L., Ray, J. P., de Boer, C. G., Jenkins, R. W., … Hacohen, N. (2018). Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell*, *175*(4), 998-1013.e20. https://doi.org/10.1016/j.cell.2018.10.038

Sakuishi, K., Apetoh, L., Sullivan, J. M., Blazar, B. R., Kuchroo, V. K., & Anderson, A. C. (2010). Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *Journal of Experimental Medicine*, *207*(10), 2187–2194. https://doi.org/10.1084/jem.20100643

Saleh, R., & Elkord, E. (2019). Treg-mediated acquired resistance to immune checkpoint inhibitors. *Cancer Letters*, *457*(April), 168–179. https://doi.org/10.1016/j.canlet.2019.05.003

Sallusto, F., Lenig, D., & Förster, R. (1999). *Pillars Article : Two Subsets of Memory T Lymphocytes with Distinct Homing Potentials and Effector Functions . Nature . 401*(October).

Santana Carrero, R. M., Beceren-Braun, F., Rivas, S. C., Hegde, S. M., Gangadharan, A., Plote, D., … Schluns, K. S. (2019). IL-15 is a component of the inflammatory milieu in the tumor microenvironment promoting antitumor responses. *Proceedings of the National Academy of Sciences*, *116*(2), 599 LP – 608. https://doi.org/10.1073/pnas.1814642116

Sasson, S. C., Gordon, C. L., Christo, S. N., Klenerman, P., & Mackay, L. K. (2020). Local heroes or villains: tissue-resident memory T cells in human health and disease. *Cellular and Molecular Immunology*, *17*(2), 113–122. https://doi.org/10.1038/s41423-019-0359-1

Sathaliyawala, T., Kubota, M., Yudanin, N., Turner, D., Camp, P., Thome, J. J. C., … Farber, D. L. (2013). Distribution and Compartmentalization of Human Circulating and Tissue-Resident Memory T Cell Subsets. *Immunity*, *38*(1), 187–197. https://doi.org/10.1016/j.immuni.2012.09.020

Savas, P., Virassamy, B., Ye, C., Salim, A., Mintoff, C. P., Caramia, F., … Loi, S. (2018). Single-cell profiling of breast cancer T cells reveals a tissue-resident memory subset associated with improved prognosis. *Nature Medicine*, *24*(7), 986–993. https://doi.org/10.1038/s41591-018-0078-7

Sawant, D. V., Yano, H., Chikina, M., Zhang, Q., Liao, M., Liu, C., … Vignali, D. A. A. (2019). Adaptive plasticity of IL-10+ and IL-35+ Treg cells cooperatively promotes tumor T cell exhaustion. *Nature Immunology*, *20*(6), 724–735. https://doi.org/10.1038/s41590-019-0346-9

Schadendorf, D., Hodi, F. S., Robert, C., Weber, J. S., Margolin, K., Hamid, O., … Wolchok, J. D. (2015). Pooled analysis of long-term survival data from phase II and phase III trials of ipilimumab in unresectable or metastatic melanoma. *Journal of Clinical Oncology*, *33*(17), 1889–1894. https://doi.org/10.1200/JCO.2014.56.2736

Scharping, N. E., Menk, A. V, Whetstone, R. D., Zeng, X., & Delgoffe, G. M. (2017). Efficacy of PD-1 Blockade Is Potentiated by Metformin-Induced Reduction of Tumor Hypoxia. *Cancer Immunology Research*, *5*(1), 9–16. https://doi.org/10.1158/2326-6066.CIR-16-0103

Schenkel, J. M., Fraser, K. A., Beura, L. K., Pauken, K. E., Vezys, V., & Masopust, D. (2014). T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science*, *346*(6205), 98–101.

https://doi.org/10.1126/science.1254536\rscience.1254536 [pii]
Schenkel, J. M., Fraser, K. A., Vezys, V., & Masopust, D. (2013). Sensing and alarm function of resident memory CD8(+) T cells. *Nature Immunology*, *14*(5), 509–513. https://doi.org/10.1038/ni.2568
Schnell, A., Bod, L., Madi, A., & Kuchroo, V. K. (2020). The yin and yang of co-inhibitory receptors: toward anti-tumor immunity without autoimmunity. *Cell Research*, *30*(4), 285–299. https://doi.org/10.1038/s41422-020-0277-x
Schuurman, H. J., van Wichen, D., & de Weger, R. A. (1989). Expression of activation antigens on thymocytes in the "common thymocyte" stage of differentiation. *Thymus*, *14*(1–3), 43–53.
Sharma, N., Benechet, A. P., Lefrançois, L., & Khanna, K. M. (2015). CD8 T Cells Enter the Splenic T Cell Zones Independently of CCR7, but the Subsequent Expansion and Trafficking Patterns of Effector T Cells after Infection Are Dysregulated in the Absence of CCR7 Migratory Cues. *Journal of Immunology (Baltimore, Md. : 1950)*, *195*(11), 5227–5236. https://doi.org/10.4049/jimmunol.1500993
Sharma, P., Hu-Lieskovan, S., Wargo, J. A., & Ribas, A. (2017). Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell*, *168*(4), 707–723. https://doi.org/10.1016/j.cell.2017.01.017
Sheridan, B. S., Pham, Q.-M., Lee, Y.-T., Cauley, L. S., Puddington, L., & Lefrancois, L. (2014). Oral infection drives a distinct population of intestinal resident memory CD8(+) T cells with enhanced protective function. *Immunity*, *40*(5), 747–757. https://doi.org/10.1016/j.immuni.2014.03.007
Shields, B. D., Koss, B., Taylor, E. M., Storey, A. J., West, K. L., Byrum, S. D., … Tackett, A. J. (2019). Loss of E-cadherin inhibits CD103 antitumor activity and reduces checkpoint blockade responsiveness in melanoma. *Cancer Research*, *79*(6), 1113–1123. https://doi.org/10.1158/0008-5472.CAN-18-1722
Shin, D. S., Zaretsky, J. M., Escuin-Ordinas, H., Garcia-Diaz, A., Hu-Lieskovan, S., Kalbasi, A., … Ribas, A. (2017). Primary resistance to PD-1 blockade mediated by JAK1/2 mutations. *Cancer Discovery*, *7*(2), 188–201. https://doi.org/10.1158/2159-8290.CD-16-1223
Shiow, L. R., Rosen, D. B., Brdicková, N., Xu, Y., An, J., Lanier, L. L., … Matloubian, M. (2006). CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature*, *440*(7083), 540–544. https://doi.org/10.1038/nature04606
Siddiqui, I., Schaeuble, K., Chennupati, V., Fuertes Marraco, S. A., Calderon-Copete, S., Pais Ferreira, D., … Held, W. (2019). Intratumoral Tcf1 + PD-1 + CD8 + T Cells with Stem-like Properties Promote Tumor Control in Response to Vaccination and Checkpoint Blockade Immunotherapy. *Immunity*, *50*(1), 195-211.e10. https://doi.org/10.1016/j.immuni.2018.12.021
Sim, G. C., Martin-Orozco, N., Jin, L., Yang, Y., Wu, S., Washington, E., … Radvanyi, L. (2014). IL-2 therapy promotes suppressive ICOS + Treg expansion in melanoma patients. *Journal of Clinical Investigation*, *124*(1), 99–110. https://doi.org/10.1172/JCI46266
Simoni, Y., Becht, E., Fehlings, M., Loh, C. Y., Koo, S. L., Teng, K. W. W., … Newell, E. W. (2018). Bystander CD8+ T cells are abundant and phenotypically distinct in human tumour infiltrates. *Nature*, *557*(7706), 575–579. https://doi.org/10.1038/s41586-018-0130-2
Simpson, T. R., Li, F., Montalvo-Ortiz, W., Sepulveda, M. A., Bergerhoff, K., Arce, F., … Quezada, S. A. (2013). Fc-dependent depletion of tumor-infiltrating regulatory t cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *Journal of*

Experimental Medicine, *210*(9), 1695–1710. https://doi.org/10.1084/jem.20130579
Skon, C. N., Lee, J.-Y., Anderson, K. G., Masopust, D., Hogquist, K. A., & Jameson, S. C. (2013). Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. *Nature Immunology*, *14*(12), 1285–1293. https://doi.org/10.1038/ni.2745
Slütter, B., Braeckel-Budimir, N. Van, Abboud, G., Varga, S. M., Salek-Ardakani, S., & Harty, J. T. (2017). Dynamics of influenza-induced lung-resident memory T cells underlie waning heterosubtypic immunity. *Science Immunology*, *2*(7). https://doi.org/10.1126/sciimmunol.aag2031
Snow, H. (1892). Melanotic cancerous disease. *Lancet*, *2*(872), 1874–1892.
Snyder, M. E., Finlayson, M. O., Connors, T. J., Dogra, P., Bush, E., Carpenter, D., … Farber, D. L. (2019). *Generation and persistence of human tissue-resident memory T cells in lung transplantation*. *4*(33). https://doi.org/10.1126/sciimmunol.aav5581.Generation
Spranger, S., Bao, R., & Gajewski, T. F. (2015). Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. *Nature*, *523*(7559), 231–235. https://doi.org/10.1038/nature14404
Spranger, S., Koblish, H. K., Horton, B., Scherle, P. A., Newton, R., & Gajewski, T. F. (2014). Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8+ T cells directly within the tumor microenvironment. *Journal for ImmunoTherapy of Cancer*, *2*(1), 1–14. https://doi.org/10.1186/2051-1426-2-3
Steinert, E. M., Schenkel, J. M., Fraser, K. A., Beura, L. K., Manlove, L. S., Igyártó, B. Z., … Masopust, D. (2015). Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. *Cell*, *161*(4), 737–749. https://doi.org/10.1016/j.cell.2015.03.031
Stuelten, C. H., & Zhang, Y. E. (2021). Transforming Growth Factor-β: An Agent of Change in the Tumor Microenvironment . *Frontiers in Cell and Developmental Biology* , Vol. 9, p. 2886. Retrieved from https://www.frontiersin.org/article/10.3389/fcell.2021.764727
Sukumar, M., Restifo, N. P., Sukumar, M., Liu, J., Ji, Y., Subramanian, M., … Finkel, T. (2013). Inhibiting glycolytic metabolism enhances CD8 + T cell memory and antitumor function Find the latest version : Inhibiting glycolytic metabolism enhances CD8 + T cell memory and antitumor function. *Journal of Clinical Investigation*, *123*(10), 4479–4488. https://doi.org/10.1172/JCI69589.tor
Sung, W. W., Wang, Y. C., Lin, P. L., Cheng, Y. W., Chen, C. Y., Wu, T. C., & Lee, H. (2013). IL-10 promotes tumor aggressiveness via upregulation of CIP2A transcription in lung adenocarcinoma. *Clinical Cancer Research*, *19*(15), 4092–4103. https://doi.org/10.1158/1078-0432.CCR-12-3439
Szeto, C., Gounder, M. M., Parulkar, R., Nguyen, A., Rabizadeh, S., & Reddy, S. K. (2020). High correlation between TMB, expressed TMB, and neoantigen load using tumor: Normal whole exome DNA and matched whole transcriptome RNA sequencing. *Journal of Clinical Oncology*, *38*(15_suppl), e15238–e15238. https://doi.org/10.1200/JCO.2020.38.15_suppl.e15238
Takamura, S. (2018). Niches for the long-term maintenance of tissue-resident memory T cells. *Frontiers in Immunology*, *9*(MAY). https://doi.org/10.3389/fimmu.2018.01214
Tang, A., Eller, M. S., Hara, M., Yaar, M., Hirohashi, S., & Gilchrest, B. a. (1994). E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro. *Journal of Cell Science*, *107*(Pt 4), 983–992.
Tauriello, D. V. F., Palomo-Ponce, S., Stork, D., Berenguer-Llergo, A., Badia-Ramentol, J., Iglesias, M., … Batlle, E. (2018). TGFβ drives immune evasion in genetically

reconstituted colon cancer metastasis. *Nature*, *554*(7693), 538–543. https://doi.org/10.1038/nature25492

Teijaro, J., Turner, D., Pham, Q., Wherry, J., Lefrançois, L., & Farber, D. (2011). *Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection*. *187*(11), 5510–5514. https://doi.org/10.1016/j.pestbp.2011.02.012.Investigations

Thom, J. T., Weber, T. C., Walton, S. M., Torti, N., & Oxenius, A. (2015). The Salivary Gland Acts as a Sink for Tissue-Resident Memory CD8(+) T Cells, Facilitating Protection from Local Cytomegalovirus Infection. *Cell Reports*, *13*(6), 1125–1136. https://doi.org/10.1016/j.celrep.2015.09.082

Thompson, J. F., Scolyer, R. A., & Kefford, R. F. (2005). Cutaneous melanoma. *Lancet*, *365*(9460), 687–701. https://doi.org/10.5124/jkma.2018.61.11.662

Tietze, J. K., Angelova, D., Heppt, M. V, Reinholz, M., Murphy, W. J., Spannagl, M., … Berking, C. (2017). The proportion of circulating CD45RO(+)CD8(+) memory T cells is correlated with clinical response in melanoma patients treated with ipilimumab. *European Journal of Cancer (Oxford, England : 1990)*, *75*, 268–279. https://doi.org/10.1016/j.ejca.2016.12.031

Tomić, S., Joksimović, B., Bekić, M., Vasiljević, M., Milanović, M., Čolić, M., & Vučević, D. (2019). Prostaglanin-E2 potentiates the suppressive functions of human mononuclear myeloid-derived suppressor cells and increases their capacity to expand IL-10-producing regulatory T cell subsets. *Frontiers in Immunology*, *10*(MAR), 1–15. https://doi.org/10.3389/fimmu.2019.00475

Topham, D. J., & Reilly, E. C. (2018). Tissue-resident memory CD8+ T cells: From phenotype to function. *Frontiers in Immunology*, *9*(MAR). https://doi.org/10.3389/fimmu.2018.00515

Trujillo, J. A., Luke, J. J., Zha, Y., Segal, J. P., Ritterhouse, L. L., Spranger, S., … Gajewski, T. F. (2019). Secondary resistance to immunotherapy associated with β-catenin pathway activation or PTEN loss in metastatic melanoma. *Journal for ImmunoTherapy of Cancer*, *7*(1), 1–11. https://doi.org/10.1186/s40425-019-0780-0

Tumeh, P. C., Harview, C. L., Yearley, J. H., Shintaku, I. P., Emma, J., Taylor, M., … Ribas, A. (2014). PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*, *515*(7528), 568–571. https://doi.org/10.1038/nature13954.PD-1

Turner, D. L., Bickham, K. L., Thome, J. J., Kim, C. Y., D'Ovidio, F., Wherry, E. J., & Farber, D. L. (2014). Lung niches for the generation and maintenance of tissue-resident memory T cells. *Mucosal Immunology*, *7*(3), 501–510. https://doi.org/10.1038/mi.2013.67

Tyrakis, P. A., Palazon, A., Macias, D., Lee, K. L., Phan, A. T., Veliça, P., … Johnson, R. S. (2016). S-2-hydroxyglutarate regulates CD8 + T-lymphocyte fate. *Nature*, *540*(7632), 236–241. https://doi.org/10.1038/nature20165

Uyttenhove, C., Pilotte, L., Théate, I., Stroobant, V., Colau, D., Parmentier, N., … Van den Eynde, B. J. (2003). Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nature Medicine*, *9*(10), 1269–1274. https://doi.org/10.1038/nm934

Van Allen, E. M., Miao, D., Schilling, B., Shukla, S. A., Blank, C., Zimmer, L., … Garraway, L. A. (2015). Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science (New York, N.Y.)*, *350*(6257), 207–211. https://doi.org/10.1126/science.aad0095

Van Braeckel-Budimir, N., Varga, S. M., Badovinac, V. P., & Harty, J. T. (2018). Repeated Antigen Exposure Extends the Durability of Influenza-Specific Lung-Resident Memory CD8(+) T Cells and Heterosubtypic Immunity. *Cell Reports*, *24*(13), 3374-3382.e3.

https://doi.org/10.1016/j.celrep.2018.08.073
van der Windt, G. J. W., Everts, B., Chang, C.-H., Curtis, J. D., Freitas, T. C., Amiel, E., … Pearce, E. L. (2012). Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. *Immunity*, *36*(1), 68–78. https://doi.org/10.1016/j.immuni.2011.12.007
Van Houdt, I. S., Sluijter, B. J. R., Moesbergen, L. M., Vos, W. M., De Gruijl, T. D., Molenkamp, B. G., … Oudejans, J. J. (2008). Favorable outcome in clinically stage II melanoma patients is associated with the presence of activated tumor infiltrating T-lymphocytes and preserved MHC class I antigen expression. *International Journal of Cancer*, *123*(3), 609–615. https://doi.org/10.1002/ijc.23543
Veronesi, U., & Cascinelli, N. (1991). Narrow Excision (1-cm Margin). *Archives of Surgery*, *126*(4), 438. https://doi.org/10.1001/archsurg.1991.01410280036004
Vieira Braga, F. A., Hertoghs, K. M. L., Kragten, N. A. M., Doody, G. M., Barnes, N. A., Remmerswaal, E. B. M., … van Lier, R. A. W. (2015). Blimp-1 homolog Hobit identifies effector-type lymphocytes in humans. *European Journal of Immunology*, *45*(10), 2945–2958. https://doi.org/10.1002/eji.201545650
Vignali, D. A. A., Collison, L. W., & Workman, C. J. (2008). How regulatory T cells work. *Nature Reviews Immunology*, *8*(7), 523–532. https://doi.org/10.1038/nri2343
Wakim, L. M., Woodward-Davis, A., & Bevan, M. J. (2010). Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(42), 17872–17879. https://doi.org/10.1073/pnas.1010201107
Wakim, L. M., Woodward-Davis, A., Liu, R., Hu, Y., Villadangos, J., Smyth, G., & Bevan, M. J. (2012). The molecular signature of tissue resident memory CD8 T cells isolated from the brain. *Journal of Immunology (Baltimore, Md. : 1950)*, *189*(7), 3462–3471. https://doi.org/10.4049/jimmunol.1201305
Waldman, A. D., Fritz, J. M., & Lenardo, M. J. (2020). A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nature Reviews Immunology*, *20*(11), 651–668. https://doi.org/10.1038/s41577-020-0306-5
Wan, P. T. C., Garnett, M. J., Roe, S. M., Lee, S., Niculescu-Duvaz, D., Good, V. M., … Marais, R. (2004). Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell*, *116*(6), 855–867. https://doi.org/10.1016/S0092-8674(04)00215-6
Wang, B., Wu, S., Zeng, H., Liu, Z., Dong, W., He, W., … Huang, J. (2015). CD103+ Tumor Infiltrating Lymphocytes Predict a Favorable Prognosis in Urothelial Cell Carcinoma of the Bladder. *Journal of Urology*, *194*(2), 556–562. https://doi.org/10.1016/j.juro.2015.02.2941
Wang, R., Lu, M., Zhang, J., Chen, S., Luo, X., Qin, Y., & Chen, H. (2011). Increased IL-10 mRNA expression in tumor-associated macrophage correlated with late stage of lung cancer. *Journal of Experimental and Clinical Cancer Research*, *30*(1), 1–9. https://doi.org/10.1186/1756-9966-30-62
Wang, Y., Sun, S. N., Liu, Q., Yu, Y. Y., Guo, J., Wang, K., … He, Y. W. (2016). Autocrine complement inhibits IL10-dependent T-cell-mediated antitumor immunity to promote tumor progression. *Cancer Discovery*, *6*(9), 1022–1035. https://doi.org/10.1158/2159-8290.CD-15-1412
Wang, Z. Q., Milne, K., Derocher, H., Webb, J. R., Nelson, B. H., & Watson, P. H. (2016). CD103 and intratumoral immune response in breast cancer. *Clinical Cancer Research*, *22*(24), 6290–6297. https://doi.org/10.1158/1078-0432.CCR-16-0732
Warner, A. B., Palmer, J. S., Shoushtari, A. N., Goldman, D. A., Panageas, K. S., Hayes, S. A., … Chapman, P. B. (2020). Long-term outcomes and responses to retreatment in

patients with melanoma treated with PD-1 blockade. *Journal of Clinical Oncology*, *38*(15), 1655–1663. https://doi.org/10.1200/JCO.19.01464

Webb, J. R., Milne, K., & Nelson, B. H. (2015). PD-1 and CD103 Are Widely Coexpressed on Prognostically Favorable Intraepithelial CD8 T Cells in Human Ovarian Cancer. *Cancer Immunology Research*, *3*(8), 926–935. https://doi.org/10.1158/2326-6066.CIR-14-0239

Webb, John R., Milne, K., Watson, P., DeLeeuw, R. J., & Nelson, B. H. (2014). Tumor-infiltrating lymphocytes expressing the tissue resident memory marker cd103 are associated with increased survival in high-grade serous ovarian cancer. *Clinical Cancer Research*, *20*(2), 434–444. https://doi.org/10.1158/1078-0432.CCR-13-1877

Weber, J. S., D'Angelo, S. P., Minor, D., Hodi, F. S., Gutzmer, R., Neyns, B., … Larkin, J. (2015). Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): A randomised, controlled, open-label, phase 3 trial. *The Lancet Oncology*, *16*(4), 375–384. https://doi.org/10.1016/S1470-2045(15)70076-8

Wei, S. C., Levine, J. H., Cogdill, A. P., Zhao, Y., Anang, N. A. S., Andrews, M. C., … Allison, J. P. (2017). Distinct Cellular Mechanisms Underlie Anti-CTLA-4 and Anti-PD-1 Checkpoint Blockade. LID - S0092-8674(17)30831-0 [pii] LID - 10.1016/j.cell.2017.07.024 [doi]. *Cell*, *170*(6), 1120–1133. https://doi.org/10.1016/j.cell.2017.07.024.Distinct

Weng, N. P., Levine, B. L., June, C. H., & Hodes, R. J. (1995). Human naive and memory T lymphocytes differ in telomeric length and replicative potential. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(24), 11091–11094. https://doi.org/10.1073/pnas.92.24.11091

Williams, J. B., Horton, B. L., Zheng, Y., Duan, Y., Powell, J. D., & Gajewski, T. F. (2017). The EGR2 targets LAG-3 and 4-1BB describe and regulate dysfunctional antigen-specific CD8+ T cells in the tumor microenvironment. *Journal of Experimental Medicine*, *214*(2), 381–400. https://doi.org/10.1084/jem.20160485

Woo, S. R., Turnis, M. E., Goldberg, M. V., Bankoti, J., Selby, M., Nirschl, C. J., … Vignali, D. A. A. (2012). Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Research*, *72*(4), 917–927. https://doi.org/10.1158/0008-5472.CAN-11-1620

Woon, H. G., Braun, A., Li, J., Smith, C., Edwards, J., Sierro, F., … Kaur, A. (2016). Compartmentalization of Total and Virus-Specific Tissue-Resident Memory CD8+ T Cells in Human Lymphoid Organs. *PLOS Pathogens*, *12*(8), e1005799. https://doi.org/10.1371/journal.ppat.1005799

Workel, H. H., Komdeur, F. L., Wouters, M. C. A., Plat, A., Klip, H. G., Eggink, F. A., … Nijman, H. W. (2016). CD103 defines intraepithelial CD8+ PD1+ tumour-infiltrating lymphocytes of prognostic significance in endometrial adenocarcinoma. *European Journal of Cancer (Oxford, England : 1990)*, *60*, 1–11. https://doi.org/10.1016/j.ejca.2016.02.026

Wu, J., Madi, A., Mieg, A., Hotz-Wagenblatt, A., Weisshaar, N., Ma, S., … Cui, G. (2020). T Cell Factor 1 Suppresses CD103+ Lung Tissue-Resident Memory T Cell Development. *Cell Reports*, *31*(1), 107484. https://doi.org/10.1016/j.celrep.2020.03.048

Wu, T., Hu, Y., Lee, Y.-T., Bouchard, K. R., Benechet, A., Khanna, K., & Cauley, L. S. (2014). Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *Journal of Leukocyte Biology*, *95*(2), 215–224. https://doi.org/10.1189/jlb.0313180

Xu, Y., Chaudhury, A., Zhang, M., Savoldo, B., Metelitsa, L. S., Rodgers, J., … Dotti, G. (2016). Glycolysis determines dichotomous regulation of T cell subsets in hypoxia.

Journal of Clinical Investigation, *126*(7), 2678–2688. https://doi.org/10.1172/JCI85834
Yarchoan, M., Hopkins, A., & Jaffee, E. M. (2017, December). Tumor Mutational Burden and Response Rate to PD-1 Inhibition. *The New England Journal of Medicine*, Vol. 377, pp. 2500–2501. https://doi.org/10.1056/NEJMc1713444
Yu, J., Du, W., Yan, F., Wang, Y., Li, H., Cao, S., … Ren, X. (2013). Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer. *Journal of Immunology (Baltimore, Md. : 1950)*, *190*(7), 3783–3797. https://doi.org/10.4049/jimmunol.1201449
Yu, J., Sun, J., Wang, S. E., Li, H., Cao, S., Cong, Y., … Ren, X. (2011). Upregulated expression of indoleamine 2, 3-dioxygenase in primary breast cancer correlates with increase of infiltrated regulatory T cells in situ and lymph node metastasis. *Clinical and Developmental Immunology*, *2011*. https://doi.org/10.1155/2011/469135
Zaretsky, J. M., Garcia-Diaz, A., Shin, D. S., Escuin-Ordinas, H., Hugo, W., Hu-Lieskovan, S., … Ribas, A. (2016). Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. *The New England Journal of Medicine*, *375*(9), 819–829. https://doi.org/10.1056/NEJMoa1604958
Zhang, D., He, W., Wu, C., Tan, Y., He, Y., Xu, B., … Jiang, J. (2019). Scoring system for tumor-infiltrating lymphocytes and its prognostic value for gastric cancer. *Frontiers in Immunology*, *10*(JAN), 1–11. https://doi.org/10.3389/fimmu.2019.00071
Zhang, L., Conejo-Garcia, J. R., Katsaros, D., Gimotty, P. a, Massobrio, M., Regnani, G., … Coukos, G. (2003). Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *The New England Journal of Medicine*, *348*(3), 203–213. https://doi.org/10.1056/NEJMoa020177
Zhang, Y., & Wang, X. (2020). Targeting the Wnt/β-catenin signaling pathway in cancer. *Journal of Hematology and Oncology*, *13*(1), 1–16. https://doi.org/10.1186/s13045-020-00990-3
Zheng, M. Z. M., & Wakim, L. M. (2021). Tissue resident memory T cells in the respiratory tract. *Mucosal Immunology*, (June), 1–10. https://doi.org/10.1038/s41385-021-00461-z
Zuber, J., Shonts, B., Lau, S.-P., Obradovic, A., Fu, J., Yang, S., … Sykes, M. (2016). Bidirectional intragraft alloreactivity drives the repopulation of human intestinal allografts and correlates with clinical outcome. *Science Immunology*, *1*(4), 139–148. https://doi.org/10.1126/sciimmunol.aah3732.Bidirectional