CTLA-4 in regulatory T cells for cancer immunotherapy

Authors: Navid Sobhani[1], Dana Rae Tardiel-Cyril[1], Aram Davtyan[2], Daniele Generali[3], Raheleh Roudi[4], Yong Li[1]

Affiliations:

[1] *Department of Medicine, Section of Epidemiology and Population Sciences, Baylor College of Medicine, Houston, TX 77030, USA.*

[2] *Atomwise, 717 Market St, San Francisco, CA 94103, USA.*

[3] *Department of Medical, Surgery and Health Sciences, University of Trieste, 34147 Trieste, Italy.*

[4] *Department of Medicine, University of Minnesota Medical School, Minneapolis, Minnesota 55455, USA.*

Simple Summary

In the fight against cancer, immunotherapies have given a great hope after the encouraging results in clinical investigations showing complete remission in some patients with melanoma. In fact, directing the immune system against cancer has been a very innovative strategy fostered during the past three decades. Despite this fact, the disease is serious and the mortality is still very high and only a minority of patients are responsive to immunotherapies. Therefore, there is a need for a better understanding of the molecular mechanisms of resistance to immune checkpoint inhibitors such as antibodies against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). In this article, we discuss the molecular mechanism of CTLA-4 in T regulatory cells inhibition, while highlighting the knowledge gap.

Abstract

Immune checkpoint inhibitors (ICIs) have obtained durable responses in many cancers, making it possible to foresee their potential in improving the health of cancer patients. However, immunotherapies are limited at the moment to a minority of patients and there is a need for a better understanding of the basic molecular mechanisms and functions of pivotal immune regulatory molecules. Immune checkpoint cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and regulatory T (T_{reg}) cells play pivotal roles in hindering the anticancer immunity. T_{reg} cells suppress antigen-presenting cells (APCs) by depleting immune stimulating cytokines, producing immunosuppressive cytokines and constitutively expressing CTLA-4. CTLA-4 molecules bind with higher affinity to CD80 and CD86 than CD28 and act as competitive inhibitors of CD28 in APCs. The purpose of this review is to summarize state-of-the-art understanding of the molecular mechanisms underlining CTLA-4 immune regulation and the correlation of ICI

response with CTLA-4 expression in T_{reg} cells from preclinical and clinical studies for possibly improving CTLA-4-based immunotherapies, while highlighting the knowledge gap.

Keywords: CTLA-4; T_{reg} cells: Immune checkpoint inhibitors; CD28; Antigen Presenting Cells.

1) Introduction

Globally, cancer remains the leading cause of mortality and morbidity, with nearly 9 million deaths every year[1]. Early diagnosis and advances in cancer treatment have improved the survival of cancer patients, but new cases of cancer in the United States were more than 1.7 million in 2019[1]. A considerable percentage of these patients manifested drug resistance, metastasis, and recurrence [2].

A promising paradigm in the dilemma and challenge of cancer therapy is immunotherapy, and the T cell population has generated considerable enthusiasm among scientists due to their ability to kill malignant tumor cells directly [3].

There are two major types of T cells: conventional adaptive T cells (including helper CD4+ T cells [Th1, Th2, Th17, Th9, and Tfh], cytotoxic CD8+ T cells, memory T cells, and regulatory CD4+ T cells [T_{reg}]) and innate-like T cells (including natural killer T cells, mucosal associated invariant T cells, and γδ T cells) [4].

T_{reg} cells are one of the most fascinating immunosuppressive subset of CD4+ (CD25+) T cells, mainly represented by master transcription factor 3 (FOXP3), and they account for nearly 5 % of the total CD4+ T cell population under normal conditions [5]. T_{reg} cells increase dramatically in response to the early stages of malignant tumor initiation and growth [6]. In the tumor microenvironment, T_{reg} cells can suppress the immune system activity of cytotoxic T lymphocytes (CTLs)[7]. A panel of immune-modulatory receptors expressed on the T_{reg} cell population include cytotoxic T lymphocyte antigen 4 (CTLA-4), vascular endothelial growth factor receptor (VEGFR), and programmed cell death protein 1 (PD1)[8]. CTLA-4 is expressed on activated T and T_{reg} cells [9]. Infiltration of T_{reg} cells by down-regulation of costimulatory molecules, CD80/86 expression, on antigen-presenting cells (APCs), can be activated by CTLA-4, and therefore this molecule has a critical role in cancer progression[9,10]. Atkins et al. showed that immune checkpoint blockade of the CTLA-4 improved the survival rate of renal cell carcinoma, melanoma, non-small cell lung cancer (NSCLC), and head and neck squamous cell cancer[11]. This protein was the second receptor for the T-cell costimulatory ligand B7 and, therefore, an immune checkpoint whose function is critical for downmodulating the immune response. In contrast to the first receptor, which is antigen-dependent, CTLA-4 is antigen-independent[12]. Ipilimumab has been the first immunotherapy drug targeting CTLA-4 receiving FDA-approval in 2011 to treat late-stage melanoma[13]. This approval came after encouraging results of a large randomized phase III clinical trial improving patients' survival compared to

standard therapy. Since then, several immunotherapies targeting the PD-1/PD-L1 axis have received FDA approval to treat multiple types of cancers[13].

This review will describe mechanisms of CTLA-4 immune checkpoint inhibition, the role of T_{reg} cells in tumorigenesis, and how anti-CTLA-4 antibodies can change T_{reg} cell CTLA-4 expressions while exerting anti-cancer therapeutic activity.

2) Mechanism of CTLA-4 immune system inhibition

A better understanding of the biological mechanisms and functions of negative and positive co-stimulatory molecules has been shown essential for improving current and potentially new anti-CTLA-4 or Programmed Cell Death 1 (PD-1) inhibitors for anti-cancer immunotherapies.

Once bound to B7-1 (CD80) or B7-2 (CD86), CTLA-4 switches-off antigen-presenting cells[14]. CTLA-4 was immediately increased after T-cell receptor (TCR) engagement, reaching its highest level of expression as a homodimer at 2-3 days after T cell activation[15,16]. CTLA-4 competes against costimulatory molecule CD28 for the B7 ligand CD80 and CD86, for which it has higher affinity and avidity [17,18]. It is necessary to inhibit interactions with both CD80 and CD86 with antibodies to optimally block CD28-depednent proliferation of Mixed Lymphocyte Reaction (MLR)-stimulated B lymphoblastoid. Since both CD80 and CD86 exert a positive costimulatory signal through CD28, the role played by CTLA-4 in competitive inhibition of CD28 is important in order to attenuate T-cell activation, thereby fine-tuning immune response[19]. A rapid binding kinetics with a very fast dissociation rate constant (k_{off}) of both CTLA-4 and CD28 to CD80 has been observed ($k_{off} \geq 1.6$ and ≥ 0.43 s-1)[20], which permits their instant competition.

Additionally, after T-cell activation by TCR, CTLA-4 within intracellular compartments is immediately transported to the immunologic synapse[21]. The stronger the TCR signaling the more CTLA-4 were transported to the immunological synapse[21]. After reaching the synapse, CTLA-4 becomes stable through its binding to the CD80 and CD86 ligands, leading to its accumulation and effective out-competition against CD28 [14]. Differences in both affinity and avidity in ligand-binding cause selective CD28 or CTLA-4 recruitment to the immunological synapse. The major ligand leading to CTLA-4 localization in synapse is CD80, while for CD28 is CD86[14]. In this way CTLA-4 attenuates the positive co-stimulation of CD28, thereby limiting the downstream signaling of CD28, which is primarily through PI3K and AKT[22,23]. This mechanism allows a fine-tuning of TCR signaling and therefore of T-cell activity. The negative co-stimulation of CTLA-4 is intrinsically linked to B7 and CD28 positive co-stimulations. CTLA-4 mainly regulates T-cell at priming sites (e.g. gut or lymphoid organs such as spleen and lymph nodes). Since CTLA-4 plays a crucial function for the activation of T-cells, its negative co-stimulation plays a critical role for tolerance. As a matter of fact, the biallelic genetic *Ctla-4* deletion in mice leads to their death at 3-4 weeks of age because of pronounced lymphoproliferation with multi-organ lymphocytic infiltration and tissue destruction, particularly with pancreatitis and myocarditis[24–26]. T-cell attenuation by CTLA-4 can occur through extrinsic mechanisms. RAG2- deficient mice with CTLA-4-deficient bone marrow had a significantly higher survival compared to mice without the CTLA-4 deficiency. Intriguingly, reconstituting RAG2-defincient mice with a mixture of normal and CTLA-4-deficient bone marrow remained healthy without developing any disease[27]. Mice lethality can therefore be

prevented by normal T cell factors. Several groups foster the idea that extrinsic cell suppressive functions of CTLA-4 are mainly mediated through T_{reg} cells[28,29]. Others support that CTLA-4 ability to inhibit T-cells was T_{reg} cells-independent[30,31]. As an argumentation to the first line of thought, it is that a particular loss of CTLA-4 in T_{reg} cells was enough to induce abnormal T-cell activation and autoimmunity[28,32]. In fact Wing et al showed that the loss of CTLA-4 in T_{reg} cells was capable of hyper producing immunoglobulin E, systemic lymphoproliferation, fatal T cell-mediated autoimmune disease and powerful tumor immunity[28]. After losing CTLA-4 subpopulation, the T_{reg} cells were not capable to exert their T cell suppressive functions, especially were not able to down-modulate the dendritic cell expressions of CD80 and CD86 [28]. It must be noted that the lack of CTLA-4 in T_{reg} cells leads also to an aberrant expression and expansion of T_{conv} cells, which can cause the latter cells to infiltrate and fatally damage nonlymphoid tissues and cells[32]. Therefore CTLA-4 in T_{reg} cells are also needed to prevent accumulations of T cells that could harm vital organs. It is possible, based on such data, that T_{reg} cells from CTLA-4 are required to have immunologic tolerance, even though it is unlikely that on its own this subpopulation could maintain T-cell mediated tolerance. As a hypothetical molecular biology explanation, it is possible that T_{reg} cells with CTLA-4 could limit the availability of B7 ligands for the positive co-stimulation of CD28 in effector T-cells. Through such mechanism the CTLA-4 would indirectly and cell-extrinsically damped T-cell activation. It is also known that CTLA-4 on effector T-cells can trans-compete for B7 ligands[33]. Another mechanism by which CTLA-4 can lower the total availability of B7 ligands is through APCs-mediated trans-endocytosis of B7 ligands[34]. The last two mechanisms explain how CTLA-4 could mediate anti-cancer immune reactions without the need for T_{reg} cells. Overall, it is noteworthy that these mechanisms are not yet fully understood and each contribution remains elusive in the context of cancer immunity and drug design.

Loss of CTLA-4 from T_{reg} cells population gives resistance to autoimmune encephalomyelitis (EAE) in mice [35]. Resistance to EAE was possible in mice by the deletion of *Ctla-4* of T_{reg} cells, which suggests that the expression of *Ctla-4* in T_{reg} cells can prevent autoimmunity. Depletion of T_{reg} cells could counter-act autoimmunity. This could improve anti-CTLA-4 antibody therapies functioning in such manner in specific cancer cells where T_{reg} cells are found at high concentrations.

Additionally, since CTLA-4 expression has been correlated with TCR signal strength, high T_{reg} cells and CTLA-4 expressions are concomitant[36,37]. The inhibition efficacy of any cell by CTLA-4 depends on the affinity between pMHC ligand to its TCR. The higher the affinity of TCRs the more those cells can be inhibited[38,39]. Of note, neither wild-type nor CTLA-4 deficient T cells can express factor forkhead box protein P3 (FOXP3) when 10^4 CD45.1$^+$ RAG2-deficient 5C.C7 T cells were injected into normal syngenic B10.A recipient mice. This suggests CTLA-4 cell-extrinsic inhibition of T responses as a function of effector T cells, independently from T_{reg} cells[33]. Additionally, in CD8-depleted splenocytes -stimulated with soluble anti-CD3 mAb, the induction of CTLA-4 restricts CD4+ T-helper clonal expansion, allowing local and temporal growth of these cells in response to the specific immunological threat Through this mechanism, strong TCR signal can be attenuated and medium TCR signal can lead to powerful T-cell activation as well.

Furthermore, a number of structures of the extracellular domain of human CTLA-4 are available in Protein Data Bank (PDB), including apo structures and various complexes. The very first structure of CTLA-4 was determined using solution NMR spectroscopy (PDB ID: 1AH1),

revealing an Ig-like V (variable)-type domain, where two beta-sheets of the V-fold are connected by two disulfide bonds (21 to 94 and 48 to 68) [40]. Another apo structure of CTLA-4 was later published in the physiological dimeric state (PDB ID: 3OSK) [41]. CTLA-4 binds its native ligands CD80 and CD86 at A 'GFCC' face, which contains a number of charged residues that are highly conserved between CTLA-4 and CD28 (and across species). The key role in those interactions is also played by the $_{99}$MYPPPY$_{104}$ loop connecting F and G strands [40]. The structures of CTLA-4 with CD80 and CD86 (PDB IDs: 1I8L and 1I85) manifested a mostly convex binding surface at CTLA-4, free of any notable cavities that could have been targeted with traditional small-molecule campaigns [42,43]. It is also interesting to note, that while the CD80-bound conformation of CTLA-4 is very similar to the apo form, CD86 binding requires some structural rearrangement, most significantly, in the FG loop [41–43]. Finally, several structures of CTLA-4 bound to monoclonal antibodies have also been reported recently (PDB IDs: 5GGV, 5TRU, 5XJ3, 6RP8) [44–46]. Those structures reveal that ipilimumab and tremelimumab directly compete with CD80 and CD86 at their binding surface, sterically displacing and preventing their interactions with CTLA-4. Moreover, subtle differences in the CTLA-4 structure, such as slightly larger distance between G and F stands, and extended interactions of antibodies with non-conserved residues on the opposite side of the FG loop, enable selectivity between CTLA-4 and CD28 [45].

While the antitumor activity and clinical benefits of antibodies like ipilimumab that block CTLA-4 interactions with its ligands have been demonstrated [45], it is always desirable to have bioavailable and cheaper options in the form of small molecules or peptides. In the cases of traditionally undruggable targets, like CTLA-4, where no suitable small-molecule binding pockets can be immediately identified at the ligand-binding interface, peptide drugs can present a viable alternative. Like antibodies, peptides can achieve high affinity and specificity by capturing a larger interaction area with the target. At the same time, they are easier to synthesize and have greater tissue penetration due to their smaller size compared to the antibodies. Moreover, peptides have recommended themselves in a variety of therapeutic areas, including cancer [47,48]. In addition, targets similar to CTLA-4 can be amenable to less-standard small molecule campaigns. One such approach is an allosteric modulation. In this case, a small molecule bound to a distant site can activate or inhibit the protein function or its interactions with other molecules as a result of structural changes that it induces at a distance [49]. However, for CTLA-4, such sites still have to be determined either through experimental or computational techniques [50,51].

3)   Regulatory T-cells and anticancer Immunity

First insights into the T$_{reg}$ cells

After T$_{reg}$ cells were discovered for the first time in the CD4+ CD25+ T cells subpopulation in 1995 [52], mutations of *FOXP3* recapitulated in impaired formation or improper functional T$_{reg}$ cells causing an immune dysregulation syndrome in mice, termed polyendocrinopathy enteropathy X-linked syndrome, which ultimately leads to multiple autoimmune disorders [53]. To corroborate the importance of T$_{reg}$ cells for functional immune response, mice carrying spontaneous alterations of *Foxp3* – that ultimately lacked T$_{reg}$ cells – died out of systemic autoimmunity[54,55]. As expected, external expression of FOXP3 bestowed naïve CD4+ T cells (T$_{conv}$, without T$_{reg}$ cells) with the same immune-suppressive capacity that was typical to T$_{reg}$ cells. Hence, FOXP3 is a master transcription factor that regulates T$_{reg}$ cells phenotypes and their function as immunosuppressant. The role of T$_{reg}$ cells in cancer is mainly played in inflammatory

sites where it migrates and destroys different types of effector T cells, such as CD4$^+$ T helper (T$_H$) cells and CD8$^+$ cytotoxic T-cells (CTLs)[56–59]. As a consequence, intervening on such molecules could evoke the immune system in the fight against cancer.

Inhibitory effects of Treg cells on APC

T$_{reg}$ cells represent a crucial component of the immune system, essential for controlling self-tolerance and thereby play essential roles in various medical conditions. T$_{reg}$ cells have a crucial role in the suppression of immune response in cancer [52,54,60–64]. T$_{reg}$ cells inhibit APC by three main mechanisms: 1) depleting immune stimulating cytokines, 2) producing immunosuppressive cytokines, 3) constitutively expressing CTLA-4. T$_{reg}$ cells express Interleukin 2 (IL2) receptors that bind to IL2, thereby limiting the amount of this cytokine available for T$_{conv}$ cells [65,66].

T$_{reg}$ cells produce immunosuppressive cytokines like TGFβ, IL10 and IL35[67–70]. Constitutive expression of CTLA-4 blocks the priming and activation of T$_{conv}$ cells to APCs[28,71]. Figure 1 summarizes the role of CTLA-4 in T$_{reg}$ cells modulating T$_{conv}$ activation.
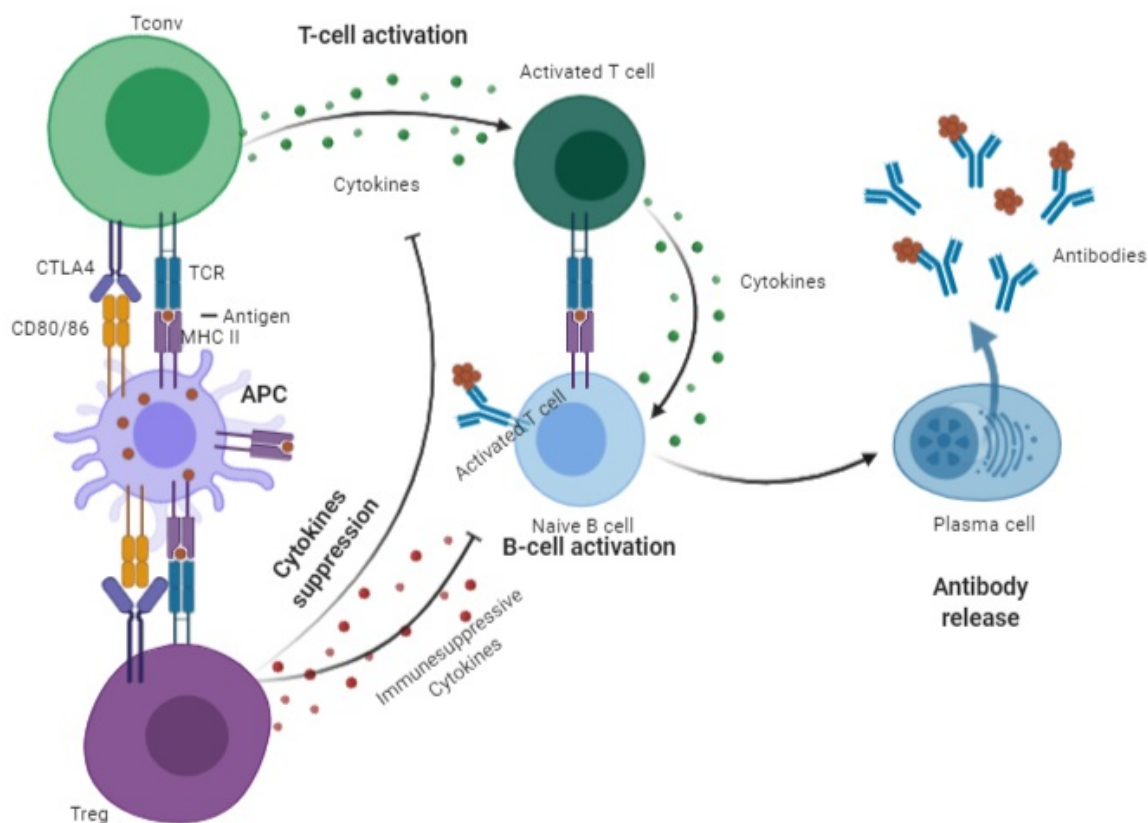
Figure 1- T$_{reg}$ cells inhibit Antigen Presenting Cells (APC) by three main mechanisms: 1) depleting immune stimulating cytokines, 2) producing immunosuppressive cytokines, 3) constitutively expressing CTLA-4.

T_{reg} cells block the normal protective immune-surveillance and inhibit the antitumor immune response in cancer patients. Thereby, if T_{conv} cells are like tumor suppressors, T_{reg} cells could be considered as oncogenes because they are suppressing antitumor immunity[60,61,72,73]. Likewise CTLA-4 and PD-1 immune checkpoints, since they are blocking the immune system's recognition of cancer cells, they could also be considered as oncogenes.

Conflicting roles of T_{reg} cells in malignant tumors

The role of T_{reg} cells in immunoncology was discovered by two Japanese groups in 1999 [72,73]. The two groups independently reported that anti-CD25 antibodies, capable of depleting CD4+CD25+ T_{reg} cells, led to a higher tumor rejection and retarded tumor growth in normal and nude mice[72,73]. CD25 is the α chain of interleukin-2 receptor. Onizuka *et al* showed that a single dose (less than 0.125 mg) of anti-CD25 was capable of causing regression of multiple tumors derived from four different inbred mouse strains (five leukemias, myeloma and two sarcomas)[72]. Similarly Shimizu et al showed that elimination of CD25-expressing T cells caused a powerful immune response in synergic tumors in mice, leading to tumor regression within 1 month, thereby allowing the host to survive > 80 days[73]. In CD4+ T cells the percentage of T_{reg} cells is higher the blood of cancer patients compared to that of healthy infividuals [62,74,75]. Expectedly, the relatively higher T_{reg} cells levels in tumor microenvironment correlated with a poor prognosis in various cancer types too, such as melanoma and non-small cell lung, ovarian and gastric cancers[61,62]. T_{reg} cell population is not high in the periphery blood of cancer patients compared with the TME, implying that T-cells interaction with tumor cells is important[75]. On the contrary, certain tumors, such as colorectal cancer (CRC), with a high level of FOXP3+ T cells is correlated with better prognosis[76]. This is because the accumulation of FOXP3+ occurred together with inflammatory cytokines, possibly implying that the T_{reg} cells play a role repressing tumor inflammation. It was brought to light that two populations of FOXP3 (+) CD4 (+) T cells had distinct roles in controlling the prognosis of CRCs contributing in opposing ways. FOXP3 (hi) T_{reg} cells are correlated with worse survival, whereas FOXP3 (lo) non-T_{reg} T cells are correlated with a better one. This is possibly because the FOXP3+ (lo) non-T_{reg} T cells population leads to an inflammatory TME against the tumor. In fact, it was observed that FOXP3+ non-T_{reg} T cells in CRCs are correlated with high levels of tumor necrosis factor (TNF), IL2 and TGFβ [74]. Depleting FOXP3 (hi) T_{reg} cells from tumor tissues could be deployed to increase antitumor immunity to treat CRC or other cancers, whereas other strategies enhancing the levels of FOXP3(lo) non-T_{reg} T cells could also be used to suppress or prevent tumorigenesis[74].

There are conflicting reports regarding the prognostic value of tumor-infiltrating T_{reg} cells. Shang et al demonstrated that FOXP3+ T_{reg} cells are correlated with shorter overall survival in breast, hepatocellular, gastric, melanoma, renal and cervical cancers, longer overall survival in head and neck, colorectal, and esophageal cancers, and no correlation for pancreatic and ovarian cancers[77].

In conclusion, T_{reg} cells inhibit anti-cancer immunity, blocking the immune surveillance of tumors that ultimately lead to cancer spread [60–62,72,73]. Immunosuppressive T_{reg} cells, producing cytokines, are observed in both human chronic inflammatory disease and in cancers, where they promote tumorigenesis through a mechanism similar to that of chronic inflammation[31,78,79]. Depletion of T_{reg} cells in mice is capable of promoting lymphocyte

recruitment and as a consequence a decrease in tumor growth rate and the presence of high endothelial venules, indicating a destruction of the tumor tissues [80,81].

T_{reg} cells and tumor microenvironment

TME is mainly constituted by a subpopulation of T_{reg} cells called bona fide T_{reg} (eT_{reg}) cells that enhance the expression of immunosuppressant molecules such as CTLA-4 and T-cell immunoreceptors with Ig and ITIM domains (called also TIGIT), whose expression is very low in naïve T_{reg} cells[62,74,82]. Transcriptome analysis of 15 human lung cancer samples and 14 colorectal cancer samples has shown that tumor-infiltrating T_{reg} cells have very high levels of different T_{reg} activation markers, like T cell immunoglobulin mucin receptor 3 (HAVCR2), glucocorticoid-induced TNFR-related protein (GIRT), lymphocyte-activation gene 3 protein (LAG3) and inducible T cell co-stimulator (ICOS). Interestingly, this phenotype was not observed in peripheral blood samples from the same patients, whose expression levels in the blood remained the same. This could indicate that T_{reg} cells become activated in TME where they exert their immune suppressive functions [83].

Cross-talking between T_{reg} cells and tumor microenvironment

It has been recently shown that apoptotic T_{reg} cells in TME exert higher immunosuppressive roles than apoptotic T_{reg} cells[7,84]. A weak NRF2-associated antioxidant pathway leads to a vulnerable system against reactive oxygen species in TME possibly causing apoptosis in T_{reg} cells, a process that has been shown to convert high ATP levels into adenosine through T-reg cell-expressed ectoenzymes CD39 and CD73. An abundance in adenosine that becomes generate in turn engages purinergic adenosine A2A receptors (also known as ARORA2A), a family of G protein-couples receptor with 7 transmembrane alpha helices whose function is to regulate oxygen demand and increase vasodilatation as well as suppressing immune cells. Apoptotic T_{reg} use the A2A pathway to suppress immune cells [7,84]. As to the mechanism postulated to explain the activation of T_{reg} cells in TME, it is that proliferating and dying tumor cells have loads of self-antigens, which are best recognized through T_{reg} cells and thereby become activated in TME[85]. Another explanation comes from results from mice experiments of two research groups showing that immune dendritic cells expressed in mice tumors activate T_{reg} cells through a TGFβ-dependent manner[85,86]. T_{reg} cells recognize specific self-antigens that can become clonally expanded in TME[87,88]. T_{reg} cells typically have a higher affinity TCRs for self-antigens than T_{conv} cells and therefore should be predominantly activated even in competition with T_{conv} cells. It must be stated however that these data comes from animal studies and that T_{reg} cells induced by TFGβ have not been fully demonstrated in humans yet. As to epigenetic profile of tumor-infiltrating T_{reg} cells still very little is understood[89–91]. Epigenetic studies of T_{reg} cells are limited and future studies could shed more light on the subject in order to better know the origin and mechanisms of activation of T_{reg} cells. T_{reg} cells move to the TME by chemotaxis via chemokines and their receptors, such as CXCL12-CXCR4, CCL5-CCR5, CCL22-CCR4 or CCL1-CCR8 [62,83,92–96]. Blocking such chemotactic signals can reduce the accumulation of T_{reg} cells inside tumors[97]. Such chemokines are produced in the TME by the tumor and/or macrophages[62,83,92–94]. Additionally, some chemokines like CCL1 and CCL22 can be produced within tumors by exhausted or dysfunctional CD8+ T-cells[97,98]. Therapies targeting chemokines could be considered to decrease levels of $T_{reg}:T_{conv}$ ratios to the tumor microenvironment. Cancers engage various immune escape mechanisms that can be dependent

sometimes to specific tumor intrinsic factors. In fact, alterations in tumor suppressor PTEN, Liver Kinase B1 (LKB1) or oncogenes WNT/ β-catenin, KRAS or basic leucine zipper transcriptional factor ATF-like 3 (BATF3), affect effector T-cells recruitment to the tumors[99–103]. On the contrary, tumor hyper-activation of FAK leads to a recruitment of T_{reg} cells together with a chemokine-driven CD8+ T cell exhaustion or down-modulation[104,105]. In fact, Jiang et al using tissues from pancreatic ductal adenocarcinoma (PDAC) patients observed that FAK was elevated and it correlated with high levels of fibrosis and poor CD8+ cytotoxic T cell infiltration, signs of an immune suppressive TME. The use of a FAK inhibitor (VS-4718) substantially limited tumor progression and doubled survival of a humanoid mice model of PDAC[104]. Serrel et al showed in squamous cell carcinoma (SCC) cells, FAK was shown to drive exhaustion of CD8+ T cells and recruitment of T_{reg} cells in TME through chemokines and cytokines as well as ligand-receptors (such as Ccl5), ultimately permitting tumor growth. FAK kinase inhibitor VS-4718 drove T_{reg} cells depletion and promoted the anti-tumor response of CD8+ T cells[105].

Treg cells and nonself antigens

At the location of tumor cells there are two types of antigens recognized by T_{reg} cells: shared antigens or neoantigens. The first one arises from highly or aberrantly expressed endogenous proteins encoded by germ line cells. The second one derives from either abnormal self-proteins formed from somatic genetic alterations or from oncogenic viral proteins. Experiments in animals have shown that T_{reg} cells priming to nonself antigens increased the affinity of the CD8+ T cells, most likely by the inhibition of T-cells carrying TCRs with low-avidity to antigens [106]. APCs makes CD8+ T cells targeting self-antigens self-tolerant through T_{reg} cells signaling reduction[107]. In fact, the authors showed that T_{reg} cells were able to make the self-reactive human CD8+ cells anergic *in vitro* upon antigen stimulation. In addition they observed the proliferative activity of Tet+CD8+ T cells in CTLA-4+ and CTLA-4- fractions. The CTLA-4+ fraction was highly proliferative, had a low expression level of BCL2 and was prone to death upon Melan-A stimulation. On the contrary, T_{reg} cells were not capable to suppress non-self-specific CD8+ T cells in humans[107]. Therefore, T_{reg} cell-mediated immunosuppression could be more diffused in shared antigen-expressing tumors compared to neoantigens. This could be a reason why tumors with neoantigens respond better to immune checkpoint and tumors with low mutational burden are nonresponsive[108,109]. One of major aims of immunotherapy research is to understand why some cancer patients respond very well to immune checkpoint inhibitions while others do not, as well as discovering new biomarkers useful for just-in-time determination of treatment-responsive patients, before administrating immunotherapies.

4) Correlation Between Anti-CTLA-4 Inhibition with T_{reg} cells expression

Anti-CTLA-4 monoclonal antibody ipilimumab (Yervoy, Bristol-Meyers Squibb) gained FDA-approval in march 2011 for the treatment of advanced melanoma, the most dangerous type of skin cancer, after the large randomized phase III clinical trial made of 676 patients elicited that ipilimumab improved overall survival (OS) of melanoma patients who did not respond to standard therapy. In fact the median OS was 10 months in 403 patients randomly assigned to receive ipilimumab 3mg/kg with investigational vaccine made of HLA-A*01201-restricted glycoprotein 100 with incomplete Freund' adjuvant was 10.0 months (gp100, 95% Confidence Interval [CI], 8.5-11.5) vs 6.4 months observed for 136 patients treated with gp100 only (Hazard Ration [HR] for death = 0.68; p=0.001). Patients treated with ipilimumab alone were 137 and had an OS of 10.1 months vs 6.4 months in the gp100 alone (95% CI, 9.0-13.8; HR for

death=0.66, p=0.003)[110]. After its approval, the drug was added as a category 1 recommendation in National Comprehensive Cancer Network (NCCN) guidelines to the systemic treatment of advanced or metastatic melanoma.

This clinical evidence shows that the antibody enhanced the ability of the immune system to attack cancer through CTLA-4 inhibition. It must be mentioned that adverse events occurred in 10-15% of patients treated with ipilimumab alone compared to patients treated with gp100 only[110].

In 2014 another pivotal phase III clinical trial (CA184-024) in 502 metastatic melanoma tested ipilimumab. The standard of care treatment for the disease currently is chemotherapy (decarbazine), which has not shown to increase OS. Interestingly, treatment of patients with 850 mg/m^2 decarbazine with 10 mg/kg ipilumab improved OS compared to an arm with only the chemotherapy with placebo. OS of patients treated with ipilimumab plus decarbazine vs decarbazine plus placebo were 47.3% vs 36.3% at the first year; 28.5% vs 17.9 % at second year; and 20.8 % vs 12.2 % at third year (HR for death with ipilimumab/decarbazine, 0.72; p< 0.001). The risk to progress through the disease decreased by 24% using ipilimumab/decarbazine vs decarbazine/placebo (HR for progression, 0.76; p= 0.006). The ratios of the disease to control were similar between the two groups (33.2% for ipilimumab/decarbazine and 30.2% for decarbazome/placebo; p=0.41). This study was important because it showed how ipilimuamb could be used as the first line treatment for metastatic melanoma.[111] The study tested a higher concentration (10 mg/kg) of ipilimuab than the approved 3 mg/kg.[112] Consequently more adverse events were observed using higher doses of anti-CTLA-4 possibly because CTLA-4 molecular degradation. In fact CTLA-4 is needed to prevent immune-related adverse reactions and its degradation can be deleterious.

Interestingly, a recent report demonstrates that the irAEs of ipilimumab and alike come from lysosomal degradation of CTLA-4 in T_{reg} cells. They used CTLA-4 mutant (Y201V), which is incapable of being recycled because it lacks of interaction with LRBA. This indicates that the specific region of CTLA4 is an essential mediator for CTLA-4 recycling. They made antibodies targeting CTLA-4 (HL12 and HL32) that they were not able to degrade CTLA-4 of T_{reg} cells. In fact, in contrast to Ipilimumab or TremeIgG1, the use of novel anti-CTLA4 antibodies had no effect on CTLA-4 level of T_{reg} cells in the same model. Additionally, HL12 and HL32 could more effectively lead to tumor rejection with fewer irAEs in mice[113]. Such knowledge is useful for the generation of novel antibodies or molecules that could inhibit CLTA4 without eliciting its degradation and could therefore be used in combination with other PD-1 or PD-L1 inhibitors with fewer toxicity.

Various studies show that consolidated or novel types of CTLA-4 therapies correlated with different expression levels of T_{reg} cells. Ji et al showed that treatment of mice with 0.25mg anti-CTLA-4 monoclonal antibody correlated with a lower level of CD25+Foxp3+ T_{reg} cells population (p<0.05)[114]. Qu et al observed that IL36-stimulated antitumor activities CTLA-4 monoclonal antibodies blocked tumors by decreasing T_{reg} cells expression in tumors[115]. Mihic-Probst et al evinced that CTLA-4 antibodies Ipilimumab, anti PD-1 antibodies nivolumab or pembrolizumab decreased CD25+ T_{reg} cells[116]. Sun et al observed that T_{reg} cells decreased after treating mice with anti-CTLA-4 or anti PD-1 antibodies in HPV16 E6/E7$^+$ syngeneic mouse tumor model [117]. Kvarnhammar et al showed that a new IgG1 bispecific anti-CTLA-4 and anti-OX40 induced activation of T-cells and T_{reg} cells depletion *in vitro* and *in vivo* in the tumor[118]. Sharma et al using samples from 19 melanoma, 17 prostate, 9 bladder cancer treated

with ipilimumab and 18 samples from melanoma cancers treated with tremelimumab, observed that the monoclonal antibodies depleted intratumoral FOXP3 T_{reg} cells in tumors[31]. Pal et al demonstrated that a tetravalent bispecific anti-CTLA-4 antibody with 2 variables joined through a short flexible linker in tandem, decreased T_{reg} cells in tumors from patients using immunohistochemistry (IHC) and Mass Cytometry CyTOF, which is a variation of flow cytometry where antibodies are tagged with heavy metal ion tags instead of fluorochromes[31]. Morris et al observed that anti-CTLA-4 antibodies IgG2a and IgG2b isotypes of 9D9 clone decreased T_{reg} cells in syngeneic murine tumors of B78 melanoma and/or Panc02 pancreatic cancer[119]. Duperret et al observed that anti CTLA-4 together in combination with a TERT DNA vaccine administered once a week for 4 rounds of immunization in C57BL/6 mice, the levels of T_{reg} cells decreased within the tumors, while it remained unchanged within the peripheral blood[120]. Tang et al observed with IHC and quantitative real-time PCR that anti-CTLA-4 monoclonal antibody decreased T_{reg} cells expression in mice tumors microenvironment, but not in peripherial lymphoid organs [121]. Son et al showed that anti-CTLA-4 antibody and radiotherapy suppressed CD25 T_{reg} cells in C57BL mice injected with lung cancer[122]. Schwarz et al investigated the effect of using different doses of anti-CTLA-4 on T_{reg} cells expression in mice. They used low dose of 0.25 mg CTLA-Ig antibody (LD, 10 mg/kg body weight), high dose of 1.25 mg CTLA-Ig antibody (HD, 50 mg/kg body weight) and very high dose of 6.25 mg CTLA-Ig antibody (VHD, 250 mg/kg body weight). T_{reg} cells decreased independently from the doses [123]. Marabelle et al using a combination of anti-CTLA-4, anti-OX40 with CpG therapy observed a reduction of T_{reg} cells in tumors[124].

Interestingly, Du et al observed that anti-CTLA4 antibodies are capable of inducing efficiently T_{reg} cells depletion and tumor regression in mice [125].

In contrast, several other groups reported an increase of T_{reg} cells in cancers after anti-CTLA-4 treatment. In fact, Sandi et al observed that high doses treatment of anti-CTLA-4 increased accumulation of T_{reg} cells in secondary lymphoid organs[126]. Kavanagh et al observed that anti-CTLA-4 antibody ipilimumab in 4 cohorts of patients did increase T_{reg} cells levels in a dose-dependent manner. The drug was administered every 28 days[127]. Quezada et al observed that CTLA-4 blockade with GM-CSF combination immunotherapy in an *in vivo* B16/BL6 mouse model of melanoma led to a self-expansion of T_{reg} cells in tumors[30]. The reason for such discrepancies between the last four studies to the majority of studies described in the previous paragraphs are still unknown. A possible explanation could be that different subpopulations of T_{reg} cells were detected by the groups, such as bona fide and naïve Treg cells, or that the organisms TME of either animals or humans were different across the different experimental settings.

Of note, CTLA-4 has two opposing and crucial properties in cancer and autoimmunity. For self-tolerance it is important to have functional CTLA-4. In adult mice it was observed that conditional deletion of CTLA-4 resulted in hypergammaglobulinemia, spontaneous lymphoproliferation and histologically evident gastritis, organi-specific autoantibodies generation, insulitis, pneumonits and sialadenitis[128]. Current antibodies developed against CTLA-4 have the property of reducing by half the levels of CTLA-4 by lysosomal degradation, which is directly responsible for their toxicity[113]. Therefore since CTLA-4 is crucial for preventing autoimmunity, the major cause of irAE triggered by monoclonal antibodies such as Ipilimumab and Tremelimumab[113], new drugs should be developed considering such gap. Encouraging results have already come from Zhang et al HL12 and HL32 anti-CTLA-4

antibodies that did not change CTLA-4 levels total or in the T_{reg} cells fraction, while exerting their powerful anti-CTLA-4 induced tumor inhibition[113]. The authors transiently transfected 293T cells with CTLA-4 cDNA and incubated the cells with control hIgGFc, Ipilimumab, TremeIgG1, HL12 and HL32 for 4 hours. They investigated the CTLA-4 proteins expression by western blot; with immunoblot for cytosolic and plasma membrane fractions of the protein; using stable CHO cell line expressing hCTLA-4 and after the treatments with Ipilimumab, TremeIgG1, HL12 or HL32 for 30 minutes detecting with flow cytometry the expression of CTLA-4 with AF488-conjugated anti-human Fc antibody; from in vivo studies they took the spleen and lung T_{reg} cells to evaluate CTLA-4 by flow cytometry; in PBMCs from human healthy donors' blood that were stimulated using anti-CD3/anti-CD28 for two days and treated with either the controls hIgG, Ipilimumab or HL12 for 4 hours. The levels of CD4+/FOXP3+/CTLA-4+ were measured with flow cytrometry [113]. Table 1 summarizes all the studies investigating anti-CTLA-4 therapies effect over T_{reg} cells levels.

Table 1. CTLA-4 inhibitors effect on levels of T_{reg} cells.

Reference	Anti-CTLA4 Therapy and Samples	Effect on T_{reg} cells Expression
Ji et al 2020	In vivo investigated effect of administration of 0.25 mg anti-CTLA4 monoclonal antibody on the CD25+Foxp3+ population in spleens and tumor tissues.	Decreased T_{reg} cells (p<0.05) in tumor. It did not in spleen
Qu et al 2020	CTLA-4 monoclonal antibodies	Decreased T_{reg} cells in tumors
Probst et al 2020	All patients received anti-CTLA-4 therapy and 4 received additional anti-PD1 therapy.	Decreased T_{reg} cells in tumors
Zhang et al 2019	anti-CTLA-4 therapy Ipililumab and TremeIgG1 standard and HL12 and HL32 experimental antibodies	Ipilimumab and TremeIgG1 downregulated cell-surface and total CTLA-4 level in T_{reg} cells from spleen and lung. In contrast, HL12 and HL32 had no effect on

		CTLA-4 level of T_{reg} cells in the same model.
Sun et al 2019	Anti –CTLA4 antibody	Downregulation of T_{reg} cells in tumors of mice
Kvarnhammar et al 2019	CTLA4 x OX40 bispecific antibody ATOR-1015 was used in vivo	Reduced the frequency of T_{reg} cells in vitro and at the tumor site in vivo
Sharma et al 2019	19 melanoma patients, 17 prostate cancer, 9 bladder cancer samples were treated with ipililumab. 18 melanoma tumors were treated with tremelimumab	mAbs depleted intratumoral FOXP3+ T_{reg} cells in tumors via Fc-dependent mechanisms.
Pal et al 2019	Anti CTLA4 DVD Ig tetravalent bispecific antibody-like antibody containing an Fc region and 2 pairs of variable domains joined in tandem by a short flexible linker	Decreased T_{reg} cells in tumors
Tang et al 2019	Anti-CTLA4 monoclonal antibody	Increase of T_{reg} cells in tumors
Morris et al 2018	Anti CTLA4 (IgG2a and IgG2b isotypes of the 9D9 clone)	Decreased T_{reg} cells in tumors
Duperret et al 2018	Anti CTLA-4 with a TERT DNA vaccine in C57BL/6 mice. Mice were immunized at 1-week intervals for a total of 4 immunizations.	Decreased T_{reg} cells frequency within the tumor. It did not in peripheral blood.

Du et al 2018	Anti-CTLA4 antibodies binding to human like Ipilimumab	No effect on T_{reg} cells
Son et al 2017	Anti-CTLA4 antibody therapy and radiotherapy in mice	Suppression of T_{reg} cells in tumors
Schwarz et al 2016	Anti-CTLA4 low dose (0.25 mg), high dose (1.25 mg) and very high dose (6.25 mg were given to mice.	CD25 T_{reg} cells were reduced independently from the doses
Sandin et al 2014	Comparison between low-dose peritumoral and high-dose systemic CTLA-4 Blockade therapy	As opposed to low-dose, high dose systemic therapy stimulated accumulation of T_{reg} cells in secondary lymphoid organs. This could counteract immunotherapeutic benefit of CTLA4 blockade
Marabelle et al 2013	Anti-CTLA-4 and anti-OX40 with CpG	Depleted T_{reg} cells in tumors
Mangsbo et al 2010	Anti-CTLA-4 or anti-PD-1 with CpG therapy.	The combinations reduced numbers of T_{reg} cells at tumor site
Kavanagh et al 2007	Anti CTLA-4 antibody dose escalation	Increased T_{reg} cells in tumors in a dose dependent manner
Quezada et al 2006	CTLA-4 blockade and GM-CSF combination immunotherapy in vivo mice model B16/BL6 melanoma	Led to self-expansion of T_{reg} cells in tumors

Moreover, in clinical routine it should also to be considered that T cells are made of multiple subpopulations with their own peculiar effects. The modulation of T_{reg} cells and/or the T_{eff} cells and pro-inflammatory responses is critical for cancer. An immunosuppressive state (increased T_{reg} and/or decreased T_{eff}) may facilitate the growth and spread of abnormal cancer cells. Therefore, the T_{reg}:T_{eff} ratio could be used in clinical setting. The new checkpoint inhibitors attempt to pharmacologically modulate the T_{reg}:T_{eff} ratio in the treatment of cancer therapy. However, in cancer progression, expression of co-inhibitory molecules by the tumors, favors the imbalance in the tumor microenvironment toward an immune suppression status by increasing T_{reg} infiltration and decreasing T_{eff} activity[129]. The anti-CTLA-4 therapies may help in the modulation of T_{reg}:T_{eff} ratio by the T_{reg} depletion in the tumour as the high expression of CTLA-4 on T_{reg} and by the increase of activated effectors. The net result may result in the potentiation of effector numbers, permitting an anti-tumour response[130]. Tremelimumab showed to improve the proliferative response of T_{eff} and to abrogated the T_{reg} suppressive ability, suggesting that the monitor of these populations may allow to select properly those responsive patients from those who would not have a benefit from immunotherapy[131]. With regards to the patients' management, it seems to be crucial to understand and monitor the "ping-pong" effect produced by treatment on the T_{reg}:T_{eff} ratio in the regulation of autoimmunity and anti-tumor immunity. The clinicians should pay attention in monitoring this effect in order to maintain the effective anti-tumor response and the immune homeostasis preventing the IRAEs onset[132].

5) Conclusive remark and future directions

In conclusion, most studies have shown that CTLA-4 antibodies mainly depleted T_{reg} cells in cancers, whereas very few observed T_{reg} cells increased or remained the same because of different experimental settings or in some cases the design of their therapeutic agents. It is generally known that T_{reg} cells inhibit anti-cancer immunity, blocking the immune surveillance of tumors, leading ultimately to cancer growth. In our opinion, antibodies or small molecules that inhibit CTLA-4, but do not alter CTLA-4 levels in T_{reg} cells could be innovative and ultimately more effective in eradicating cancer cells. In fact, such drugs would not cause degradation of CTLA-4 and consequently do not interfere with T_{reg} cells' function in preventing autoimmunity. Consequently inhibition of CTLA-4 could be achieved without the degradation of CTLA-4 and adverse related events caused by toxicity. Testing their efficiency together with other checkpoint inhibitors such as anti-PD1 and anti-PD-L1 could further improve therapy efficacy.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Miller, K.D.D.; Fidler-Benaoudia, M.; Keegan, T.H.H.; Hipp, H.S.S.; Jemal, A.; Siegel, R.L.L. Cancer statistics for adolescents and young adults, 2020. *CA. Cancer J. Clin.* **2020**, *70*, 443–459, doi:10.3322/caac.21637.

2. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2019. *CA. Cancer J. Clin.* **2019**, *69*, 7–34, doi:10.3322/caac.21551.

3. Speiser, D.E.E.; Ho, P.C.C.; Verdeil, G. Regulatory circuits of T cell function in cancer. *Nat. Rev. Immunol.* 2016, *16*, 599–611.

4. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 2006, *6*, 295–307.

5. Mougiakakos, D.; Johansson, C.C.C.; Trocme, E.; All-Ericsson, C.; Economou, M.A.A.; Larsson, O.; Seregard, S.; Kiessling, R. Intratumoral forkhead box p3-positive regulatory t cells predict poor survival in cyclooxygenase-2-positive uveal melanoma. *Cancer* **2010**, *116*, 2224–2233, doi:10.1002/cncr.24999.

6. Piccirillo, C.A.A. Regulatory T cells in health and disease. *Cytokine* 2008, *43*, 395–401.

7. Maj, T.; Wang, W.; Crespo, J.; Zhang, H.; Wang, W.; Wei, S.; Zhao, L.; Vatan, L.; Shao, I.; Szeliga, W.; et al. Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor. *Nat. Immunol.* **2017**, *18*, 1332–1341, doi:10.1038/ni.3868.

8. Vanamee, É.S.S.; Faustman, D.L.L. TNFR2: A Novel Target for Cancer Immunotherapy. *Trends Mol. Med.* 2017, *23*, 1037–1046.

9. Togashi, Y.; Shitara, K.; Nishikawa, H. Regulatory T cells in cancer immunosuppression — implications for anticancer therapy. *Nat. Rev. Clin. Oncol.* 2019, *16*, 356–371.

10. Zeng, G.; Jin, L.; Ying, Q.; Chen, H.; Thembinkosi, M.C.C.; Yang, C.; Zhao, J.; Ji, H.; Lin, S.; Peng, R.; et al. Regulatory t cells in cancer immunotherapy: Basic research outcomes and clinical directions. *Cancer Manag. Res.* 2020, *12*, 10411–10421.

11. Atkins, M.B.B.; Clark, J.I.I.; Quinn, D.I.I. Immune checkpoint inhibitors in advanced renal cell carcinoma: experience to date and future directions. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* 2017, *28*, 1484–1494.

12. Foell, J.; Hewes, B. T Cell Costimulatory and Inhibitory Receptors as Therapeutic Targets for Inducing Anti-Tumor Immunity. *Curr. Cancer Drug Targets* **2007**, *7*, 55–70, doi:10.2174/156800907780006841.

13. Wei, S.C.; Duffy, C.R.; Allison, J.P.; S.C., W.; C.R., D.; J.P., A. Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discov.* **2018**, *8*, 1069–1086, doi:10.1158/2159-8290.CD-18-0367 LK - http://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=21598290&id=doi:10.1158%2F2159-8290.CD-18-0367&atitle=Fundamental+mechanisms+of+immune+checkpoint+blockade+therapy&stitle=Cancer+Discov.&title=Cancer+Discovery&volume=8&issue=9&spage=1069&epage=1086&aulast=Wei&aufirst=Spencer+C.&auinit=S.C.&aufull=Wei+S.C.&coden=&isbn=&pages=1069-1086&date=2018&auinit1=S&auinitm=C.

14.   Pentcheva-Hoang, T.; Egen, J.G.; Wojnoonski, K.; Allison, J.P. B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse. *Immunity* **2004**, *21*, 401–413, doi:10.1016/j.immuni.2004.06.017.

15.   Walunas, T.L.; Lenschow, D.J.; Bakker, C.Y.; Linsley, P.S.; Freeman, G.J.; Green, J.M.; Thompson, C.B.; Bluestone, J.A. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* **1994**, *1*, 405–413, doi:10.1016/1074-7613(94)90071-X.

16.   Brunner MC, Chambers CA, Chan FK, Hanke J, Winoto A, A.J. CTLA-4-Mediated inhibition of early events of T cell proliferation - PubMed Available online: https://pubmed.ncbi.nlm.nih.gov/10229815/ (accessed on Nov 18, 2020).

17.   Linsley, P.S.; Brady, W.; Urnes, M.; Grosmaire, L.S.; Damle, N.K.; Ledbetter, J.A. CTLA4 is a second receptor for the b cell activation antigen B7. *J. Exp. Med.* **1991**, *174*, 561–569, doi:10.1084/jem.174.3.561.

18.   Linsley, P.S.; Greene, J.A.L.; Brady, W.; Bajorath, J.; Ledbetter, J.A.; Peach, R. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* **1994**, *1*, 793–801, doi:10.1016/S1074-7613(94)80021-9.

19.   Lanier, L.L.; O'Fallon, S.; Somoza, C.; Phillips, J.H.; Linsley, P.S.; Okumura, K.; Ito, D.; Azuma, M. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. *J. Immunol.* **1995**, *154*.

20.   Van Der Merwe, P.A.; Bodian, D.L.; Daenke, S.; Linsley, P.; Davis, S.J. CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J. Exp. Med.* **1997**, *185*, 393–403, doi:10.1084/jem.185.3.393.

21.   Egen, J.G.; Allison, J.P. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity* **2002**, *16*, 23–35, doi:10.1016/S1074-7613(01)00259-X.

22.   Pagès, F.; Ragueneau, M.; Rottapel, R.; Truneh, A.; Nunes, J.; Imbert, J.; Olive, D. Binding of phosphatidyl-inositol-3-OH kinase to CD28 is required for T-cell signalling. *Nature* **1994**, *369*, 327–329, doi:10.1038/369327a0.

23.   Kane, L.P.; Andres, P.G.; Howland, K.C.; Abbas, A.K.; Weiss, A. Akt provides the CD28 costimulatory signal for up-regulation of IL-2 and IFN-γ but not TH2 cytokines. *Nat. Immunol.* **2001**, *2*, 37–44, doi:10.1038/83144.

24.   Chambers, C.A.; Cado, D.; Truong, T.; Allison, J.P. Thymocyte development is normal in CTLA-4-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 9296–9301, doi:10.1073/pnas.94.17.9296.

25.   Waterhouse, P.; Penninger, J.M.; Timms, E.; Wakeham, A.; Shahinian, A.; Lee, K.P.; Thompson, C.B.; Griesser, H.; Mak, T.W. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science (80-.).* **1995**, *270*, 985–8, doi:10.1126/science.270.5238.985.

26.   Tivol, E.A.; Borriello, F.; Schweitzer, A.N.; Lynch, W.P.; Bluestone, J.A.; Sharpe, A.H. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* **1995**, *3*, 541–7, doi:10.1016/1074-7613(95)90125-6.

27.   M F Bachmann, G Köhler, B Ecabert, T W Mak, M.K. Cutting edge: lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous - PubMed Available online: https://pubmed.ncbi.nlm.nih.gov/10415006/ (accessed on Nov 19, 2020).

28.   Wing, K.; Onishi, Y.; Prieto-Martin, P.; Yamaguchi, T.; Miyara, M.; Fehervari, Z.; Nomura, T.; Sakaguchi, S. CTLA-4 control over Foxp3+ regulatory T cell function. *Science (80-.).* **2008**, *322*, 271–275, doi:10.1126/science.1160062.

29.   Friedline, R.H.; Brown, D.S.; Nguyen, H.; Kornfeld, H.; Lee, J.; Zhang, Y.; Appleby, M.; Der, S.D.; Kang, J.; Chambers, C.A. CD4+ regulatory T cells require CTLA-4 for the maintenance of systemic tolerance. *J. Exp. Med.* **2009**, *206*, 421–434, doi:10.1084/jem.20081811.

30.   Quezada, S.A.; Peggs, K.S.; Curran, M.A.; Allison, J.P. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J. Clin. Invest.* **2006**, *116*, 1935–1945, doi:10.1172/JCI27745.

31.   Sharma, A.; Subudhi, S.K.; Blando, J.; Scutti, J.; Vence, L.; Wargo, J.; Allison, J.P.; Ribas, A.; Sharma, P. Anti-CTLA-4 immunotherapy does not deplete Foxp3 þ regulatory T cells (Tregs) in human cancers. *Clin. Cancer Res.* **2019**, *25*, 1233–1238, doi:10.1158/1078-0432.CCR-18-0762.

32.   Jain, N.; Nguyen, H.; Chambers, C.; Kang, J. Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 1524–1528, doi:10.1073/pnas.0910341107.

33.   Corse, E.; Allison, J.P. Cutting Edge: CTLA-4 on Effector T Cells Inhibits In Trans . *J. Immunol.* **2012**, *189*, 1123–1127, doi:10.4049/jimmunol.1200695.

34.   Qureshi, O.S.; Zheng, Y.; Nakamura, K.; Attridge, K.; Manzotti, C.; Schmidt, E.M.; Baker, J.; Jeffery, L.E.; Kaur, S.; Briggs, Z.; et al. Trans-endocytosis of CD80 and CD86: A molecular basis for the cell-extrinsic function of CTLA-4. *Science (80-.).* **2011**, *332*, 600–603, doi:10.1126/science.1202947.

35.   Paterson, A.M.; Lovitch, S.B.; Sage, P.T.; Juneja, V.R.; Lee, Y.; Trombley, J.D.; Arancibia-Cárcamo, C. V.; Sobel, R.A.; Rudensky, A.Y.; Kuchroo, V.K.; et al. Deletion of CTLA-4 on regulatory T cells during adulthood leads to resistance to autoimmunity. *J. Exp. Med.* **2015**, *212*, 1603–1621, doi:10.1084/jem.20141030.

36.   Doyle, A.M.; Mullen, A.C.; Villarino, A. V.; Hutchins, A.S.; High, F.A.; Lee, H.W.; Thompson, C.B.; Reiner, S.L. Induction of cytotoxic T lymphocyte antigen 4 (CTLA-4) restricts clonal expansion of helper T cells. *J. Exp. Med.* **2001**, *194*, 893–902, doi:10.1084/jem.194.7.893.

37.   Egen, J.G.; Kuhns, M.S.; Allison, J.P. CTLA-4: New insights into its biological function and use in tumor immunotherapy. *Nat. Immunol.* 2002, *3*, 611–618.

38.   Busch, D.H.; Pamer, E.G. T cell affinity maturation by selective expansion during infection. *J. Exp. Med.* **1999**, *189*, 701–709, doi:10.1084/jem.189.4.701.

39.   Savage, P.A.; Boniface, J.J.; Davis, M.M. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity* **1999**, *10*, 485–492, doi:10.1016/S1074-7613(00)80048-5.

40.   Metzler, W.J.; Bajorath, J.; Fenderson, W.; Shaw, S.Y.; Constantine, K.L.; Naemura, J.; Leytze, G.; Peach, R.J.; Lavoie, T.B.; Mueller, L.; et al. Solution structure of human CTLA-4 and delineation of a CD80/CD86 binding site conserved in CD28. *Nat. Struct. Biol.* **1997**, *4*, 527–531, doi:10.1038/nsb0797-527.

41.   Yu, C.; Sonnen, A.F.P.; George, R.; Dessailly, B.H.; Stagg, L.J.; Evans, E.J.; Orengo, C.A.; Stuart, D.I.; Ladbury, J.E.; Ikemizu, S.; et al. Rigid-body ligand recognition drives cytotoxic T-lymphocyte antigen 4 (CTLA-4) receptor triggering. *J. Biol. Chem.* **2011**, *286*, 6685–6696, doi:10.1074/jbc.M110.182394.

42.   Stamper, C.C.; Zhang, Y.; Tobin, J.F.; Erbe, D. V.; Ikemizu, S.; Davis, S.J.; Stahl, M.L.; Seehra, J.; Somers, W.S.; Mosyak, L. Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses. *Nature* **2001**, *410*, 608–611, doi:10.1038/35069118.

43.   Schwartz, J.C.D.; Zhang, X.; Fedorov, A.A.; Nathenson, S.G.; Almo, S.C. Structural basis for co-stimulation by the human CTLA-4/B7-2 complex. *Nature* **2001**, *410*, 604–608, doi:10.1038/35069112.

44.   Zhang, F.; Qi, X.; Wang, X.; Wei, D.; Wu, J.; Feng, L.; Cai, H.; Wang, Y.; Zeng, N.; Xu, T.; et al. Structural basis of the therapeutic anti-PD-L1 antibody atezolizumab. *Oncotarget* **2017**, *8*, 90215–90224, doi:10.18632/oncotarget.21652.

45.   Ramagopal, U.A.; Liu, W.; Garrett-Thomson, S.C.; Bonanno, J.B.; Yan, Q.; Srinivasan, M.; Wong, S.C.; Bell, A.; Mankikar, S.; Rangan, V.S.; et al. Structural basis for cancer immunotherapy by the first-in-class checkpoint inhibitor ipilimumab. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, E4223–E4232, doi:10.1073/pnas.1617941114.

46.   He, M.; Chai, Y.; Qi, J.; Zhang, C.W.H.; Tong, Z.; Shi, Y.; Yan, J.; Tan, S.; Gao, G.F. Remarkably similar CTLA-4 binding properties of therapeutic ipilimumab and tremelimumab antibodies. *Oncotarget* **2017**, *8*, 67129–67139, doi:10.18632/oncotarget.18004.

47.   Wang, H.; Sun, Y.; Zhou, X.; Chen, C.; Jiao, L.; Li, W.; Gou, S.; Li, Y.; Du, J.; Chen, G.; et al. CD47/SIRPα blocking peptide identification and synergistic effect with irradiation for cancer immunotherapy. *J. Immunother. Cancer* **2020**, *8*,

905, doi:10.1136/jitc-2020-000905.

48. Kaspar, A.A.; Reichert, J.M. Future directions for peptide therapeutics development. *Drug Discov. Today* 2013, *18*, 807–817.

49. Xie, J.; Si, X.; Gu, S.; Wang, M.; Shen, J.; Li, H.; Li, D.; Fang, Y.; Liu, C.; Zhu, J. Allosteric Inhibitors of SHP2 with Therapeutic Potential for Cancer Treatment. *J. Med. Chem.* **2017**, *60*, 10205–10219, doi:10.1021/acs.jmedchem.7b01520.

50. Song, K.; Liu, X.; Huang, W.; Lu, S.; Shen, Q.; Zhang, L.; Zhang, J. Improved Method for the Identification and Validation of Allosteric Sites. *J. Chem. Inf. Model.* **2017**, *57*, 2358–2363, doi:10.1021/acs.jcim.7b00014.

51. Roca, C.; Requena, C.; Sebastián-Pérez, V.; Malhotra, S.; Radoux, C.; Pérez, C.; Martinez, A.; Antonio Páez, J.; Blundell, T.L.; Campillo, N.E. Identification of new allosteric sites and modulators of AChE through computational and experimental tools. *J. Enzyme Inhib. Med. Chem.* **2018**, *33*, 1034–1047, doi:10.1080/14756366.2018.1476502.

52. Sakaguchi, S.; Sakaguchi, N.; Asano, M.; Itoh, M.; Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **1995**, *155*.

53. Bennett, C.L.; Christie, J.; Ramsdell, F.; Brunkow, M.E.; Ferguson, P.J.; Whitesell, L.; Kelly, T.E.; Saulsbury, F.T.; Chance, P.F.; Ochs, H.D. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* **2001**, *27*, 20–21, doi:10.1038/83713.

54. Fontenot, J.D.; Gavin, M.A.; Rudensky, A.Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *J. Immunol.* **2017**, *198*, 986–992, doi:10.1038/ni904.

55. Brunkow, M.E.; Jeffery, E.W.; Hjerrild, K.A.; Paeper, B.; Clark, L.B.; Yasayko, S.A.; Wilkinson, J.E.; Galas, D.; Ziegler, S.F.; Ramsdell, F. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* **2001**, *27*, 68–73, doi:10.1038/83784.

56. Linterman, M.A.; Pierson, W.; Lee, S.K.; Kallies, A.; Kawamoto, S.; Rayner, T.F.; Srivastava, M.; Divekar, D.P.; Beaton, L.; Hogan, J.J.; et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat. Med.* **2011**, *17*, 975–982, doi:10.1038/nm.2425.

57. Koch, M.A.; Tucker-Heard, G.; Perdue, N.R.; Killebrew, J.R.; Urdahl, K.B.; Campbell, D.J. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat. Immunol.* **2009**, *10*, 595–602, doi:10.1038/ni.1731.

58. Chung, Y.; Tanaka, S.; Chu, F.; Nurieva, R.I.; Martinez, G.J.; Rawal, S.; Wang, Y.H.; Lim, H.; Reynolds, J.M.; Zhou, X.H.; et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat. Med.* **2011**, *17*, 983–988, doi:10.1038/nm.2426.

59. Chaudhry, A.; Rudra, D.; Treuting, P.; Samstein, R.M.; Liang, Y.; Kas, A.; Rudensky, A.Y. CD4+ regulatory T cells control TH17 responses in a stat3-dependent manner. *Science (80-.).* **2009**, *326*, 986–991, doi:10.1126/science.1172702.

60. Wing, K.; Sakaguchi, S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat. Immunol.* 2010, *11*, 7–13.

61. Sakaguchi, S.; Miyara, M.; Costantino, C.M.; Hafler, D.A. FOXP3 + regulatory T cells in the human immune system. *Nat. Rev. Immunol.* 2010, *10*, 490–500.

62. Togashi, Y.; Nishikawa, H. Regulatory T cells: Molecular and cellular basis for immunoregulation. In *Current Topics in Microbiology and Immunology*; Springer Verlag, 2017; Vol. 410, pp. 3–27.

63. Hori, S.; Nomura, T.; Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *J. Immunol.* **2017**, *198*, 981–985, doi:10.1126/science.1079490.

64. Khattri, R.; Cox, T.; Yasayko, S.A.; Ramsdell, F. An essential role for Scurfin in CD4+CD25+T regulatory cells. *J.*

Immunol. **2017**, *198*, 993–998, doi:10.1038/ni909.

65.   Takahashi, T.; Kuniyasu, Y.; Toda, M.; Sakaguchi, N.; Itoh, M.; Iwata, M.; Shimizu, J.; Sakaguchi, S. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: Induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* **1998**, *10*, 1969–1980, doi:10.1093/intimm/10.12.1969.

66.   Thornton, A.M.M.; Shevach, E.M.M. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* **1998**, *188*, 287–296, doi:10.1084/jem.188.2.287.

67.   Steinbrink, K.; Wölfl, M.; Jonuleit, H.; Knop, J.; Enk, A.H.H. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* **1997**, *159*.

68.   Collison, L.W.W.; Workman, C.J.J.; Kuo, T.T.T.; Boyd, K.; Wang, Y.; Vignali, K.M.M.; Cross, R.; Sehy, D.; Blumberg, R.S.S.; Vignali, D.A.A.A.A. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **2007**, *450*, 566–569, doi:10.1038/nature06306.

69.   Jarnicki, A.G.G.; Lysaght, J.; Todryk, S.; Mills, K.H.G.H.G. Suppression of Antitumor Immunity by IL-10 and TGF-β-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*, 896–904, doi:10.4049/jimmunol.177.2.896.

70.   Turnis, M.E.E.; Sawant, D.V. V.; Szymczak-Workman, A.L.L.; Andrews, L.P.P.; Delgoffe, G.M.M.; Yano, H.; Beres, A.J.J.; Vogel, P.; Workman, C.J.J.; Vignali, D.A.A.A.A. Interleukin-35 Limits Anti-Tumor Immunity. *Immunity* **2016**, *44*, 316–329, doi:10.1016/j.immuni.2016.01.013.

71.   Perez, V.L.L.; Van Parijs, L.; Biuckians, A.; Zheng, X.X.X.; Strom, T.B.B.; Abbas, A.K.K. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* **1997**, *6*, 411–417, doi:10.1016/S1074-7613(00)80284-8.

72.   Onizuka, S.; Tawara, I.; Shimizu, J.; Sakaguchi, S.; Fujita, T.; Nakayama, E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. *Cancer Res.* **1999**, *59*, 3128–3133.

73.   J Shimizu, S.Y.S.S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity - PubMed. *J. Immunol.* **1999**, *163*, 5211–5218.

74.   Saito, T.; Nishikawa, H.; Wada, H.; Nagano, Y.; Sugiyama, D.; Atarashi, K.; Maeda, Y.; Hamaguchi, M.; Ohkura, N.; Sato, E.; et al. Two FOXP3 + CD4 + T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat. Med.* **2016**, *22*, 679–684, doi:10.1038/nm.4086.

75.   Tada, Y.; Togashi, Y.; Kotani, D.; Kuwata, T.; Sato, E.; Kawazoe, A.; Doi, T.; Wada, H.; Nishikawa, H.; Shitara, K. Targeting VEGFR2 with Ramucirumab strongly impacts effector/ activated regulatory T cells and CD8+ T cells in the tumor microenvironment. *J. Immunother. Cancer* **2018**, *6*, 106, doi:10.1186/s40425-018-0403-1.

76.   Fridman, W.H.; Pagès, F.; Sautès-Fridman, C.; Galon, J. The immune contexture in human tumours: Impact on clinical outcome. *Nat. Rev. Cancer* 2012, *12*, 298–306.

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

78.   Kryczek, I.; Wu, K.; Zhao, E.; Wei, S.; Vatan, L.; Szeliga, W.; Huang, E.; Greenson, J.; Chang, A.; Roliński, J.; et al. IL-17 + Regulatory T Cells in the Microenvironments of Chronic Inflammation and Cancer . *J. Immunol.* **2011**, *186*, 4388–4395, doi:10.4049/jimmunol.1003251.

79.   Kryczek, I.; Liu, R.; Wang, G.; Wu, K.; Shu, X.; Szeliga, W.; Vatan, L.; Finlayson, E.; Huang, E.; Simeone, D.; et al. FOXP3 defines regulatory t cells in human tumor and autoimmune disease. *Cancer Res.* **2009**, *69*, 3995–4000, doi:10.1158/0008-5472.CAN-08-3804.

80.   Colbeck, E.J.; Jones, E.; Hindley, J.P.; Smart, K.; Schulz, R.; Browne, M.; Cutting, S.; Williams, A.; Parry, L.; Godkin, A.; et al. Treg depletion licenses T cell–driven HEV neogenesis and promotes tumor destruction. *Cancer Immunol. Res.* **2017**, *5*, 1005–1015, doi:10.1158/2326-6066.CIR-17-0131.

81.   Hindley, J.P.; Jones, E.; Smart, K.; Bridgeman, H.; Lauder, S.N.; Ondondo, B.; Cutting, S.; Ladell, K.; Wynn, K.K.; Withers, D.; et al. T-cell trafficking facilitated by high endothelial venules is required for tumor control after regulatory T-cell depletion. *Cancer Res.* **2012**, *72*, 5473–5482, doi:10.1158/0008-5472.CAN-12-1912.

82.   Sugiyama, D.; Nishikawa, H.; Maeda, Y.; Nishioka, M.; Tanemura, A.; Katayama, I.; Ezoe, S.; Kanakura, Y.; Sato, E.; Fukumori, Y.; et al. Anti-CCR4 mAb selectively depletes effector-Type FoxP3+CD4+ regulatory T cells, evoking antitumor immune responses in humans. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 17945–17950, doi:10.1073/pnas.1316796110.

83.   Facciabene, A.; Peng, X.; Hagemann, I.S.; Balint, K.; Barchetti, A.; Wang, L.P.; Gimotty, P.A.; Gilks, C.B.; Lal, P.; Zhang, L.; et al. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T reg cells. *Nature* **2011**, *475*, 226–230, doi:10.1038/nature10169.

84.   Togashi, Y.; Nishikawa, H. Suppression from beyond the grave. *Nat. Immunol.* 2017, *18*, 1285–1286.

85.   Nishikawa, H.; Kato, T.; Tawara, I.; Saito, K.; Ikeda, H.; Kuribayashi, K.; Allen, P.M.; Schreiber, R.D.; Sakaguchi, S.; Old, L.J.; et al. Definition of target antigens for naturally occurring CD4+ CD25+ regulatory T cells. *J. Exp. Med.* **2005**, *201*, 681–686, doi:10.1084/jem.20041959.

86.   Ghiringhelli, F.; Puig, P.E.; Roux, S.; Parcellier, A.; Schmitt, E.; Solary, E.; Kroemer, G.; Martin, F.; Chauffert, B.; Zitvogel, L. Tumor cells convert immature myeloid dendritic cells into TGF-β-secreting cells inducing CD4 +CD25 + regulatory T cell proliferation. *J. Exp. Med.* **2005**, *202*, 919–929, doi:10.1084/jem.20050463.

87.   Hindley, J.P.; Ferreira, C.; Jones, E.; Lauder, S.N.; Ladell, K.; Wynn, K.K.; Betts, G.J.; Singh, Y.; Price, D.A.; Godkin, A.J.; et al. Analysis of the T-cell receptor repertoires of tumor-infiltrating conventional and regulatory T cells reveals no evidence for conversion in carcinogen-induced tumors. *Cancer Res.* **2011**, *71*, 736–746, doi:10.1158/0008-5472.CAN-10-1797.

88.   Sainz-Perez, A.; Lim, A.; Lemercier, B.; Leclerc, C. The T-cell receptor repertoire of tumor-infiltrating regulatory T lymphocytes is skewed toward public sequences. *Cancer Res.* **2012**, *72*, 3557–3569, doi:10.1158/0008-5472.CAN-12-0277.

89.   Morikawa, H.; Ohkura, N.; Vandenbon, A.; Itoh, M.; Nagao-Sato, S.; Kawaji, H.; Lassmann, T.; Carninci, P.; Hayashizaki, Y.; Forrest, A.R.R.; et al. Differential roles of epigenetic changes and Foxp3 expression in regulatory T cell-specific transcriptional regulation. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 5289–5294, doi:10.1073/pnas.1312717110.

90.   Wei, G.; Wei, L.; Zhu, J.; Zang, C.; Hu-Li, J.; Yao, Z.; Cui, K.; Kanno, Y.; Roh, T.Y.; Watford, W.T.; et al. Global Mapping of H3K4me3 and H3K27me3 Reveals Specificity and Plasticity in Lineage Fate Determination of Differentiating CD4+ T Cells. *Immunity* **2009**, *30*, 155–167, doi:10.1016/j.immuni.2008.12.009.

91.   Ohkura, N.; Hamaguchi, M.; Morikawa, H.; Sugimura, K.; Tanaka, A.; Ito, Y.; Osaki, M.; Tanaka, Y.; Yamashita, R.; Nakano, N.; et al. T Cell Receptor Stimulation-Induced Epigenetic Changes and Foxp3 Expression Are Independent and Complementary Events Required for Treg Cell Development. *Immunity* **2012**, *37*, 785–799, doi:10.1016/j.immuni.2012.09.010.

92.   Curiel, T.J.; Coukos, G.; Zou, L.; Alvarez, X.; Cheng, P.; Mottram, P.; Evdemon-Hogan, M.; Conejo-Garcia, J.R.; Zhang, L.; Burow, M.; et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* **2004**, *10*, 942–949, doi:10.1038/nm1093.

93.   Wei, S.; Kryczek, I.; Edwards, R.P.; Zou, L.; Szeliga, W.; Banerjee, M.; Cost, M.; Cheng, P.; Chang, A.; Redman, B.; et al. Interleukin-2 administration alters the CD4+FOXP3+ T-cell pool and tumor trafficking in patients with ovarian carcinoma. *Cancer Res.* **2007**, *67*, 7487–7494, doi:10.1158/0008-5472.CAN-07-0565.

94.   Tan, M.C.B.; Goedegebuure, P.S.; Belt, B.A.; Flaherty, B.; Sankpal, N.; Gillanders, W.E.; Eberlein, T.J.; Hsieh, C.-S.; Linehan, D.C. Disruption of CCR5-Dependent Homing of Regulatory T Cells Inhibits Tumor Growth in a Murine Model of Pancreatic Cancer. *J. Immunol.* **2009**, *182*, 1746–1755, doi:10.4049/jimmunol.182.3.1746.

95.   Hoelzinger, D.B.; Smith, S.E.; Mirza, N.; Dominguez, A.L.; Manrique, S.Z.; Lustgarten, J. Blockade of CCL1 Inhibits T Regulatory Cell Suppressive Function Enhancing Tumor Immunity without Affecting T Effector Responses. *J. Immunol.* **2010**, *184*, 6833–6842, doi:10.4049/jimmunol.0904084.

96.   Sen, D.R.; Kaminski, J.; Barnitz, R.A.; Kurachi, M.; Gerdemann, U.; Yates, K.B.; Tsao, H.W.; Godec, J.; LaFleur, M.W.; Brown, F.D.; et al. The epigenetic landscape of T cell exhaustion. *Science (80-.).* **2016**, *354*, 1165–1169, doi:10.1126/science.aae0491.

97.   Spranger, S.; Spaapen, R.M.; Zha, Y.; Williams, J.; Meng, Y.; Ha, T.T.; Gajewski, T.F. Up-regulation of PD-L1, IDO, and Tregs in the melanoma tumor microenvironment is driven by CD8+ T cells. *Sci. Transl. Med.* 2013, *5*, 200ra116.

98.   Williams, J.B.; Horton, B.L.; Zheng, Y.; Duan, Y.; Powell, J.D.; Gajewski, T.F. 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**, *214*, 381–400, doi:10.1084/jem.20160485.

99.   Zakiryanova, G.K.; Wheeler, S.; Shurin, M.R. Oncogenes in immune cells as potential therapeutic targets. *ImmunoTargets Ther.* **2018**, *Volume 7*, 21–28, doi:10.2147/itt.s150586.

100.   Spranger, S.; Bao, R.; Gajewski, T.F. Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. *Nature* **2015**, *523*, 231–235, doi:10.1038/nature14404.

101.   Peng, W.; Chen, J.Q.; Liu, C.; Malu, S.; Creasy, C.; Tetzlaff, M.T.; Xu, C.; McKenzie, J.A.; Zhang, C.; Liang, X.; et al. Loss of PTEN promotes resistance to T cell–mediated immunotherapy. *Cancer Discov.* **2016**, *6*, 202–216, doi:10.1158/2159-8290.CD-15-0283.

102.   Spranger, S.; Gajewski, T.F. Impact of oncogenic pathways on evasion of antitumour immune responses. *Nat. Rev. Cancer* 2018, *18*, 139–147.

103.   Hamarsheh, S.; Groß, O.; Brummer, T.; Zeiser, R. Immune modulatory effects of oncogenic KRAS in cancer. *Nat. Commun.* **2020**, *11*, doi:10.1038/s41467-020-19288-6.

104.   Jiang, H.; Hegde, S.; Knolhoff, B.L.; Zhu, Y.; Herndon, J.M.; Meyer, M.A.; Nywening, T.M.; Hawkins, W.G.; Shapiro, I.M.; Weaver, D.T.; et al. Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. *Nat. Med.* **2016**, *22*, 851–860, doi:10.1038/nm.4123.

105.   Serrels, A.; Lund, T.; Serrels, B.; Byron, A.; McPherson, R.C.; Von Kriegsheim, A.; Gómez-Cuadrado, L.; Canel, M.; Muir, M.; Ring, J.E.; et al. Nuclear FAK Controls Chemokine Transcription, Tregs, and Evasion of Anti-tumor Immunity. *Cell* **2015**, *163*, 160–173, doi:10.1016/j.cell.2015.09.001.

106.   Pace, L.; Tempez, A.; Arnold-Schrauf, C.; Lemaitre, F.; Bousso, P.; Fetler, L.; Sparwasser, T.; Amigorena, S. Regulatory T cells increase the avidity of primary CD8+ T cell responses and promote memory. *Science (80-.).* **2012**, *338*, 532–536, doi:10.1126/science.1227049.

107.   Maeda, Y.; Nishikawa, H.; Sugiyama, D.; Ha, D.; Hamaguchi, M.; Saito, T.; Nishioka, M.; Wing, J.B.; Adeegbe, D.; Katayama, I.; et al. Detection of self-reactive CD8+ T cells with an anergic phenotype in healthy individuals. *Science (80-.).* **2014**, *346*, 1536–1540, doi:10.1126/science.aaa1292.

108.   Snyder, A.; Makarov, V.; Merghoub, T.; Yuan, J.; Zaretsky, J.M.; Desrichard, A.; Walsh, L.A.; Postow, M.A.; Wong, P.; Ho, T.S.; et al. Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N. Engl. J. Med.* **2014**, *371*, 2189–2199, doi:10.1056/nejmoa1406498.

109.   Rizvi, N.A.; Hellmann, M.D.; Snyder, A.; Kvistborg, P.; Makarov, V.; Havel, J.J.; Lee, W.; Yuan, J.; Wong, P.; Ho, T.S.; et al. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science (80-.).* **2015**, *348*, 124–128, doi:10.1126/science.aaa1348.

110.   Hodi, F.S.; O'Day, S.J.; McDermott, D.F.; Weber, R.W.; Sosman, J.A.; Haanen, J.B.; Gonzalez, R.; Robert, C.; Schadendorf, D.; Hassel, J.C.; et al. Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. *N. Engl. J. Med.* **2010**, *363*, 711–723, doi:10.1056/NEJMoa1003466.

111.   Robert, C.; Thomas, L.; Bondarenko, I.; O'Day, S.; Weber, J.; Garbe, C.; Lebbe, C.; Baurain, J.-F.; Testori, A.; Grob, J.-J.; et al. Ipilimumab plus Dacarbazine for Previously Untreated Metastatic Melanoma. *N. Engl. J. Med.* **2011**, *364*, 2517–2526, doi:10.1056/NEJMoa1104621.

112.   Fellne, C. Ipilimumab (Yervoy) prolongs survival in advanced melanoma: Serious side effects and a hefty price tag may limit its use. *P T* **2012**, *37*, 503.

113.   Zhang, Y.; Du, X.; Liu, M.; Tang, F.; Zhang, P.; Ai, C.; Fields, J.K.; Sundberg, E.J.; Latinovic, O.S.; Devenport, M.; et al. Hijacking antibody-induced CTLA-4 lysosomal degradation for safer and more effective cancer immunotherapy. *Cell Res.* **2019**, *29*, 609–627, doi:10.1038/s41422-019-0184-1.

114.   Ji, D.; Song, C.; Li, Y.; Xia, J.; Wu, Y.; Jia, J.; Cui, X.; Yu, S.; Gu, J. Combination of radiotherapy and suppression of Tregs enhances abscopal antitumor effect and inhibits metastasis in rectal cancer. *J Immunother Cancer* **2020**, *8*, 826, doi:10.1136/jitc-2020-000826.

115.   Qu, Q.; Zhai, Z.; Xu, J.; Li, S.; Chen, C.; Lu, B. IL36 Cooperates With Anti-CTLA-4 mAbs to Facilitate Antitumor Immune Responses. *Front. Immunol.* **2020**, *11*, doi:10.3389/fimmu.2020.00634.

116.   Mihic-Probst, D.; Reinehr, M.; Dettwiler, S.; Kolm, I.; Britschgi, C.; Kudura, K.; Maggio, E.M.; Lenggenhager, D.; Rushing, E.J. The role of macrophages type 2 and T-regs in immune checkpoint inhibitor related adverse events. *Immunobiology* **2020**, *225*, 152009, doi:10.1016/j.imbio.2020.152009.

117.   Sun, N.Y.; Chen, Y.L.; Lin, H.W.; Chiang, Y.C.; Chang, C.F.; Tai, Y.J.; Chen, C.A.; Sun, W.Z.; Chien, C.L.; Cheng, W.F. Immune checkpoint Ab enhances the antigen-specific anti-tumor effects by modulating both dendritic cells and regulatory T lymphocytes. *Cancer Lett.* **2019**, *444*, 20–34, doi:10.1016/j.canlet.2018.11.039.

118.   Kvarnhammar, A.M.; Veitonmäki, N.; Hägerbrand, K.; Dahlman, A.; Smith, K.E.; Fritzell, S.; Von Schantz, L.; Thagesson, M.; Werchau, D.; Smedenfors, K.; et al. The CTLA-4 x OX40 bispecific antibody ATOR-1015 induces anti-tumor effects through tumor-directed immune activation. *J. Immunother. Cancer* **2019**, *7*, 103, doi:10.1186/s40425-019-0570-8.

119.   Morris, Z.S.; Guy, E.I.; Werner, L.R.; Carlson, P.M.; Heinze, C.M.; Kler, J.S.; Busche, S.M.; Jaquish, A.A.; Sriramaneni, R.N.; Carmichael, L.L.; et al. Tumor-specific inhibition of in situ vaccination by distant untreated tumor sites. *Cancer Immunol. Res.* **2018**, *6*, 825–834, doi:10.1158/2326-6066.CIR-17-0353.

120.   Duperret, E.K.; Wise, M.C.; Trautz, A.; Villarreal, D.O.; Ferraro, B.; Walters, J.; Yan, J.; Khan, A.; Masteller, E.; Humeau, L.; et al. Synergy of Immune Checkpoint Blockade with a Novel Synthetic Consensus DNA Vaccine Targeting TERT. *Mol. Ther.* **2018**, *26*, 435–445, doi:10.1016/j.ymthe.2017.11.010.

121.   Tang, F.; Du, X.; Liu, M.; Zheng, P.; Liu, Y. Anti-CTLA-4 antibodies in cancer immunotherapy: Selective depletion of intratumoral regulatory T cells or checkpoint blockade? *Cell Biosci.* **2018**, *8*, 30, doi:10.1186/s13578-018-0229-z.

122.   Son, C.H.; Bae, J.; Lee, H.R.; Yang, K.; Park, Y.S. Enhancement of antitumor immunity by combination of anti-CTLA-4 antibody and radioimmunotherapy through the suppression of Tregs. *Oncol. Lett.* **2017**, *13*, 3781–3786, doi:10.3892/ol.2017.5933.

123.   Schwarz, C.; Unger, L.; Mahr, B.; Aumayr, K.; Regele, H.; Farkas, A.M.; Hock, K.; Pilat, N.; Wekerle, T. The Immunosuppressive Effect of CTLA4 Immunoglobulin Is Dependent on Regulatory T Cells at Low But Not High Doses. *Am. J. Transplant.* **2016**, *16*, 3404–3415, doi:10.1111/ajt.13872.

124.   Marabelle, A.; Kohrt, H.; Sagiv-Barfi, I.; Ajami, B.; Axtell, R.C.; Zhou, G.; Rajapaksa, R.; Green, M.R.; Torchia, J.; Brody, J.; et al. Depleting tumor-specific Tregs at a single site eradicates disseminated tumors. *J. Clin. Invest.* **2013**, *123*, 2447–2463, doi:10.1172/JCI64859.

125.   Du, X.; Tang, F.; Liu, M.; Su, J.; Zhang, Y.; Wu, W.; Devenport, M.; Lazarski, C.A.; Zhang, P.; Wang, X.; et al. A reappraisal of CTLA-4 checkpoint blockade in cancer immunotherapy. *Cell Res.* **2018**, *28*, 416–432, doi:10.1038/s41422-018-0011-0.

126.   Sandin, L.C.; Eriksson, F.; Ellmark, P.; Loskog, A.S.I.; Tötterman, T.H.; Mangsbo, S.M. Local CTLA4 blockade

effectively restrains experimental pancreatic adenocarcinoma growth in vivo. *Oncoimmunology* **2014**, *3*, doi:10.4161/onci.27614.

127.   Kavanagh, B.; O'Brien, S.; Lee, D.; Hou, Y.; Weinberg, V.; Rini, B.; Allison, J.P.; Small, E.J.; Fong, L. CTLA4 blockade expands FoxP3+ regulatory and activated effector CD4 + T cells in a dose-dependent fashion. *Blood* **2008**, *112*, 1175–1183, doi:10.1182/blood-2007-11-125435.

128.   Tannock, I.F.; de Wit, R.; Berry, W.R.; Horti, J.; Pluzanska, A.; Chi, K.N.; Oudard, S.; Théodore, C.; James, N.D.; Turesson, I.; et al. Docetaxel plus Prednisone or Mitoxantrone plus Prednisone for Advanced Prostate Cancer. *N. Engl. J. Med.* **2004**, *351*, 1502–1512, doi:10.1056/nejmoa040720.

129.   Francisco, L.M.; Salinas, V.H.; Brown, K.E.; Vanguri, V.K.; Freeman, G.J.; Kuchroo, V.K.; Sharpe, A.H. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J. Exp. Med.* **2009**, *206*, 3015–3029, doi:10.1084/jem.20090847.

130.   Li, C.; Jiang, P.; Wei, S.; Xu, X.; Wang, J. Regulatory T cells in tumor microenvironment: New mechanisms, potential therapeutic strategies and future prospects. *Mol. Cancer* **2020**, *19*, 1–23, doi:10.1186/s12943-020-01234-1.

131.   Khan, S.; Burt, D.J.; Ralph, C.; Thistlethwaite, F.C.; Hawkins, R.E.; Elkord, E. Tremelimumab (anti-CTLA4) mediates immune responses mainly by direct activation of T effector cells rather than by affecting T regulatory cells. *Clin. Immunol.* **2011**, *138*, 85–96, doi:10.1016/j.clim.2010.09.011.

132.   Kumar, P.; Saini, S.; Prabhakar, B.S. Cancer immunotherapy with check point inhibitor can cause autoimmune adverse events due to loss of Treg homeostasis. *Semin. Cancer Biol.* **2020**, *64*, 29–35, doi:10.1016/j.semcancer.2019.01.006.