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The clinically relevant role tregs play in establishing an immunosuppressive tumor microenvironment in melanoma

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THE CLINICALLY RELEVANT ROLE TREGS PLAY IN ESTABLISHING AN IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENT IN MELANOMA

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ABSTRACT

The study of regulatory T cells (Tregs) is a relatively new field. Within the past few decades, research on Tregs has greatly deepened scientists' understanding of the link between the immune system and cancer. The study of melanoma is one such cancer that has benefited greatly from this area of study. Tregs are a subset of CD4+ T cells (TCs) that are either generated in the thymus or in the periphery. The main role of Tregs in normal immune physiology is to suppress immune cells. This is an essential component in the prevention of autoimmunity. In melanoma, however, Tregs prevent components of the immune system from mounting a robust response to cancerous lesions and tumors.

Tregs have been observed to infiltrate melanoma tumors due to chemokines and other soluble signaling molecules such as CCL1 and CCL22. Once Tregs accumulate inside melanoma tumors, they generate an immunosuppressive microenvironment in a contact-dependent and contact-independent manner. IL-10 secretion and use of the CTLA-4

pathway were observed to be the most well characterized modes of suppression but other mechanisms are still being discovered.

Clinicians can take advantage of new therapeutics to modulate the activity of Tregs. Exogenous administration of antibodies that bind to CTLA-4, PD-1, CCR4 and other receptors and molecules can prevent Treg development and action. Preventing Tregs from carrying out their suppressive function may allow other elements of the immune system, such as CD8+ TCs, to target and destroy melanoma cells. Clinicians can also measure the relative abundance of Tregs or use the ratio of effector TCs (Teff) and Tregs to predict patient outcomes and survival.

More research is needed to determine that precise mechanisms of Treg infiltration and accumulation within the tumor and the mode of Treg suppression. This paper finds that there is no standard Treg identification marker. This can lead to aberrant results and failures such as the inability to distinguish Tregs from melanoma cells that also express Treg-like markers or a failure to identify other Treg subtypes. Lack of consensus also extends to the prognostic value of Tregs due, in part, to small sample sizes and the inability to accurately identify Tregs *in vivo*. Future research must focus on Treg identification, action, and the elucidation of therapeutic mechanisms. These future studies will ensure that clinicians have the correct information to choose the proper melanoma treatment that will target the specific Treg populations found within patients' melanoma tumors.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIRE ..... Autoimmune Regulator

APC ..... Antigen Presenting Cell

BC ..... B Cell

BCR ..... B Cell Receptor

CD ..... Cluster of Differentiation

cTEC ..... Cortical Thymic Epithelial Cell

CTL ..... Cytotoxic T Lymphocyte

IL ..... Interleukin

KO ..... Knockout

MHC ..... Major Histocompatibility Complex

mTEC ..... Medullary Thymic Epithelial Cell

NK ..... Natural Killer

PD-1 ..... Program Cell Death Protein 1

TC ..... T Cell

TCR ..... T Cell Receptor

Teff ..... Teffector Cell

Tnaive ..... Naïve T Cell

Treg ..... Regulatory T Cell

UV ..... Ultraviolet

INTRODUCTION

Brief overview of the adaptive immune system

The adaptive immune system has evolved to mount a large and long-lasting humoral and cellular response towards pathogenic threats. The two principle cell types that are involved in this process are B cell (BC) lymphocytes and T cell (TC) lymphocytes, although there are many other players. Both types of lymphocytes arise from the common lymphoid progenitor in the bone marrow, but their sites of maturation differ: BCs stay in the bone marrow during the maturation process while TCs move to the thymus. Once in the thymus, TCs can differentiate into different subtypes which are often characterized by distinct surface receptors and co-receptors and/ or transcription factors. TCs which express the CD4 co-receptor on their surface become CD4+ TCs, also known as helper TCs. Likewise, TCs that express CD8 are classified as CD8+ TCs, commonly referred to as either cytotoxic TCs or cytotoxic lymphocytes (CTLs). Furthermore, TCs that express CD25, CD4 and the transcription factor FoxP3 are classified as CD4+ CD25+ FoxP3+ TCs, which, henceforth will be notated as regulatory TCs (Tregs). BCs recognize soluble antigens using a membrane bound antibody called the BC receptor (BCR). In contrast, the TC receptor (TCR) of TCs only recognizes peptide antigens that are presented by Major Histocompatibility Complex (MHC) molecules found on nearly all nucleated somatic cells. Despite these differences, BCs and TCs work together with elements of the innate immune system to quickly identify and respond to internally- and externally-

derived pathogenic antigens. As effective as the mammalian immune system is at targeting and destroying dangerous antigens, left unchecked, the immune system has great potential to aberrantly recognize and damage self-tissue.

Regulatory T Cells

Regulatory T cells (Tregs) are a subset of CD4+ TCs that, as the name suggests, have a regulatory function in the immune response. Since their discovery in 1969 by Japanese researchers Nishizuka and Sakakura, Tregs have been the focus of much interest not only because of their unique function but also because of their clinical adaptability in autoimmune diseases and cancer.[1] Even within the Treg subset there exists multiple different variations of Tregs. In general, however, there are two separate origins of Treg cells which give rise to two of the best defined Treg subtypes. Tregs originating in the thymus are often called thymic Tregs (tTregs). Tregs that originate in the periphery, or extrathymically, are called peripheral Tregs (pTregs) (Figure 1).[2] Currently, Tregs are classified as CD4+ CD25+ TCs that express Forkhead box protein 3 (FoxP3+). In humans, a condition known as IPEX demonstrates the autoreactive environment that is caused by a lack of Tregs.[3] In IPEX, patients have a mutation in the Treg marker FoxP3. This gene codes for the master transcription factor that directs the development of Treg cells.[4] As a result of this mutation, Treg cells fail to develop which leads to massive autoimmunity, and early death.[5]

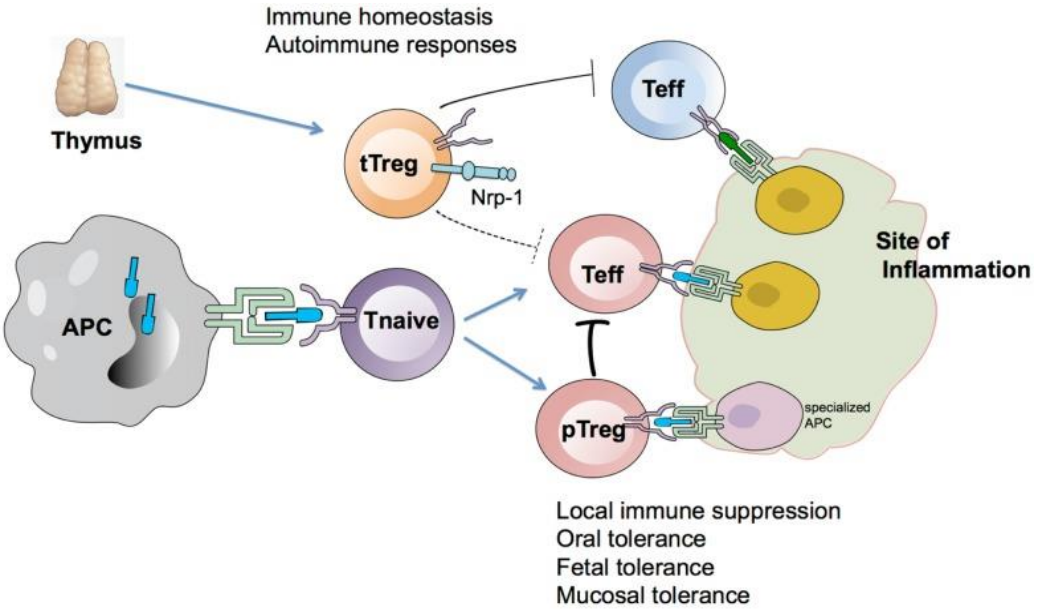

Figure 1. tTreg and pTreg generation occur in different compartments but perform overlapping function.
tTregs are generated in the thymus due to thymic-dependent selection mechanisms. They suppress activated TCs, also known as Teffector cells (Teff). pTregs are generated extrathymically from naive CD4+ TCs (Tnaive) in sites of inflammation. Like tTregs, pTregs can suppress Teff (Adapted from Yadav et al., 2013).[2]

Mechanism of Treg immunosuppression

Interleukin 2 (IL-2) is an essential cytokine for lymphocyte regulation and an important growth factor for all TCs. Tregs express high levels of CD25, a component of the IL-2 receptor (IL-2R), but express low levels of IL-2. This allows Tregs to bind exogenous IL-2, effectively starving the surrounding conventional TCs of this cytokine (Table 1).[4] In

addition, through unknown mechanism, Tregs can downregulate IL-2 mRNA levels in conventional TCs.[6] Thus, in the presence of Tregs, TCs, especially CD4+ TCs, are starved of IL-2 and may die via cytokine deprivation-mediated apoptosis.[7]

Another mechanism of suppression is through Treg cytokine secretion of IL-10, TGF-B, and IL-35. IL-10 is a potent suppressor of inflammation via the downregulation of proinflammatory cytokines and cell surface receptors such as co-stimulatory receptors on multiple cell types (Table 1).[8] TGF-B is not only needed to maintain the immunosuppressive function of Tregs through sustaining FoxP3 expression but is also thought to have immunosuppressive abilities itself through suppression of IL-2 production and decreasing cell proliferation.[9] IL-35 is a newly discovered cytokine. Although it shares subunits with pro-inflammatory cytokines, it is exclusively anti-inflammatory.[10] At this point, Tregs are the only known source of IL-35.[10] It has been observed that IL-35 can reduce effector TC function and induce TC exhaustion (Table 1).[10] Furthermore, IL-34, recently discovered in 2008, has been shown to be expressed in certain types of FoxP3+ Tregs. This cytokine has less pronounced immunosuppressive effects than the other cytokines; however, IL-34 plays a role in rheumatoid arthritis and other autoimmune diseases in which Tregs are involved (Table 1).[10]

Adenosine secretion by Tregs is another important mechanism of immunosuppression (Table 1). Adenosine is a nucleoside that is formed by the extracellular hydrolysis of ATP. In Tregs, this is accomplished through membrane bound CD39 and CD73

ectonucletidases. When bound to A2 receptors on immune cells such as conventional TCs, DCs, and NK cells, expansion and function of those immune cells is reduced.[11] Although adenosine can be produced by many different cells, Treg-specific knockout of CD39 or CD73 is associated with decreased proliferation of TCs and an increase in pro-inflammatory cytokine secretion from CD4+ conventional TCs.[12,13]

Tregs can also use their surface receptors to exert immunosuppressive effects in a contact-dependent manner. One of these receptors is CTLA-4 which is constitutively expressed on CD4+ FoxP3+ Tregs. Although CTLA-4 is also expressed on conventional TCs, its effect in Tregs is to promote immunosuppression. A study from 2000 showed that a Treg specific knockout of CTLA-4 in mice causes severe autoimmunity.[14] One immunosuppressive mechanism mediated by CTLA-4 expressed on Tregs is to suppress the actions of antigen presenting cells (APCs) by downregulating the expression of B7 receptors in these cells.[15] Therefore, CTLA-4's effect on APCs hinders their ability to effectively present antigens to conventional TCs and thus limits an adaptive immune response (Table 1).[4] It is still unknown, however, whether Treg expression of CTLA-4 is essential for their suppressive effects.[16]

Another surface receptor that Tregs use is PD-1. There are two main ligands for PD-1: PD-L1, which is mainly expressed on APCs, and PD-L2 which has a much varied expression pattern. Interestingly, Tregs express both the receptor PD-1 and its ligand PD-L1.[4] Unlike CTLA-4, PD-1 signaling plays a less critical role in immunosuppression and

tolerance. While CTLA-4 knockout mice die at an early age, mice without functional PD-1 receptors do not die, although they have severe autoimmunity with symptoms similar to lupus (Table 1).[17,18]

Other mechanisms of Treg function include direct cytotoxicity, inhibiting CTLs and ICOS expression (Table 1).[19] In particular, ICOS has proven to be the subject of much research. ICOS is a surface co-stimulatory molecule that is essential for Tregs function. Anti-ICOS Ab antagonists have shown to block Treg suppressive function. ICOS is also important for Treg survival as it confers increased sensitivity to IL-2.[20]

Table 1. Multiple methods of Treg suppression that establishes an immune suppressive environment.

Growth factor starvation	High expression of CD25 coupled with minimum secretion of IL-2 allows Tregs to starve conventional TCs of IL-2
Cytokine secretion	IL-10 – anti-inflammatory
	TGF-B – immunosuppressive and generates pTregs
	IL-35 – anti-inflammatory and promotes TC exhaustion
	IL-34 – unknown function
Adenosine generation	Tregs catalytically hydrolyze adenosine from ATP. Adenosine reduces function of TCs, DCs, and NK cells
Surface receptors	CTLA-4 – immunosuppressive via the binding of B7 family of receptors
	PD-1 and PD-L – immunosuppression via binding to conventional TCs and via generating pTregs
	ICOS – increased survival of Tregs
Direct cytotoxicity	Destruction of conventional TCs via perforin and granzyme secretion.

Cancer immunology

It has been known for quite some time that cancer and the immune system are linked. In early experiments, it was discovered that immunodeficient mice had higher rates of cancer occurrence.[21] This is also true in human patients with primary immunodeficiencies such as hyper-IgE syndrome who are susceptible to certain malignancies.[22] This opened up the door to the possibility that the presence and growth of cancer may be regulated by the host immune system. The very nature of cancer, however, poses a roadblock to researchers' understanding of this possibility. Typically, the immune system is thought to protect against foreign compounds and pathogens. Cancer; however, is neither explicitly foreign nor an exogenous pathogen. Therefore, how does the immune system recognize cancer and effectively mount a specific response to it?

Cancers can produce two main types of antigens which the immune system can respond to: tumor-specific antigens and tumor-associated antigens. Tumor-specific antigens are antigens that are only produced by tumor cells and not by host cells. They are often produced by an accumulation of point and missense mutations. Because these mutations produce different proteins from the host proteins, they can be targeted by immune cells, such as CD8+ TCs.[23] On the other hand, tumor-associated antigens are antigens that are produced by both the tumor and host cells. They can be immunogenic in cases where, for instance, they are upregulated above normal physiological levels or are differentially expressed. Finally, self-antigens can be targeted by autoreactive TCs; however, not much

is known of the importance of this mechanism in the tumor clearance process. It is thought that strongly autoreactive TCs undergo clonal deletion during positive and negative selection. The TCs that remain, however, still weakly recognize self-antigens. If conditions are right, these autoreactive TCs can mount a limited response toward these self-antigens.[23]

Both tumor-specific and tumor-associated antigens, as well as self-antigens, are displayed on MHC class I molecules on the surface of cancer cells which can flag them to be targeted by CTLs. This represents the most important immune-mediated mechanism of tumor clearance. The effects of CD4+ TCs on tumor clearance are less well understood. It is generally recognized that Th1 cells are the most important helper TCs involved in this process. Th1 cells are able to activate macrophages and DCs using IFN-g which can help with tumor clearance and aid in further presentation of tumor antigen on MHC class II molecules. Th2 cells are also seen to be important as well.[24]

As a result of tumor-specific and tumor-associated antigens, an immune response can be mounted directed toward the tumor. Unfortunately, however, cancer cells have developed mechanisms that prevent an effective immune response. Tumor cells can downregulate MHC I molecules or upregulate certain inhibitory receptors such as PD-1 which will inhibit TC targeting. Tumors cells can also express immunosuppressive cytokines such as IL-10 and TGF-B, and also recruit Treg cells into the tumor environment.[25] Melanoma tumors use these types of suppressive mechanisms which makes this cancer a good model

to study the connection between cancer and the immune system. Another potential difficulty in the immunological clearance of tumors is an excessive immune response which can cause autoimmunity. In melanoma, vitiligo, an autoimmune skin disorder, is associated with an immune response toward the tumor, showing the importance of not only mounting a response toward tumor antigens, but also mounting an appropriate and limited response.[26] Tregs are thought to be involved in limiting the immune response.[26]

Role of Tregs in cancer

In addition to their role in immunological tolerance and prevention of autoimmunity, Tregs also have an important role in regulating tumor immunity. Tregs have been observed to infiltrate and accumulate in solid tumors of all different types of cancers.[27] Evidence for how Tregs invade tumors is relatively scarce but researchers have generally seen that the chemokine CCL22 is secreted by both tumor cells and by intratumoral macrophages. Tregs follow the gradients established by CCL22 and other chemokines which will lead them into the tumor.[27] Tregs are also attracted to the inflammatory environment present in many tumors. In addition to thymic Treg infiltration, conventional CD4+ TCs can be induced to differentiate into pTregs due to a cytokine milieu high in TGF-B.[27] Once in the tumor, Tregs will use their TCRs to bind to self-antigens or tumor-associated antigens as Tregs were selected during TC differentiation due to their ability to bind to self-peptides with moderate-high intensity. As a result, Tregs become activated in the tumor environment in response to self-antigens that are released by tumor cells and

presented in the context of MHC receptors (Figure 2).[27] Once activated, Tregs will generate a cytokine milieu which will suppress effector cells that are attempting to target and destroy the tumor. As a result, the tumor is less targeted by the immune system and is allowed to survive. In mice, Treg depletion results in higher rates of tumor rejection.[28] The lack of Tregs in these models are associated with altered intratumoral cytokine environments and increased CD8+ TC destruction of the tumors, demonstrating the essential role of Tregs in the regulation of cancer clearance by the immune system.[28]

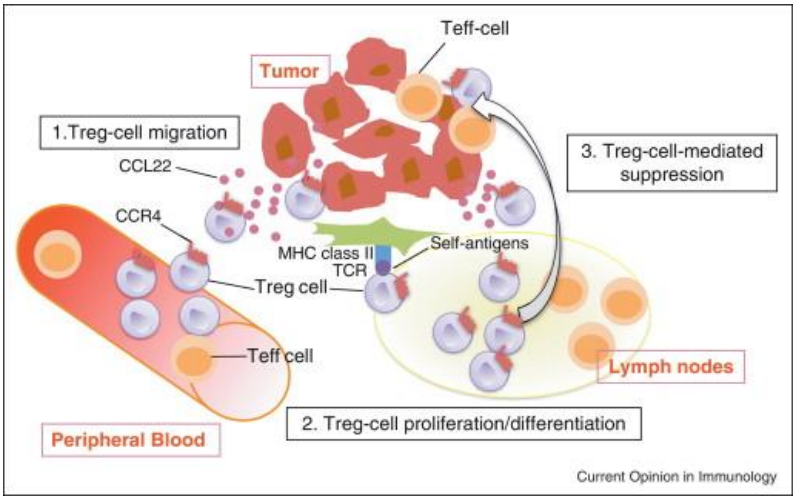

Figure 2. Tregs mediated the establishment of a suppressive tumor microenvironment.
Tregs infiltrate the tumor environment by expressing CCR4 and binding to CCL22 which is secreted by tumor cells. Tregs then bind to self-antigen peptides that are released by tumor cells and then subsequently expressed on MHC class II receptors on the surface of APCs, thereby activating Tregs. These activated Tregs then either stay in the tumor or go to nearby lymph nodes and suppress effector TCs (Adapted from Nishikawa and Sakaguchi, 2014).[27]

Melanoma

Epidemiology and risk factors

Melanoma is an important cancer model because of this cancer can be consistently recreated in different animals. For immunologists, melanoma is also important because tumors can undergo spontaneous regression, showing that the cancer is able to be targeted by the immune system and cleared. For these reasons, this paper will focus on melanoma and the role Tregs have in its immunogenicity and clearance.

Melanomas are neoplasms of melanin producing melanocytes, the cells that give skin its dark color. Melanoma is a common and fatal cancer in certain populations. In the US, melanoma is the sixth most common fatal cancer among Caucasian populations.[29] In populations that have higher levels of UV exposure, such as populations in Australia, melanoma is even more common as it is the fourth most common cancer among males and the third most common among females.[30] Making matters worse, melanoma rates are quickly increasing, especially for the Caucasian population. Diagnoses of melanoma doubles roughly every 10 to 20 years.[30] Besides its high rate of occurrence and mortality, this cancer also proves to be a financial hardship for patients and the healthcare system with an annual cost of almost $1 billion in just the USA.[31] When melanoma metastasize, the cancer becomes dramatically harder to treat and the response rate to treatment drops to as low as 5%.[32]

The biggest risk factor for developing melanoma is exposure to ultraviolet (UV) radiation. Surprisingly, even a single serious sunburn event due to UV exposure in childhood can double the risk of developing melanoma as an adult.[32] The other major risk factor is the presence of melanocytic nevi, more commonly referred to as moles. Diagnosis of melanoma can be made through visual inspection of skin by looking for skin lesions that are asymmetric, irregular, and large. Additionally, clinicians can use the presence of microphthalmia transcription factor, a melanocyte marker, in blood tests to aid in diagnosis.[33]

Pathogenesis

Melanoma is a cancer that is associated with some of the highest rates of mutations. These mutations will effect multiple levels of cellular function including cell cycle and apoptotic regulation, proliferation, metabolism, and signal cascades.[34] Melanoma arises due to genetic mutations caused by UV induced damage or other mutagenic factors. The most common genetic mutations in melanoma are mutations in the genes for *BRAF*, *NF1*, and *NRAS*. Melanoma normally consists of neoplastic tissue that has multiple different genetic mutations and not just a single one. For instance, 80% of benign nevi have the *BRAF* mutation but do not normally progress to melanoma lesions unless subsequent mutations are accumulated such as mutations in *TERT* or *CDKN2A* (Figure 3).[34] As mutations accumulate in a stepwise function, pre-melanoma melanocytes grow in a radial

growth phase. After this, cancerous melanocytes enter a vertical growth phase in which they start to invade areas of the dermis and hypodermis.[35] From here, melanocytes can enter lymphatics and capillaries and spread to secondary locations.

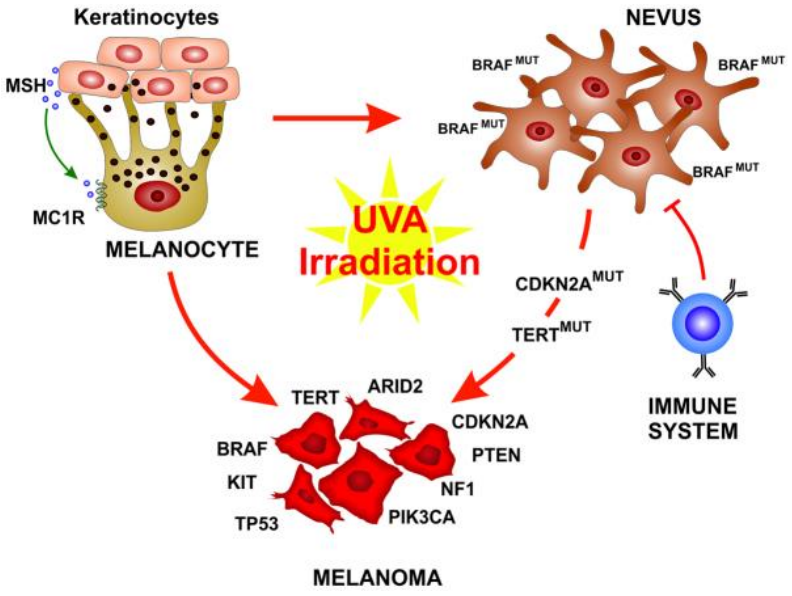

Figure 3. Melanoma generation is due to multiple genomic mutations caused by UV irradiation.
When normal melanocytes are irradiated with UVA light a single mutation can occur in BRAF, thereby creating a pre-melanomic nevi. At this stage, the immune system can prevent its excessive growth. Once more mutations accumulate, however, melanoma can develop and uncontrolled growth can occur (Adapted from Leonardi *et al*., 2018).[34]

SPECIFIC AIMS

Melanoma is a deadly skin cancer which takes the lives of thousands of people each year. Traditional therapies such as excision and radiation are not effective for all patients. A different approach is to take advantage of a patient's own immune system to combat this cancer; however, the effectiveness of such a plan may be blunted by Tregs. Therefore, this paper aims to determine what role Tregs play in melanoma immunology and whether these cells may have a therapeutic role. To accomplish this aim, this paper will:

- Review the recent literature about the evidence of Regulatory T cells in the development, progression, and prognosis of melanoma. Focus will be put on the infiltration of regulatory T cells into melanoma tumors and the ability of regulatory T cells to generate an immunosuppressive tumor microenvironment.

- Investigate the role that the new generation of immunomodulating drugs have on the ability of regulatory T cells to establish a suppressive microenvironment within melanoma tumors.

- Discuss the effectiveness of regulatory T cells to establish an immunosuppressive tumor microenvironment and identify areas for future studies to better define regulatory T cells and their clinical relevance in cancer treatment.

LITERATURE REVIEW

Treg infiltration of melanoma tumors

It is well supported that Treg cells can infiltrate tumors and the surrounding areas.[36-38] In melanoma specifically, there are two times more CD4+ CD25+ Tregs in the lymph nodes of patients with metastatic melanoma when compared to melanoma-free lymph nodes.[39] Furthermore, Tregs that are in cancer sites have been shown to be in direct contact with DCs and conventional TCs which signifies their potential involvement in tumor immunology. These observations underscore the importance of elucidating the mechanism for Treg infiltration in melanoma tumors.

Skin tissue affected by melanoma expresses increased levels of the chemokines CCL22 and CCL1.[40] These chemokines interact with the CCR4 and CCR8 receptors on the surface of Tregs. Ligand binding leads to Treg migration to the tumor site.[40-43] In addition, CCL22 also leads to infiltration of CD8+ CTLs into the tumor and CTL-secreted cytokines may further enhance Treg recruitment into the tumor.[44]

Further studies on chemokines show that CCL21 signaling via CCR7 is involved in Treg recruitment to melanoma tumors. A study in 2010 showed that overexpression of CCL21 in melanoma tumors causes an increase in Treg infiltration when compared to malanomas that have normal or low levels of CCL21.[43] More research is needed to further elucidate

whether chemokines are absolutely needed for Treg migration or whether they keep Tregs inside the tumor

Function of Tregs in melanoma

A strong immune response toward cancer requires the presence and action of Th1 and CTLs.[25] Tregs have multiple methods of suppressing these cells and other immune responses. Not all methods, however, are used or favored in all scenarios. Examining the property of Tregs within melanoma environments can shed light on the mechanism of Treg action. In a study of Tregs taken from patients with metastatic melanoma, Tregs showed high expression of membrane bound CTLA-4 and production of IL-10.[39] Interestingly, however, most Treg populations did not have a high expression of TGF-B. When these Tregs were isolated alongside CD4+ and CD8+ conventional TCs, a dose-dependent inhibition in proliferation of these TCs was observed. In addition, IFN-g and IL-2 secretion from conventional TCs was significantly reduced.[39] In this same study, Treg suppression of CD4+ conventional TCs was observed in both a cytokine- and cell contact-dependent manner.[39] These results support previous evidence that Tregs generate an immunosuppressive environment via IL-10 and CTLA-4.[45]

Adenosine secretion is also an important component of Treg function in melanoma. Tregs that express CD39 and CD73 – the surface bound ectonucletidases – generate high levels of extracellular adenosine inside melanoma tumors, which promotes immunosuppression

of CD4+ and CD8+ TCs. However, Tregs are not the only cells within melanoma tumors to secrete adenosine. Both melanoma cells and CD4+ conventional TCs can secrete adenosine in a CD39-dependent and CD73-dependent manner.[46] In addition, Tregs upregulate CD39 and CD73 levels in response to exogenous adenosine, suggesting a positive feedback loop in which adenosine released from melanocytes causes Tregs to secrete more adenosine which in turn creates an immunosuppressive environment.[47] However, the exact role of Tregs in establishing or maintaining the adenosine milieu in melanoma requires further research.

Treg T cell receptor repertoire for melanoma

Little is known about exactly what antigens are recognized by TCRs on Tregs. Part of the problem is that Tregs can be either thymically derived (tTregs) or peripherally induced from conventional TCs (pTregs), which leads to differences in the repertoire of TCRs expressed by each subtype. However, because these two Treg subtypes are phenotypically similar to each other, it is difficult to isolate subtype-specific Tregs. In the context of cancer and melanoma, tTregs contain TCRs that mostly recognize self-antigens while pTregs contain TCRs that recognize non-self-, tumor-associated, and tumor- specific antigens (Figure 4).[48,49]

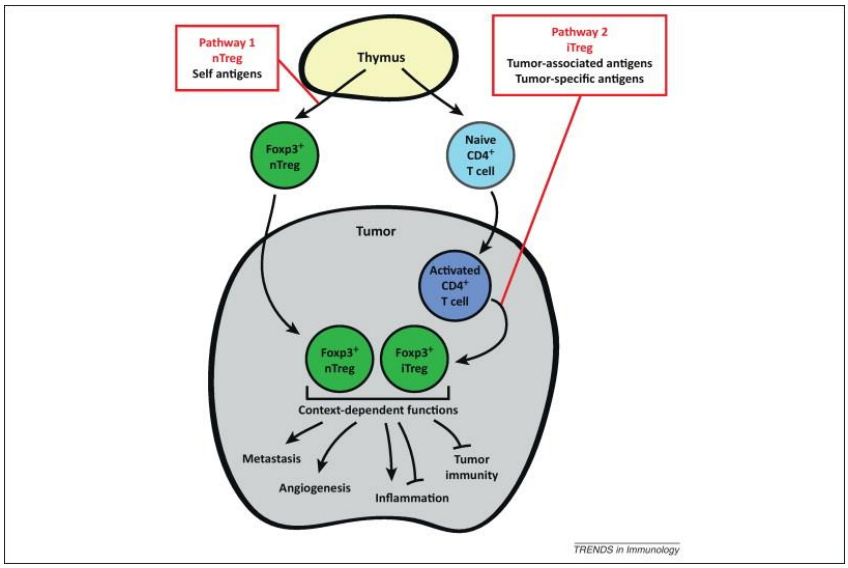

Figure 4. Thymically derived Tregs and peripherally induced Tregs represent separate TCR repertoire populations but perform overlapping function. nTregs, or tTregs, are directly thymically derived and express TCRs that preferentially recognize self-antigens. Once they bind to self-antigens inside the tumor environment, Tregs will promote a pro-tumor microenvironment. Peripherally derived pTregs, or iTregs, express TCRs that preferentially bind to tumor-associated and tumor-specific antigens. When iTregs bind to their cognate antigens, they will help tTregs to promote a pro-tumor environment (Adapted from Séralini *et al.*, 2012).[49]

Many of the antigens associated with melanoma are endogenous self-made proteins, such as tyrosinases. During TC differentiation, TCs are given survival signals if their TCRs bind very weakly to self-antigens. This weak binding results in conventional TCs that are not able to mount a strong response against self-antigens and melanoma-associated antigens. On the other hand, TCs that bind self-antigens with higher affinity will either die by apoptosis or develop into Tregs. One of the most important regulators of this selection mechanism is a protein called autoimmune regulator (AIRE). This protein

regulates the expression of self-antigens in specialized thymic epithelial cells called medullary thymic epithelial cells (mTECs). As a result, these antigens can be displayed to TCs during the negative selection step in TC differentiation.[50] In AIRE-deficient mice, mTECs are unable to express self-antigens, such as tyrosinase related protein-1 (TRP-1).[51] This results in defective negative selection and lack of elimination of self-reactive TCs. In an AIRE-deficient melanoma mouse model, tumor growth is slowed principally due to the increase in effector TCs that are autoreactive and target self-antigens and melanoma-associated antigens on melanoma cells.[51] In addition, an increased Teff:Treg ratio established by a deficiency in AIRE was correlated with reduced tumor size (Figure 5).[51]

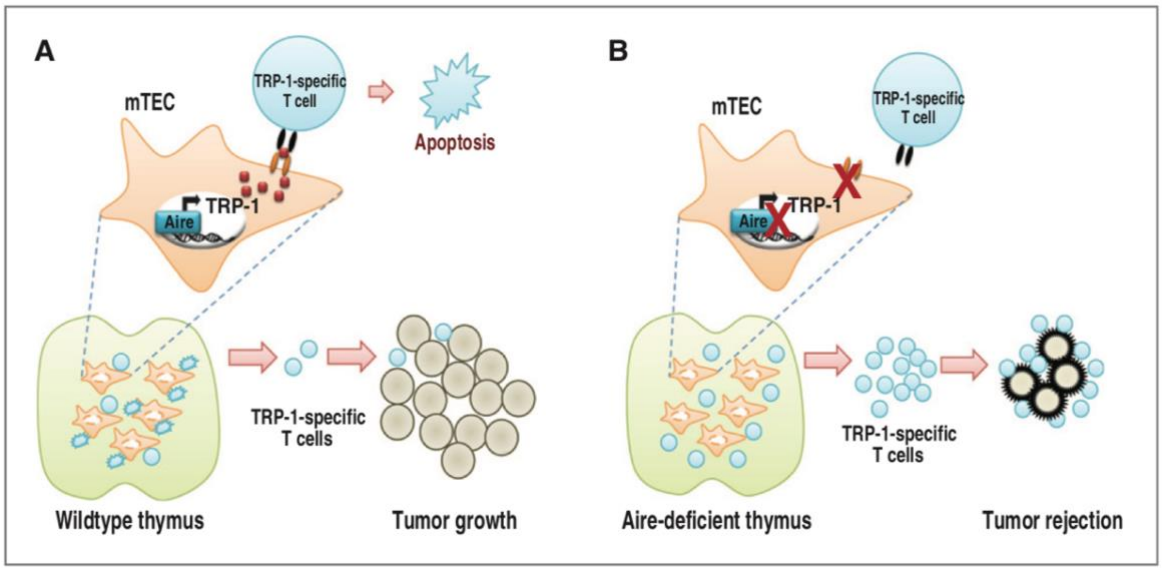

Figure 5. AIRE deficiency promotes tumor rejection by allowing tumor-specific TCs to survive TC development in the thymus.
(A) During TC development in the thymus, AIRE expression allows for peripheral proteins, such as TRP-1 to be expressed by mTECs. When TCs with TCRs specific for TRP-1 bind to receptors on mTEC that express TRP-1, these TRP-1-specific TCs will die via apoptosis. As a result of the lack of TRP-1-specific TCs, TRP-1-expressing tumors are allow to survive and grow. (B) In AIRE KO models, TRP-1 is not expressed by mTEC, thereby allowing TRP-1-specific TCs to survive. This will allow these TRP-1-specific TCs to attack TRP-1-expressing tumor cells, thereby resulting in tumor destruction and rejection (Adapted from Zhu *et al*., 2013).[51]

Modulating Treg activity to increase immune response to Tumor cells

The use of IL-2 in modifying Treg activity

IL-2 is an important cytokine initially characterized in 1976 as a TC growth factor.[52] Administration of high dose IL-2 therapy was approved in 1998 to treat late stage and metastatic melanoma. IL-2 is produced mostly by CD4+ TCs but is also produced by CD8+ TCs and other lymphocytes. The receptor for IL-2 is IL-2R and it is expressed on the surface of a myriad of different cell types including TCs, BCs, and endothelial cells. In the most stereotypical case, IL-2R is upregulated in response to TCR stimulation and costimulation.[53] IL-2R signaling increases effector cytotoxic functions of TCs and promotes differentiation and development of memory and effector cells.[53] These and other effects of IL-2R signaling increase immune response and function.[53] In mice deficient in IL-2, severe autoimmunity developed, suggesting that a lack of IL-2 causes an increase, not a decrease in immune response.[54] These observations open up the possibility that IL-2, in addition to promoting a robust immune response, also induces immune regulatory function. It is believed that this IL-2-dependent regulatory function is mediated through Tregs.[55]

IL-2 is essential for Treg development as IL-2 deficiency in mice results in low Treg numbers.[56] IL-2 promotes the survival and proliferation of thymically derived tTregs.[57] Also, in conjunction with TGF-B, IL-2 supports the differentiation of conventional TCs

to pTregs.[58] Thus, high dose IL-2 administration used in melanoma and other cancer therapy may not only aid in increasing the antitumor effects of conventional TCs by increasing their activity and survival, but may also help establish an pro-tumor immunosuppressive environment by enhancing Treg survival and function. In melanoma, high dose IL-2 therapy not only increases the overall Treg population, but also significantly increases a highly suppressive ICOS+ Treg subset.[59] The increase in ICOS+ Tregs may be particularly detrimental in melanoma patients because melanoma cells express the ligand for ICOS. In fact, patients that had increased ICOS+ Tregs due to IL-2 therapy had worse clinical outcomes than patients that had a less expanded ICOS+ Treg population.[59]

CTLA-4 modification to inhibit Treg function

CTLA-4 is an important immune checkpoint inhibitor (ICI) that is expressed mainly on TCs. Its primary function is to inhibit TC function by outcompeting CD28 for B7 molecules that are expressed on APCs such as DCs (Figure 6).[60] Tregs constitutively express CTLA-4, therefore it is accepted that Tregs have some role in the mechanism of action of ICIs, but whether ICIs have a direct or indirect effect on Tregs is under debate.

Modulating Treg function using the CTLA-4 pathway is accomplished through the administration of antibodies that target CTLA-4. The proposed mechanism for how these antibodies effect Tregs is that they promote antibody-dependent cellular cytotoxicity

(ADCC) directed towards Tregs.[61] Two anti-CTLA-4 mAbs, Ipilimumab and tremelimumab, have been shown to target Tregs using FcgR-mediated ADCC in melanoma mouse models as well as in *ex vivo* experiments in melanoma patients.[61] This Treg depletion was associated with an increased Teff:Treg ratio and reduced Treg infiltration into tumors.[62] ADCC is thought to be accomplished via NK cells and macrophages that express at least some form of Fcg receptor (FcgR) which recognizes the IgG antibody molecules.[63]

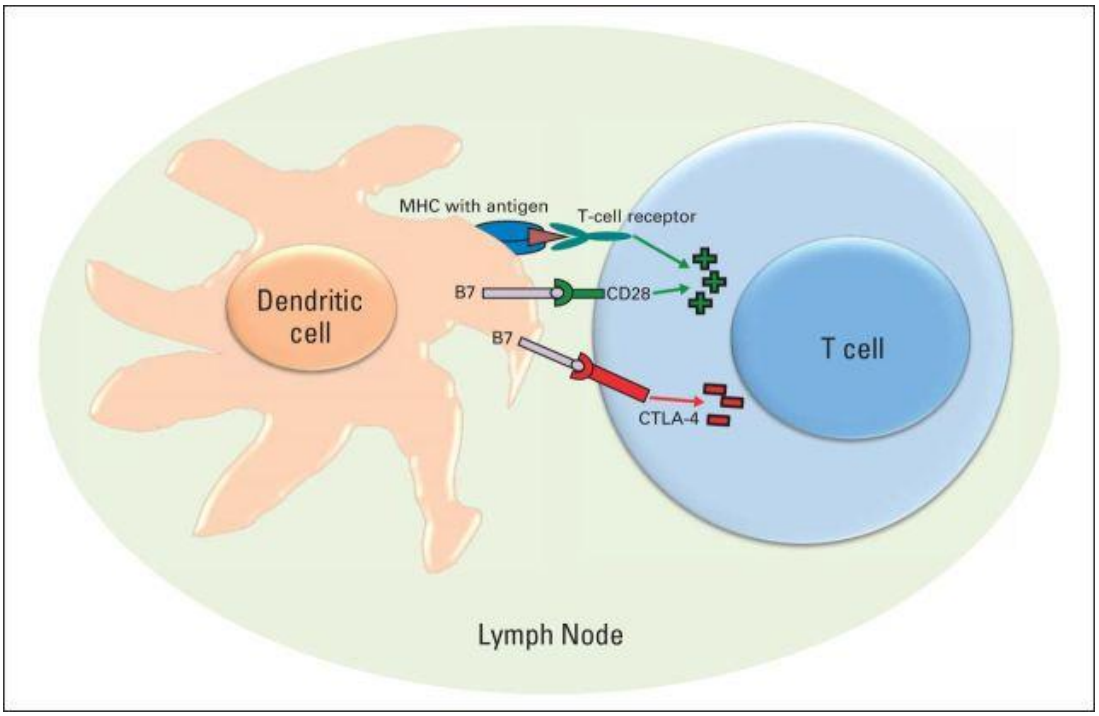

Figure 6. CTLA-4 promotes inhibitory signals in TCs thereby suppressing TC activity and effector function.
Both CD28 and CTLA-4, expressed on TCs, bind to B7 on APCs, such as DCs. CD28 promotes activation signals to TCs, allowing the TC to be activated. CTLA-4, on the other hand, has a higher affinity for B7 and, thus will outcompete CD28 for B7 binding. CTLA-4 sends inhibitory signals to TCs which inhibits TC activation (Adapted from Postow et al., 2015).[60]

Use of PD-1 antibody antagonists to reduce Treg function and induction

The normal immunological role of PD-1 is to prevent autoimmunity or excessive immune response.[4] PD-1 does this by inhibiting TC function and by increasing the survival and function of Tregs via its binding to PD-L1 and PD-L2.[4] Through this binding, PD-1

activation on effector TCs leads to TCR signal inhibition and is associated with TC exhaustion which is a state of reduced TC effector function.[64,65]

PD-1 is involved in the immune-suppressive role of Tregs in melanoma. In a 2009 study, anti-PD-1 mAb was observed to effect Treg-mediated immunosuppression.[66] When CTL cells which recognize melanoma antigens were cultured *in vitro* with Tregs, CTL proliferation was, as expected, hindered.[66] Treatment with anti-PD-1 Ab, however, resulted in both an increase in frequency and total number of CTLs, suggesting that PD-1 somehow mediates Treg suppression of CTLs.[66] Clues to the mechanism of how Tregs use the PD-1 pathway to restrain CTL generation come with further observations that Tregs cause an upregulation of PD-1 and PD-L1 in CTLs when the two cell lines are cultured together. Addition of Anti-PD-1 mAb to the co-culture was able to prevent this upregulation.[66] These observations suggests that in melanoma, Tregs may use the PD-1 pathway to carry out suppressive function by both upregulating and binding to PD-1 receptors on the surface of effector TCs. Further studies show that nivolumab, an FDA approved anti-PD-1 mAb, can rescue Treg-mediated suppression of CD4+ TCs as well as reverse Treg-mediated reduction of CD4+ TC IFN-g production.[67]

Another effect that the PD-1 pathway has on Treg function is that PD-1 plays a large role in peripheral Treg induction from CD4+ conventional TCs. As stated previously, pTregs can be generated from CD4+ conventional TCs in response to certain signaling molecules such as TGF-B. In addition to a TGF-B rich milieu, PD-1 signaling has been observed to

promote pTreg induction when its ligand, PD-L1, is expressed on DCs.[68] Blocking PD-L1 specifically on DCs and by using systemic anti-PD-L1 Ab caused a decrease in pTreg development in mice.[69] In addition, using PD-L1-Ig agonist to stimulate PD- increased FoxP3 expression in pTregs.[70] These observations show that the current array of anti-PD-1 mAb therapy used for melanoma can reduce Treg infiltration into tumors by preventing peripheral Treg conversion via a downregulation of FoxP3.

Modulating LAG-3 receptors inhibits LAG3+ Treg populations

While the CTLA-4 and PD-1 pathways are promising therapeutic targets, researchers have continued to look for the next generation of immunotherapeutic agents. Leukocyte activation gene-3 (LAG-3) is a recently discovered receptor which has profound immunomodulating effects.[71] Like the other checkpoint receptors CTLA-4 and PD-1, LAG-3 can suppress TC immune response toward cancer cells through multiple mechanisms. One of these mechanisms includes the use of Tregs.[72]

High expression of LAG-3 is seen in some Tregs of melanoma patients both in the periphery and around tumor sites. These LAG3+ Tregs have high levels of IL-10 and TGF-B suppressive cytokine secretion and decrease the proliferation of CD4+ TCs.[72] Thus, in melanoma, LAG-3 is associated with a Treg population which performs immunosuppressive functions.[72] Blocking LAG-3 on Tregs decreases their suppression activity as seen in both *in vitro* and *in vivo* experiments.[73] Unfortunately, the mechanism

underlying the role of LAG3 in Treg function remains unknown. However, preliminary clinical trial data shows promising results for LAG525 (Novartis), an IgG4 anti-LAG-3 mAb, either alone or in combination with a PD-1 blocker.[71] Therefore, the LAG-3 pathway is a potentially exciting melanoma immunotherapeutic target which involves Tregs.

Effects of Anti-CCR4 monoclonal antibodies on Treg function

Although CCR4 is expressed on many cell types, its expression is upregulated on Tregs.[41] Because CCR4 signaling is potentially involved in Treg chemotaxis, and because the ligands for CCR4 are upregulated in melanoma, this and other related receptors may be a valuable therapeutic target.

Anti-CCR4 mAb has been approved for use to treat many different types of cancers. In Japan, mogamulizumab was approved in 2012 to treat leukemia and lymphoma. Mogamulizumab was shown to reduce peripheral and intratumoral levels of Tregs and increased CTL response.[73] Another anti-CCR4 mAb, KM2760, has been observed to reduce Tregs inside tumors and also promoted an ADCC response in a Hodgkin's lymphoma mouse model. Similar to melanoma, Hodgkin's lymphoma tumors secrete CCR4 ligands which influence Treg intratumoral invasion.[74] Specifically within melanoma, anti-CCR4 mAb can effectively deplete Tregs in melanoma tumors. In addition, melanoma-specific CD4+ and CD8+ TCs were induced.[75] Whether the latter

observation is due to a reduction in Treg presence cannot be determined. The proposed mechanism for CCR4 Ab antagonists is that the antibodies will block CCR4 on the surface of Tregs. This blockage will prevent CCR4 from binding to its multiple ligands and therefore will prevent the chemotaxis of Tregs into the melanoma tumor environment.

Modulation of Tregs via the GITR pathway

Glucocorticoid-induced tumor necrosis factor-related receptor (GITR) is constitutively expressed at high levels on Tregs and is upregulated in conventional TCs upon activation. It is therefore a good Treg marker. GITR ligand (GITR-L) has a very diverse expression profile but is mainly expressed on APCs and endothelial cells.[76]

In mice, GITR stimulation results in moderately increased CD4+ and CD8+ TC activation, proliferation and cytokine secretion.[77] More significantly, however, is its effect on Tregs. Administration of Fc-GITR-L, an agonist recombinant protein that fuses the Fc region of an Ab and the GITR-L, results in increased Treg numbers.[78] Additionally, in GITR KO mice, very low levels of Tregs are observed.[76] These results suggests that GITR is important for Treg development and expansion. A possible mechanism for this is that GITR increases IL-2 sensitivity. As previously stated, Tregs need an exogenous source of IL-2 and in the absence of this cytokine, Tregs fail to survive. If GITR can increase Treg sensitivity to IL-2, then Tregs are more likely to

receive the signals necessary to survive. Supporting this are studies in IL-2 deficient mice, such as a 2015 experiment which showed that Treg death can be reversed by the administration of an anti-GITR mAb agonist.[76] Importantly, although GITR stimulation causes increased Treg expansion, it also limits the suppressive abilities of Tregs. It does this by downregulating FoxP3 expression and activating non-immunosuppressive pathways such as activating the proinflammatory transcription factor, NF-kB.[78] This makes GITR an interesting pharmacological target.

Anti-GITR Ab agonists have shown promising results at clearing solid tumors. In a mouse melanoma model, anti-GITR Ab was observed to deplete FoxP3 levels in the tumor environment.[27] However, this decrease may not reflect a loss of FoxP3-positive Tregs. Instead, the reduction in FoxP3 expression could represent Tregs that have been effectively shut off. Additionally, anti-GITR Ab can cause intratumoral Treg depletion via Fc-mediated ADCC.[76] Both of these drug actions are extremely desirable because they result in Treg depletion mainly within the tumor, thus allowing for peripheral Treg populations to be preserved so that systemic autoimmunity is less likely to occur. Anti-GITR Abs have shown promising results in mice and are currently tested in clinical trials.[79]

DISCUSSION

Treg infiltration into melanoma tumors

It is widely accepted that Tregs rely on CCR4 and CCR8 expression to chemotactically migrate to sites of inflammation and to resident APCs.[41] On the other hand, Treg infiltration into tissue affected by melanoma needs more investigation. Little is known about what the exact signals and chemokines are that Tregs use to locate melanoma cells and move toward the tissue environment. Complicating matters more, Treg infiltration into melanoma tumors may be dependent on the stage of tumor growth. Possibly at earlier stages of melanoma development, the chemokine CCL2, not CCL22 or CCL1, acting though CCR4 may be the responsible for Treg chemotaxis.[80] Not all researchers agree on this, however.

There is great controversy as to whether chemokines, such as CCL22 and CCL1, working through CCR4 and CCR8 actually promote migration or just prevent Tregs from leaving the tumor environment. In fact, much recent research has been focused on finding CCL-independent and CCR-independent mechanisms for Treg infiltration. This lead to research on vascular endothelial growth factor (VEGF) and its gradient established by melanoma tumors as a critical factor in causing the migration of Tregs. Neuropilin 1 (NRP1), a co-receptor for VEGF, has been shown to be expressed on the surface of Treg cells. nrp1+ Tregs are seen in high concentrations inside VEGF secreting tumors.[81]

NRP1's involvement in Treg migration is not limited to melanoma, as research suggests that 90% of all Tregs found within cancers are nrp1+.[81]

TGF-B-independent Treg function

There is a consensus that IL-10 and TGF-B are important for Treg immunosuppression in a normal physiological environment. In the melanoma tumor microenvironment, however, the role TGF-B plays, is much less agreed upon. In a previously mentioned study where CTLA-4+ Tregs were isolated from melanoma patients, and cocultured alongside CD4+ TCs, the administration of anti-TGF-B antibodies reduced the suppression of conventional TCs.[61] This suggests that Tregs use TGF-B as an immunosuppressive agent. As previously stated, however, Tregs were not the main source of TGF-B in this study. Instead, CD4+ conventional TCs were observed to secrete TGF-B. This suggests that IL-10, not TGF-B plays a large role in Treg mediated immunosuppression in melanoma. Treg-independent sources of TGF-B have been observed before in melanoma. CD4+ conventional Tregs have been observed to secrete TGF-B and, surprisingly, melanoma cells themselves have been shown to express FoxP3 and secrete TGF-B.[44] These observations open the door to a more complicated picture of how Tregs modulate a immunosuppressive microenvironment. This fact also goes contrary to much published literature that suggests that TGF-B is an important immunosuppressive cytokine that is secreted by Tregs.

Tregs as a prognosis marker

Although it is widely accepted that Tregs hamper the immune response toward cancer, in reality, solely looking at Treg infiltration is an inconsistent prognostic marker when comparing different types of cancers. Treg presence in colorectal tumors, for instance, is associated with better survivability but not in lung cancer.[82] When confining the comparison between cancers of the same type, the presence of FoxP3+ TCs was still not necessarily a clear prognostic tool that could predict patient survival.[83] For instance, in one study, FoxP3 staining did not correlate with frequency of metastasis or survival of patients with melanoma.[84] Another study, however, showed a significant correlation between intramelanonic Tregs and patient survival.[85] For melanoma, this variation can be partly explained by the heterogeneity present in melanoma tumor microenvironments and by the different stages of tumor development. Some melanoma tissues, for example, have high expression of COX-2, an important enzyme in the generation of prostaglandins. In patients that have high melanoma tumor expression of COX-2, the presence of Tregs was associated with a better prognosis.[85] Other melanoma tumors express the *BRAF* mutation, while others do not. These differences in melanoma expression profiles emphasizes the importance of distinguishing the specific tumor microenvironment. Therefore, although there is a general consensus that Treg infiltration is correlated with worse prognosis, differences in local tumor environments will prevent the usage of Treg infiltration as an essential prognostic marker for overall survival. With that being said, although the presence of Tregs in a tumor cannot accurately predict patient survival, it can still provide

clinician's with valuable information. In stage III melanoma, for instance, the presence of Tregs could predict cancer recurrence and is an independent marker for melanoma progression.[86] These observations are supported by other studies which link the presence of Tregs with primary tumor progression.[87] This type of information is valuable to determine what type of immune response the body is having toward the tumor. In turn, this will aid in the decision making of what treatment is best.

Problems with Treg identification in melanoma

Once in the tumor, Tregs are maintained by high levels of IL-10 and TGF-B produced by tumor cells.[87] Levels of IL-10 and TGF-B are elevated in melanomas, therefore these cytokines are likely the reason why Treg populations are maintained. Furthermore, as stated previously, a prevalent IL-10 and TGF-B milieu promotes induction of conventional TCs to Tregs. This fact makes it difficult to determine if the Tregs found within tumor environment are composed of tTregs that migrated in, or are pTregs that are derived from conventional TCs that were already present inside the tumor.

Even a cursory review of the available literature reveals variations between study techniques. For instance, there are large variations in Treg identification techniques employed by researchers. Some studies use CD4 and CD25 staining, while others use FoxP3 staining. Relying on only a single marker, such as FoxP3, could lead to aberrant results because, as was explained earlier, FoxP3 and other markers can be expressed on

non-Treg cells. This underscores the need for more reliable Treg markers such as neuropilin-1 which has just recently been discovered and, at least in one occasion, has been correlated with poor prognosis.[10] Other studies attempt to identify the types of cells that are expressing Treg specific markers while other studies do not. Further still, there is no uniformity in where melanoma samples are taken. These variations, on top of small sample sizes, prevent a clear consensus.

Tregs as therapeutic targets

High dose IL-2 therapy may not block Tregs

There is no consensus as to whether high dose IL-2 therapy is beneficial in melanoma patients that have high numbers of intratumoral Tregs. As stated previously, although IL-2 therapy may increase the immune response toward tumors, its effectiveness is limited by its ability to stimulate immunosuppressive Tregs. It is not yet precisely known what the clinical effects of this limitation has on IL-2 therapy. Nevertheless, IL-2 therapy has numerus adverse side effects and has been shown to have limited effectiveness in treating melanoma. In a study by Rosenberg, just 10% of melanoma patients saw some form of tumor regression.[88] Therefore, IL-2 therapy may have limited usefulness.

Possibly a more efficient and efficacious therapy would be not to administer exogenous IL-2 but to target part of its receptor by using antibodies. As stated previously, the IL-2R

has a CD25 component. This can be targeted using anti-CD25 mAb. Unlike in conventional TCs, IL-2R is constitutively expressed on Tregs in the form of CD25. This makes it a good target for Treg modulation because the antibodies will bind more readily to CD25 on the surface of Tregs. Studies have shown that, in both mice and humans, administration of antibodies that target CD25 result in a depletion – in some studies, up to 99% -- of Tregs in melanoma models. This depletion, however, failed to consistently reduce tumor growth in mice and did not have an impact on survival in humans.[89] These results may be due to a cross reactivity of the antibody not only with Tregs but of anti-tumor conventional TCs that also express CD25. The limited effectiveness of anti-CD25 Abs could also be due to differences between peripheral environments and the environment within the tumor. *In vivo* experiments in mice show that anti-CD25 Abs can eliminate Tregs via a FcgRIII-mediated ADCC mechanism; however, this mainly occurs in the periphery. Inside the tumor, although FcgRIII is present, its ability to promote ADCC of Tregs is abrogated by an inhibitory IgG constant region receptor found in high concentrations inside melanoma tumors, FcgRIIb.[90] Researchers have the ability to change the affinity of antibody-FcgR binding via modulating Fc regions. Because of this, further research is needed to determine the most effective class of anti-CD25 Ab which will elicit the strongest response both in the periphery and within the tumor environment.

Another interesting approach is to administer a fusion protein that consists of IL-2 and diphtheria toxin. Cell death occurs when the fusion protein is endocytosed. This type of drug will preferentially effect cells that express high levels of IL-2R, such as Tregs. One

version of this, Denileukin diftitox, has been shown to reduce peripheral Tregs in melanoma patients, but it is not yet approved for market use.[28] Researchers do not agree that this is a safe therapy as it may aberrantly destroy beneficial immune cells.

The PD-1 pathway may not be involved in Treg suppression

The exact role that the PD-1 pathway plays in Tregs is still under debate. Some researchers believe that Treg's suppressive abilities involve PD-1 while other researchers observe no difference in Treg suppressive actions in PD-1 KO mice. In one study, for instance, PD-L1 was observed to induce pTreg induction. When wildtype APCs expressing PD-L1 were cocultured with CD4+ TCs in the presence of TGF-B, conversion of CD4+ TCs to pTregs was observed.[91] In this same study, PD-L1 was also able to maintain and support the Treg population by allowing FoxP3 expression to persist during and after activation of Tregs. This evidence is in direct contrast to other studies that show no link between Treg abundance and PD-1.[91] In a 2014 study, although researchers concluded that PD-1 is highly constitutively expressed on Tregs, they did not observe a change in FoxP3 expression in PD-1 KO mice.[69] Therefore, although there is a consensus that the PD-1 pathway is involved in immunosuppression in other cell types, it is still unknown whether it’s involved in Tregs. As a result, anti-PD-1 Ab therapy may aid melanoma patients in a Treg-independent fashion.

Efficacy of CCR4 targeted therapy

Although there is a consensus that CCR4 is involved in the chemotaxis of conventional TCs and Tregs into peripheral sites, whether CCR4 is involved in Treg migration specifically into tumors is still debated. As previously discussed, many studies show that anti-CCR4 Abs can reduce Treg abundance in melanoma tumors. Other studies, on the other hand, have shown that blocking CCR4 in mice is not associated with reduced Treg suppression. In fact, in a study in 2016, researchers found that CCR4-deficient mice did not have reduced intratumoral Tregs. They also showed enhanced tumor growth.[92] A possible explanation for these results is that, by blocking CCR4, Th17 populations were not able to expand in the regional lymph nodes. Because Th17 cells have been shown to have anti-melanoma properties, reduced Th17 populations established a more pro-tumor environment.[92] This may suggest that drugs that target CCR4 may work in a Treg-independent manner. In addition, this latter study underlines the urgency for more research into this therapy because while anti-CCR4 therapy may work in one patient and tumor type, it may have the opposite effect in other patients.

Role of Treg heterogeneity on melanoma immunity

As previously stated, Tregs can be put into two broad categories, or subtypes: tTregs and pTregs. In addition to these, there are many more different types of Tregs that are quickly

gaining attention.[93] Unfortunately there is little consensus on the distinction between certain Treg subtypes. This reflects the novel nature of this research field. Here, this paper will discuss the different Treg types that are hotly debated in the field.

Multiple activation states of Tregs

Just like conventional TCs, Tregs can exist in different activation states – naïve, effector, and memory Tregs. Naïve Tregs have relatively lower levels of FoxP3 expression, while effector Tregs are antigen activated and, as a result, express and secrete numerous inhibitory molecules which have been previously stated.[94] Memory Tregs have not been well characterized. There is no clear evidence that Tregs can persist for long periods of times without constant antigen stimulation although there are reports of Tregs that have elevated levels of CTLA-4 that persist in skin tissue long antigen exposure.[95] These long-lived CTLA4+ Tregs may be important in the melanoma immunosurveillance process.

Distinction between tTregs, pTregs, and iTregs

By far the largest subset of Tregs is the CD4+ CD25+ FoxP3+ TCs which this paper has categorized as tTregs. These cells arise from the thymus in response to the process of negative selection. They are capable of secreting IL-10 and carry out many of the immunosuppressive functions that have been previously outlined. pTregs make up another sizeable proportion of Tregs and use many of the same suppressive mechanisms

as tTregs. Another subtype of Tregs that has been characterized is the induced Treg (iTreg). These cells were CD4+ TCs which have been induced *in vitro*, principally with TGF-B and IL-2, to become Treg-like cells. Some researchers do not put iTregs in their own category.

iTregs may share many of the same suppressive mechanisms as tTregs and pTregs; however, their main mode of action is to prevent DCs from activating effector TCs. In a 2014 study carried out by Shevach and Thornton, iTregs were co-cultured with antigen primed DCs.[2] These DCs acted as normal APCs whereby they engulfed and processed the antigen and expressed it on MHC class II molecules. Co-culturing these DCs with iTregs allowed the two cell types to interact; however, after the interactions, the DCs were isolated and then re-cultured with naïve antigen-specific TCs. It was found that the naïve antigen-specific TCs had reduced levels of activation which suggests that the iTregs somehow inhibited the DCs from performing the antigen presenting function.[2] Upon further inspection, IL-10 secretion from iTregs caused an upregulation of the ubiquitin ligase MARCH1 in DCs. MARCH1 is thought to cause the destruction of peptide-MHC class II complexes on the surface of DCs, effectively preventing DCs from activating effector TCs (Figure 7). This IL-10- and MARCH-1-dependent mechanism is the main suppressive action of iTregs, although it may also occur in limited fashion in other Treg subsets.[2] Although there is a consensus that Tregs can be induced, and many researchers put iTregs in the their own category, there is still debate on whether they have a unique suppression mechanism.

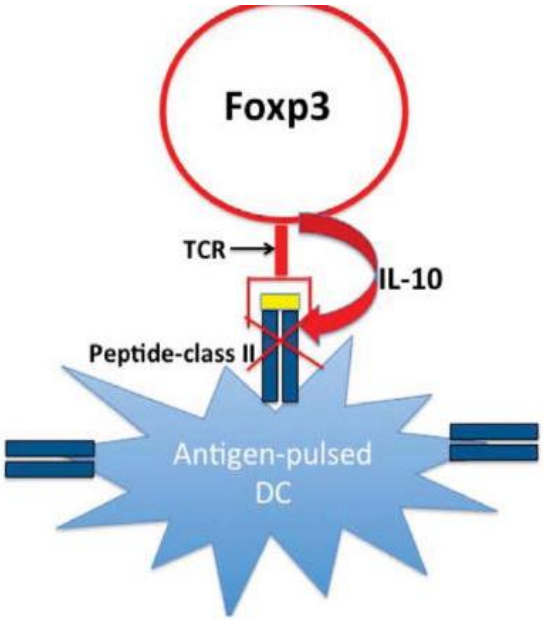

Figure 7. iTregs inhibit APCs using an IL-10-dependent MARCH1 mechanism. iTregs, donated by the FoxP3 cell, secrete IL-10 which will cause the upregulation of the ubiquitin ligase MARCH1. This ligase causes the destruction of MHC class II molecules on APCs. This will prevent antigen presentation via the APCs and therefore hinder APC activity (Adapted from Shevach and Thornton, 2014).[2]

Tr1 cells

Tr1 cells are unique Tregs because they do not have high levels of expression of FoxP3. Despite this, they are able to exert suppressive effects on TCs in a FoxP3-independent manner.[96] Tr1 cells can be differentiated from other Treg cells by their high excretion of IL-10 and TGF-B but lack of FoxP3 expression. Tr1 cells are similar to pTregs because they descend from CD4+ conventional TCs outside of the thymus. The mechanism for

how CD4+ TCs differentiate into Tr1 cells has not been fully elucidated; however, evidence suggests that it is due to chronic stimulation of TCR on CD4+ TCs in the presence of IL-10.[97] Once differentiation occurs, Tr1 cells express LAG-3, PD-1, CTLA-4, GITR, and other normal markers that are associated with Tregs. The role of Tr1 cells is similar to that of nTregs; however, unlike nTregs which govern systemic immunosuppression, T1r cells are mainly associated with suppression of tissue-specific microenvironments. Within the melanoma tumor environment, there are extremely few published sources that have elucidated the role that Tr1 cells play. What evidence there is suggests a potentially surprising role for Tr1 cells. Recent evidence proposes that there is a possibility for Tr1 cells to promote and not suppress an antitumor environment in melanoma patients. For instance, Tr1 cells have the ability to synthesize granzyme B and perforin to destroy macrophages that promote tumor growth.[98] Whether this can be replicated has yet to be determined. Although little is known about this cell type, research in this area has exploded in recent years.

IL-17 Tregs

Another subset of Tregs is the CD4+ CCR6+ FoxP3+ TCs that can secrete IL-17. This might represent a Th17 to Treg, or vice versa, conversion. This type of Treg plasticity is not understood but it could have something to do with the similarity in stimuli needed to induce differentiation. CD4+ conventional TCs require TGF-B for both Th17 and Treg differentiation.[94] Evidence for IL-17 secreting Tregs in melanoma is limited but

proinflammatory cytokine secretion, such as IL-17, have been observed by Tregs inside melanoma tissue in some studies but is often refuted.[99] Other studies in melanoma *in vivo* models have suggested that Tregs can be induced to secrete IL-17 in response to IL-6, another important cytokine for Th17 differentiation.[100] There is no clear consensus on what role these cells play. It is important to note, however, that these IL-17 Tregs still maintain their suppressive abilities *in vitro*.[99]

High ICOS expressing Tregs

ICOS is a costimulatory molecule, similar to CD28, expressed on TCs that are activated in response to TCR stimulation. It has a role in increasing IL-10 secretion from activated TCs and therefore contributes to a suppressive environment.[101] ICOS is expressed on TCs and there are Treg populations that have very high expression of ICOS – donated as $ICOS^{high}$ Tregs – which have recently been the focus of research. $ICOS^{high}$ Tregs have been shown to be present inside certain tumors. In melanoma, $ICOS^{high}$ Tregs reside almost exclusively within the tumor. High ICOS expression is associated with nTregs that have a hyper-suppression characteristic.[102] In addition, the ligand for ICOS, ICOS-L, is perfectly positioned to stimulate $ICOS^{high}$ Tregs. It has been shown in numerous studies that ICOS-L is expressed on melanoma cells and within TCs and DCs within melanoma tumors.[113]

ICOS^{high} Tregs may play an important role when treating melanoma patients. In high dose IL-2 therapy, the ICOS+ Treg population increased more than any other Treg population and these Tregs showed a high activation phenotype. This increase in Treg population may have clinical ramification; after receiving high dose IL-2 therapy it was shown that melanoma patients' clinical outcome was negatively correlated with ICOS+ Treg population expansion.[104]

CONCLUSION

Research supports the finding that Tregs play an important role in melanoma immunity. Treg cells can accumulate in melanoma tumors. Although it is not known whether Tregs can migrate into melanoma tumors due to specific signals or are simply prevented from leaving. The chemokines CCL22 and CCL1 are greatly upregulated in melanoma cells and this chemokine milieu is associated an upregulation of the cognate receptor CCR4 on Tregs. Previous studies have shown that CCL22 binding to CCR4 is used for chemotaxis toward normal skin cells. This highly suggests that Tregs chemotactically migrate from peripheral sites and into the melanoma tumor environment. Once inside the tumor, other mechanism which have not been elucidated yet, might be responsible to prevent Treg escape. The role that Tregs play inside melanoma tumors is similar to their traditional immunoregulatory functions that have been well documented. In melanoma, It is likely that IL-10 and CTLA-4 are the main modes of Treg contact-independent and contact-dependent immunosuppression. It is unlikely, however, that Treg secretion of TGF-B and adenosine play a major role in establishing an immunosuppressive microenvironment because these molecules have been observed to be secreted by other cells within melanoma tumors. Much more research is needed to determine the melanoma-specific modes of Treg immunosuppression, both *in vitro* and *in vivo*. Additionally, more research is needed to elucidate how other Treg subsets, such as Tr1 cells, contribute to the melanoma tumor microenvironment.

Tregs can be modulated by administration of exogenous drugs which can limit this cell type's effectiveness in promoting immunosuppression. This paper does not recommend that high dose IL-2 therapy, which was once seen as a standard treatment, as an effective strategy to combat melanoma. Although IL-2 can enhance the body's immune response toward melanoma, it also stimulates Tregs. This dangerous and delicate balancing act can be circumvented with the use of newer generation immunotherapeutics such as PD-1, CTLA-4, LAG-3, GITR antibodies. These agents work to block the development and action of Tregs using multiple different mechanism of actions. Anti-CTLA-4 mAb seems to be the most well researched and well established immunotherapeutic that targets Tregs. More research is needed to find alternative therapeutic agents. More research is also needed into establishing the mechanism of action of current therapeutics. Many drugs have positive and clear clinical effects on melanoma patients but have poorly defined effects on Treg cell populations. This is true of, for example, PD-1 antibodies. Better defining the mechanism of action, as it relates to Tregs, for these therapeutics will help researchers in developing combination therapies that can target melanoma from multiple different directions. This paper recommends that CCR4-targeted therapy in conjunction with CTLA-4-targeted therapy may be sufficient to block Treg action. By preventing Treg recruitment and function, melanoma tumors will be preferentially invaded by antitumor immune cells.

The prognostic value of Tregs is hotly debated in the field. As previously described, there is little consensus as to whether the presence of Tregs in the melanoma tumor

environment can accurately predict patient survival. This is mostly due to poor Treg identification. There is no consensus as to the most accurate technique to stain for Treg populations. General identification methods such as FoxP3 and CD25 that are employed by researchers is insufficient to label all the different Treg subtypes such as Tr1 cells which do not express FoxP3. The lack of consensus is also due to a deficiency in studies that take into account the different types and stages of melanoma cancers. More research is needed to establish identification methods that can predictably and accurately label Tregs within the tumor environment. Sample sizes should also be large enough to draw clear conclusions which is something that is lacking in many of the published clinical studies. A more accurate predictor for patient survival may not be the mere presence of Tregs but instead may be the Teff:Treg ratio within the tumor.

Although Tregs have been the focus of much recent research, more work is needed to deepen our understanding of this cell type. Only in this way can the scientific community understand the deep and complex connections between Tregs and melanoma immunology. Despite the lack of consensus and research on many areas, Tregs have proven to be an important part of melanoma pathophysiology and treatment.

LIST OF JOURNAL ABBREVIATIONS

Am J Physiol	The American journal of physiology
Am J Prev Med	The American Journal of Preventative Medicine
Ann N Y Acad Sci	Annals of the New York Academy of Sciences
Annu Rev Immunol	Annual review of immunology
Biochim Biophys	Biochimica et Biophysica Acta
Br J Dermatol	The British Journal of Dermatology
Cancer Immunol Immunother	Cancer Immunology, Immunotherapy
Cancer Immunol Res	Cancer immunology research
Cancer Res	Cancer Research
Cell Mol Life Sci	Cellular and Molecular Life Sciences
Clin Cancer Res	Clinical Cancer Research
Curr Med Chem	Current Medicinal Chemistry
Curr Opin Immunol	Current Opinion in Immunology
Eur J Immunol	European Journal of Immunology
Front Immunol	Frontiers in Immunology
Immune Netw	Immune Network
Immuno Rev	Immunological Reviews
Immunol Res	Journal of Immunology Research
Int Immunopharmacol	International Immunopharmacology
J Autoimmun	Journal of Autoimmunity

J Clin Invest	Journal of Clinical Investigation
J Exp Med	Journal of Experimental Medicine
J Immunol	The Journal of Immunology
J Invest Dermatol	The Journal of Translational Dermatology
J Med Genet	Journal of medical genetics
J Transl Med	The Journal of Translational Medicine
JAMA	The Journal of the American Medical Association
Lancet Oncol	The Lancet Oncology
Melanoma Res	Melanoma Research
Mol Cell Pharmacol	Molecular and Cellular Pharmacology
N Engl J Med	New England Journal of Medicine
Nat Immunol	Nature Immunology
Nat Rev Immunol	Nature Reviews Immunology
Natl Acad Sci	Proceedings of the National Academy of Sciences of the United States of America
Ochsner J	The Ochsner Journal
Oncol Rep	Oncology Reports
PNAS	Proceedings of the National Academy of Sciences of the United States of America
Pancreas	Pancreas
Pathol Oncol Res	Pathology & Oncology Research
Proc Natl Acad Sci	Proceedings of the National Academy of Sciences of the United States of America
Sci Rep	Scientific Reports

Sci Transl Med	Science translational medicine
Tumour Bio	Tumour Biology
Vacines	Vaccines

REFERENCES

1. Nishizuka Y, Sakakura T. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science*. 1969;166(3906):753-755.
2. Yadav M, Stephan S, Bluestone JA. Peripherally induced tregs - role in immune homeostasis and autoimmunity. *Front Immunol*. 2013;4:232. doi:10.3389/fimmu.2013.00232
3. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J Med Genet*. 2002;39(8):537-545. doi:10.1136/jmg.39.8.537
4. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev*. 2010;236:219–242. doi:10.1111/j.1600-065X.2010.00923.x
5. Gambineri E, Ciullini Mannurita S, Hagin D, et al. Clinical, Immunological, and Molecular Heterogeneity of 173 Patients With the Phenotype of Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX) Syndrome. *Front Immunol*. 2018;9:2411. Published 2018 Nov 1. doi:10.3389/fimmu.2018.02411
6. Barthlott T. CD25 CD4 T cells compete with naive CD4 T cells for IL-2 and exploit it for the induction of IL-10 production. *International Immunology*. 2005;17(3):279-288. doi:10.1093/intimm/dxh207

7. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol*. 2007;8(12):1353-1362. doi:10.1038/ni1536
8. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the Interleukin-10 Receptor. *Annu Rev Immunol*. 2001;19(1):683-765. doi:10.1146/annurev.immunol.19.1.683
9. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-β1 maintains suppressor function and Foxp3 expression in CD4 CD25 regulatory T cells. *The Journal of Experimental Medicine*. 2005;201(7):1061-1067. doi:10.1084/jem.20042276
10. Zhao H, Liao X, Kang Y. Tregs: Where We Are and What Comes Next? *Front Immunol*. 2017;8:1578. doi:10.3389/fimmu.2017.01578
11. 52 Whiteside TL, Mandapathil M, Schuler P. The role of the adenosinergic pathway in immunosuppression mediated by human regulatory T cells (Treg)**.** *Curr Med Chem*. 2011;18(34):5217-23.
12. Romio M, Reinbeck B, Bongardt S, Hüls S, Burghoff S, Schrader J. Extracellular purine metabolism and signaling of CD73-derived adenosine in murine Treg and Teff cells. *Am J Physiol, Cell Physiol*. 2011;301(2):C530-539. doi:10.1152/ajpcell.00385.2010
13. Deaglio S, Dwyer KM, Gao W, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med*. 2007;204(6):1257-1265. doi:10.1084/jem.20062512

14. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*. 2008;322(5899):271-275. doi:10.1126/science.1160062

15. Qureshi OS, Zheng Y, Nakamura K, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*. 2011;332(6029):600-603. doi:10.1126/science.1202947

16. Walker LSK. Treg and CTLA-4: two intertwining pathways to immune tolerance. *J Autoimmun*. 2013;45:49-57. doi:10.1016/j.jaut.2013.06.006

17. Okazaki T, Honjo T. The PD-1–PD-L pathway in immunological tolerance. *Trends in Immunology*. 2006;27(4):195-201. doi:10.1016/j.it.2006.02.001

18. Klocke K, Sakaguchi S, Holmdahl R, Wing K. Induction of autoimmune disease by deletion of CTLA-4 in mice in adulthood. *Proc Natl Acad Sci USA*. 2016;113(17):E2383-2392. doi:10.1073/pnas.1603892113

19. Mempel TR, Pittet MJ, Khazaie K, et al. Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. *Immunity*. 2006;25(1):129-141. doi:10.1016/j.immuni.2006.04.015

20. 111 Kornete M, Sgouroudis E, Piccirillo CA. ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. *J Immunol*. 2012;188(3):1064-1074. doi:10.4049/jimmunol.1101303

21. Mortaz E, Tabarsi P, Mansouri D, et al. Cancers Related to Immunodeficiencies: Update and Perspectives. *Front Immunol*. 2016;7:365. Published 2016 Sep 20. doi:10.3389/fimmu.2016.00365

22. Mortaz E, Tabarsi P, Mansouri D, et al. Cancers Related to Immunodeficiencies: Update and Perspectives. *Front Immunol*. 2016;7:365. Published 2016 Sep 20. doi:10.3389/fimmu.2016.00365

23. Jung MK, Kwak JE, Shin EC. IL-17A-Producing Foxp3^{+} Regulatory T Cells and Human Diseases. *Immune Netw*. 2017;17(5):276–286. doi:10.4110/in.2017.17.5.276

24. Ostroumov D, Fekete-Drimusz N, Saborowski M, Kühnel F, Woller N. CD4 and CD8 T lymphocyte interplay in controlling tumor growth. *Cell Mol Life Sci*. 2017;75(4):689-713.

25. Finn OJ. Cancer immunology. *N Engl J Med*. 2008;358(25):2704-2715. doi:10.1056/NEJMra072739

26. Houghton AN, Gold JS, Blachere NE. Immunity against cancer: lessons learned from melanoma. *Curr Opin Immunol*. 2001;13(2):134-140.

27. Nishikawa H, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Curr Opin Immunol*. 2014;27:1-7. doi:10.1016/j.coi.2013.12.005

28. Facciabene A, Motz GT, Coukos G. T-regulatory cells: key players in tumor immune escape and angiogenesis. *Cancer Res*. 2012;72(9):2162-71.

29. Riker AI, Zea N, Trinh T. The epidemiology, prevention, and detection of melanoma. *Ochsner J*. 2010;10(2):56-65

30. Lens MB, Dawes M. Global perspectives of contemporary epidemiological trends of cutaneous malignant melanoma. *Br J Dermatol*. 2004;150(2):179-185.

31. Guy GP, Ekwueme DU, Tangka FK, Richardson LC. Melanoma treatment costs: a systematic review of the literature, 1990-2011. *Am J Prev Med*. 2012;43(5):537-
32. Liu Y, Sheikh MS. Melanoma: Molecular Pathogenesis and Therapeutic Management. *Mol Cell Pharmacol*. 2014;6(3):228.
33. Naffouje S, Naffouje R, Bhagwandin S, Salti GI. Microphthalmia transcription factor in malignant melanoma predicts occult sentinel lymph node metastases and survival. *Melanoma Res*. 2015;25(6):496-502. doi:10.1097/CMR.0000000000000195
34. Leonardi GC, Falzone L, Salemi R, et al. Cutaneous melanoma: From pathogenesis to therapy (Review). *Int J Oncol*. 2018;52(4):1071-1080.
35. Paluncic J, Kovacevic Z, Jansson PJ, et al. Roads to melanoma: Key pathways and emerging players in melanoma progression and oncogenic signaling. *Biochim Biophys Acta*. 2016;1863(4):770-784. doi:10.1016/j.bbamcr.2016.01.025
36. Ha TY. The role of regulatory T cells in cancer. *Immune Netw*. 2009;9(6):209–235. doi:10.4110/in.2009.9.6.209
37. Linehan DC, Goedegebuure PS. CD25+CD4+ regulatory T-cells in cancer. *Immunol Res*. 2005;32(1):155-168. doi:10.1385/IR:32:1-3:155
38. Tang Y, Xu X, Guo S, et al. An Increased Abundance of Tumor-Infiltrating Regulatory T Cells Is Correlated with the Progression and Prognosis of Pancreatic Ductal Adenocarcinoma. *PLOS ONE*. 2014;9(3):e91551. doi:10.1371/journal.pone.0091551

39. Viguier M, Lemaître F, Verola O, et al. Foxp3 Expressing CD4+CD25high Regulatory T Cells Are Overrepresented in Human Metastatic Melanoma Lymph Nodes and Inhibit the Function of Infiltrating T Cells. *J Immunol*. 2004;173(2):1444-1453. doi:10.4049/jimmunol.173.2.1444

40. Klarquist J, Tobin K, Farhangi Oskuei P, et al. Ccl22 Diverts T Regulatory Cells and Controls the Growth of Melanoma. *Cancer Res*. 2016;76(21):6230-6240. doi:10.1158/0008-5472.CAN-16-0618

41. Iellem A, Mariani M, Lang R, et al. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J Exp Med*. 2001;194(6):847-53.

42. Eby JM, Kang H-K, Tully ST, et al. CCL22 to Activate Treg Migration and Suppress Depigmentation in Vitiligo. *J Invest Dermatol*. 2015;135(6):1574-1580. doi:10.1038/jid.2015.26

43. Ondondo B, Jones E, Godkin A, Gallimore A. Home sweet home: the tumor microenvironment as a haven for regulatory T cells. *Front Immunol*. 2013;4:197. Published 2013 Jul 16. doi:10.3389/fimmu.2013.00197

44. Niu J, Jiang C, Li C, et al. Foxp3 expression in melanoma cells as a possible mechanism of resistance to immune destruction. *Cancer Immunol Immunother*. 2011;60(8):1109-1118. doi:10.1007/s00262-011-1025-3

45. Jarnicki AG, Lysaght J, Todryk S, Mills KHG. Suppression of antitumor immunity by IL-10 and TGF-beta-producing T cells infiltrating the growing

tumor: influence of tumor environment on the induction of CD4+ and CD8+ regulatory T cells. *J Immunol*. 2006;177(2):896-904.

46. Fabio M, Alberto H, Barbara M, et al. Melanoma cells produce adenosine through a CD38/CD73 pathway and suppress CD4 T cell proliferation. *Front Immunol*. 2013;4. doi:10.3389/conf.fimmu.2013.02.00868
47. Leone RD, Emens LA. Targeting adenosine for cancer immunotherapy. *JITC*. 2018;6(1):57. doi:10.1186/s40425-018-0360-8
48. Wyss L, Stadinski BD, King CG, et al. Affinity for self antigen selects Treg cells with distinct functional properties. *Nat Immunol*. 2016;17(9):1093-101
49. Séralini G-E, Clair E, Mesnage R, et al. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Food and Chemical Toxicology*. 2012;50(11):4221-4231. doi:10.1016/j.fct.2012.08.005
50. Träger U, Sierro S, Djordjevic G, et al. The Immune Response to Melanoma Is Limited by Thymic Selection of Self-Antigens. *PLOS ONE*. 2012;7(4):e35005. doi:10.1371/journal.pone.0035005
51. Zhu M-L, Nagavalli A, Su MA. Aire deficiency promotes TRP-1-specific immune rejection of melanoma. *Cancer Res*. 2013;73(7):2104-2116. doi:10.1158/0008-5472.CAN-12-3781
52. Morgan DA, Ruscetti FW, Gallo R. Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science*. 1976;193(4257):1007-1008

53. 130 Rollings CM, Sinclair LV, Brady HJM, Cantrell DA, Ross SH. Interleukin-2 shapes the cytotoxic T cell proteome and immune environment–sensing programs. *Sci Signal*. 2018;11(526):eaap8112. doi:10.1126/scisignal.aap8112

54. Sharma R, Fu SM, Ju S-T. IL-2: a two-faced master regulator of autoimmunity. *J Autoimmun*. 2011;36(2):91-97. doi:10.1016/j.jaut.2011.01.001

55. Chinen T, Kannan AK, Levine AG, et al. An essential role for the IL-2 receptor in Treg cell function. *Nat Immunol*. 2016;17(11):1322-1333. doi:10.1038/ni.3540

56. Malek TR, Castro I. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity*. 2010;33(2):153-65.

57. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med*. 2005;201(5):723-735. doi:10.1084/jem.20041982

58. Zorn E, Nelson EA, Mohseni M, et al. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood*. 2006;108(5):1571–1579. doi:10.1182/blood-2006-02-004747

59. Sim GC, Martin-Orozco N, Jin L, et al. IL-2 therapy promotes suppressive ICOS+ Treg expansion in melanoma patients. *J Clin Invest*. 2013;124(1):99-110.

60. Postow MA, Callahan MK, Wolchok JD. Immune Checkpoint Blockade in Cancer Therapy. *J Clin Oncol*. 2015;33(17):1974–1982. doi:10.1200/JCO.2014.59.4358

61. Arce Vargas F, Furness AJS, Litchfield K, et al. Fc Effector Function Contributes to the Activity of Human Anti-CTLA-4 Antibodies. *Cancer Cell*. 2018;33(4):649–663.e4. doi:10.1016/j.ccell.2018.02.010

62. Simpson TR, Li F, Montalvo-Ortiz W, 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*. 2013;210(9):1695-1710. doi:10.1084/jem.20130579

63. Romano E, Kusio-Kobialka M, Foukas PG, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells ex vivo by nonclassical monocytes in melanoma patients. *Proc Natl Acad Sci USA*. 2015;112(19):6140-6145. doi:10.1073/pnas.1417320112

64. Spranger S, Spaapen RM, Zha Y, et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. *Sci Transl Med*. 2013;5(200):200ra116. doi:10.1126/scitranslmed.3006504

65. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nature Reviews Immunology*. 2015;15(8):486-499. doi:10.1038/nri3862

66. Wang W, Lau R, Yu D, Zhu W, Korman A, Weber J. PD1 blockade reverses the suppression of melanoma antigen-specific CTL by CD4+ CD25(Hi) regulatory T cells. *Int Immunol*. 2009;21(9):1065–1077. doi:10.1093/intimm/dxp072

67. Wang C, Thudium KB, Han M, et al. In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human

primates. *Cancer Immunol Res*. 2014;2(9):846-856. doi:10.1158/2326-6066.CIR-14-0040

68. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proc Natl Acad Sci USA*. 2008;105(27):9331-9336. doi:10.1073/pnas.0710441105
69. Chen X, Fosco D, Kline DE, et al. PD-1 regulates extrathymic regulatory T-cell differentiation. *Eur J Immunol*. 2014;44(9):2603-2616. doi:10.1002/eji.201344423
70. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev*. 2010;236(1):219-242. doi:10.1111/j.1600-065X.2010.00923.x
71. Andrews LP, Marciscano AE, Drake CG, Vignali DA. LAG3 (CD223) as a cancer immunotherapy target. *Immunol Rev*. 2017;276(1):80-96.
72. Camisaschi C, Casati C, Rini F, et al. LAG-3 expression defines a subset of CD4(+)CD25(high)Foxp3(+) regulatory T cells that are expanded at tumor sites. *J Immunol*. 2010;184(11):6545-6551. doi:10.4049/jimmunol.0903879
73. Goldberg MV, Drake CG. LAG-3 in Cancer Immunotherapy. In: Dranoff G, ed. *Cancer Immunol and Immun*. Current Topics in Microbiology and Immunology. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011:269-278. doi:10.1007/82_2010_114

74. Ito A, Ishida T, Yano H, et al. Defucosylated anti-CCR4 monoclonal antibody exercises potent ADCC-mediated antitumor effect in the novel tumor-bearing humanized NOD/Shi-scid, IL-2Rgamma(null) mouse model. *Cancer Immunol Immunother*. 2009;58(8):1195-1206. doi:10.1007/s00262-008-0632-0

75. Sugiyama D, Nishikawa H. CCR4 – a potential marker for effector-type regulatory T cells. 2018:3.

76. Ronchetti S, Ricci E, Petrillo MG, et al. Glucocorticoid-induced tumour necrosis factor receptor-related protein: a key marker of functional regulatory T cells. *J Immunol Res*. 2015;2015:171520.

77. Olffen RW van, Koning N, Gisbergen KPJM van, et al. GITR Triggering Induces Expansion of Both Effector and Regulatory CD4+ T Cells In Vivo. *J Immuno*. 2009;182(12):7490-7500. doi:10.4049/jimmunol.0802751

78. Ephrem A, Epstein AL, Stephens GL, Thornton AM, Glass D, Shevach EM. Modulation of Treg cells/T effector function by GITR signaling is context–dependent. *Eur J of Immunol*. 2013;43(9):2421-2429. doi:10.1002/eji.201343451

79. Knee DA, Hewes B, Brogdon JL. Rationale for anti-GITR cancer immunotherapy. *Eur J Cancer*. 2016;67:1-10. doi:10.1016/j.ejca.2016.06.028

80. Kimpfler S, Sevko A, Ring S, et al. Skin melanoma development in ret transgenic mice despite the depletion of CD25+Foxp3+ regulatory T cells in lymphoid organs. *J Immunol*. 2009;183(10):6330-6337. doi:10.4049/jimmunol.0900609

81. 35 Hansen W. Neuropilin 1 guides regulatory T cells into VEGF-producing melanoma. *Oncoimmunology*. 2013;2(2):e23039. doi:10.4161/onci.23039

82. Shang B, Liu Y, Jiang S, Liu Y. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep*. 2015;5:15179. doi:10.1038/srep15179

83. deLeeuw RJ, Kost SE, Kakal JA, Nelson BH. The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature. *Clin Cancer Res*. 2012;18(11):3022-3029. doi:10.1158/1078-0432.CCR-11-3216

84. Ladányi A, Mohos A, Somlai B, et al. FOXP3+ cell density in primary tumor has no prognostic impact in patients with cutaneous malignant melanoma. *Pathol Oncol Res*. 2010;16(3):303-309. doi:10.1007/s12253-010-9254-x

85. Mougiakakos D, Johansson CC, Trocme E, et al. Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma. *Cancer*. 2010;116(9):2224-2233. doi:10.1002/cncr.24999

86. Knol AC, Nguyen JM, Quéreux G, Brocard A, Khammari A, Dréno B. Prognostic value of tumor-infiltrating Foxp3+ T-cell subpopulations in metastatic melanoma. *Experimental Dermatology*. 2011;20(5):430-434. doi:10.1111/j.1600-0625.2011.01260.x

87. Gerber AL, Münst A, Schlapbach C, et al. High expression of FOXP3 in primary melanoma is associated with tumour progression. *Br J Dermatol*. 2014;170(1):103-109. doi:10.1111/bjd.12641

88. Rosenberg SA, Yang JC, Topalian SL, et al. Treatment of 283 Consecutive Patients With Metastatic Melanoma or Renal Cell Cancer Using High-Dose Bolus

Interleukin 2. *JAMA*. 1994;271(12):907-913. doi:10.1001/jama.1994.03510360033032

89. Jacobs JFM, Nierkens S, Figdor CG, de Vries IJM, Adema GJ. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? *Lancet Oncol*. 2012;13(1):e32-42. doi:10.1016/S1470-2045(11)70155-3
90. Arce Vargas F, Furness AJS, Solomon I, et al. Fc-Optimized Anti-CD25 Depletes Tumor-Infiltrating Regulatory T Cells and Synergizes with PD-1 Blockade to Eradicate Established Tumors. *Immunity*. 2017;46(4):577-586. doi:10.1016/j.immuni.2017.03.013
91. Francisco LM, Salinas VH, Brown KE, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009;206(13):3015-3029. doi:10.1084/jem.20090847
92. Matsuo K, Itoh T, Koyama A, et al. CCR4 is critically involved in effective antitumor immunity in mice bearing intradermal B16 melanoma. *Cancer Lett*. 2016;378(1):16-22. doi:10.1016/j.canlet.2016.04.039
93. Han Y, Guo Q, Zhang M, Chen Z, Cao X. CD69+CD4+CD25− T Cells, a New Subset of Regulatory T Cells, Suppress T Cell Proliferation through Membrane-Bound TGF-β1. *J Immunol*. 2009;182(1):111-120. doi:10.4049/jimmunol.182.1.111
94. Ward-Hartstonge KA, Kemp RA. Regulatory T-cell heterogeneity and the cancer immune response. *Clin Transl Immunology*. 2017;6(9):e154. Published 2017 Sep 15. doi:10.1038/cti.2017.43

95. Rosenblum MD, Way SS, Abbas AK. Regulatory T cell memory. *Nat Rev Immunol*. 2015;16(2):90-101.

96. Almanan MA, Raynor J, Chougnet C, et al. Type 1 regulatory T cells (Tr1) homeostasis and function in aging. *J Immunol*. 2017;198(1 Supplement):154.10-154.10. http://www.jimmunol.org/content/198/1_Supplement/154.10. Accessed March 31, 2019

97. Zeng H, Zhang R, Jin B, Chen L. Type 1 regulatory T cells: a new mechanism of peripheral immune tolerance. *Cell Mol Immunol*. 2015;12(5):566-71.

98. Yan H, Zhang P, Kong X, et al. Primary Tr1 cells from metastatic melanoma eliminate tumor-promoting macrophages through granzyme B- and perforin-dependent mechanisms. *Tumour Biol*. 2017;39(4):1010428317697554. doi:10.1177/1010428317697554

99. Jung MK, Kwak JE, Shin EC. IL-17A-Producing Foxp3^{+} Regulatory T Cells and Human Diseases. *Immune Netw*. 2017;17(5):276–286. doi:10.4110/in.2017.17.5.276

100. Diller ML, Kudchadkar RR, Delman KA, Lawson DH, Ford ML. Complete response to high-dose IL-2 and enhanced IFNγ+Th17 : TREG ratio in a melanoma patient. *Melanoma Res*. 2016;26(5):535-9.

101. Witsch EJ, Peiser M, Hutloff A, et al. ICOS and CD28 reversely regulate IL-10 on re-activation of human effector T cells with mature dendritic cells. *Eur J Immunol*. 2002;32(9):2680-2686. doi:10.1002/1521-4141(200209)32:9<2680::AID-IMMU2680>3.0.CO;2-6

102. Strauss L, Bergmann C, Szczepanski MJ, Lang S, Kirkwood JM, Whiteside TL. Expression of ICOS on human melanoma-infiltrating CD4+CD25highFoxp3+ T regulatory cells: implications and impact on tumor-mediated immune suppression. *J Immunol*. 2008;180(5):2967-2980.

103. Martin-Orozco N, Li Y, Wang Y, et al. Melanoma cells express ICOS ligand to promote the activation and expansion of T-regulatory cells. *Cancer Res*. 2010;70(23):9581-90

104. Sim GC, Martin-Orozco N, Jin L, et al. IL-2 therapy promotes suppressive ICOS+ Treg expansion in melanoma patients. *J Clin Invest*. 2014;124(1):99-110. doi:10.1172/JCI46266

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