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Current Medicinal Chemistry, **2019**, *26*, **1-24**

REVIEW ARTICLE

Molecular Markers of Regulatory T Cells in Cancer Immunotherapy with Special Focus on Acute Myeloid Leukemia (AML) - A Systematic Review

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Abstract: The next-generation immunotherapy can only be effective if researchers have an in-depth understanding of the function and regulation of Treg cells in antitumor immunity combined with the discovery of new immunity targets. This can enhance clinical efficacy of future and novel therapies and reduces any adverse reactions arising from the latter. This review discusses tumor treatment strategies using regulatory T (Treg) cell therapy in a tumor microenvironment (TME). It also discusses factors affecting TME instability as well as relevant treatments to prevent future immune disorders. It is prognosticated that PD-1 inhibitors are risky and their adverse effects should be taken into account when they are administered to treat acute myeloid leukemia (AML), lung adenocarcinoma, and prostate adenocarcinoma. In contrast, Treg molecular markers FoxP3 and CD25 analyzed here have stronger expression in almost all kinds of cancers compared with normal people. However, CD25 inhibitors are more effective compared to FoxP3 inhibitors, especially in combination with TGF-β blockade, in predicting patient survival. According to the data obtained from the Cancer Genome Atlas, we then concentrate on AML immunotherapy and discuss different therapeutic strategies including anti-CD25/IL-2, anti-CTLA-4, anti-IDO, anti-tyrosine kinase receptor, and anti-PI3K therapies and highlight the recent advances and clinical achievements in AML immunotherapy. In order to prognosticate the risk and adverse effects of key target inhibitors (namely against CTLA-4, FoxP3, CD25, and PD-1), we finally analyzed and compared the Cancer Genome Atlas derived from ten common cancers. This review shows that Treg cells are strongly increased in AML and the comparative review of key markers shows that Treg-based immunotherapy is not effective for all kinds of cancer. Therefore, blocking CD25(+)FoxP3(+) Treg cells is suggested in AML more than other kinds of cancer; meanwhile, Treg markers studied in other cancers have also great lessons for AML immunotherapy.

ARTICLE HISTORY

Received: June 04, 2019
Revised: August 13, 2019
Accepted: August 28, 2019

DOI:
10.2174/0929867326666191004164041

Keywords: Regulatory T cells, tumor microenvironment, cancer immunotherapy, CD25, FoxP3, PD-1, CTLA-4.

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0929-8673/19 $58.00+.00

1. INTRODUCTION

Regulatory T (Treg) cells play a dominant role in the management and control of autoimmune diseases. Specifically, they are crucial in managing chronic inflammation and cancer by limiting immune activation and specific immune response. It is important to identify different subtypes of T cells using molecular markers on the surface of Treg cells to study the sources of immunosuppression and interactions between their different subtypes. There has been an ongoing debate on the phenotype of these cells. Treg cells were divided into six subsets: natural Treg (nTreg), inducible Tregs (iTreg), inducible co-stimulator Treg (ICOS Treg), type 1 Treg (Tr1 Treg), CD8(+) Treg, and interleukin (IL)-17-producing Treg cells [1].

Sakaguchi (1995) for the first time discussed nTreg cells [2], namely a small group of CD4(+) T lymphocyte (about 5% ~ 10%) with sustained expression of CD25 (IL-2 receptor A chain). Originally marked nTreg cells also express the forkhead box P3 (FoxP3). Studies have found that FoxP3 is also expressed in tumor cells. Therefore, the role of FoxP3 as a specific marker of Treg cells is questionable and warrants further discussion [3–5]. The CD25 has been successfully isolated from tissues and peripheral blood and it has been used to identify Treg cells. CD4(+) CD25 T cells are generally considered to be immunosuppressive. The CD25 is also commonly expressed in activated CD4(+) and CD8(+) T cells (without immunosuppressive function). Therefore, detecting CD25 expression levels is the key to distinguishbetween Treg cells and normal T cells [6]. Due to high expression of CD25, Treg cells are more sensitive to IL-2 than conventional T cells, and phosphorylation of STAT5 in response to low dose of IL-2 leads to proliferation of Treg cell population [7]. It is important to evaluate the capacity of Treg cells to phosphorylate STAT5 in response to IL-2 to indicate their capacity to proliferate [8].

nTreg cells are responsible for maintaining peripheral tolerance and immune balance among healthy individuals whereas iTreg cells are highly suppressive and therapy resistant. iTreg cells down-regulate antitumor immune responses and promote tumor growth [9] while peripheral Treg cells may be more flexible than Treg cells originating from thymus [10]. However, it is possible that both thymus and peripheral subgroups contribute to down-regulating antitumor immune responses. Therefore, thymus and peripheral subgroups are identified as a pool [11]. Though Treg cells are an important contributor to tumor microenvironment (TME), their therapeutic targets have been difficult to determine, thus, new therapies are urgently required.

During the last ten years (2009-2019), great attention has been drawn towards the Treg-based cancer immunotherapy (Fig. **1**). In the recent years, adoptive cell immunotherapy (T-cell based therapies) has been developed and promoted, such as KYMRIAH, using chimeric antigen receptor (CAR), which has recently been approved by the United States (US) Food and Drug Administration (FDA) [12,13]. As Treg cells have the potential to combat certain types of cancer, Treg cells would be a promising immunotherapy against cancer with less harmful effects on healthy cells for the next generation [14]. Over the last five years, many reviews have been published on the role of Tregs in graft rejection and transplantation, and their efficacy on liver diseases, dendritic cell-based and cytokine-based immunotherapy [15–18]. However, it is interesting to note there has not been any comprehensive review written on the anticancer therapeutic role of Treg cells during the last five years. Therefore, there is an urgent need to examine the role and impact of Tregs cells in cancer immunotherapy to highlight its adverse effects and efficacy in different kinds of cancer.

Acute myeloid leukemia (AML) is the most incurable kind of blood cancer which has over 20000 new cases in the US annually. Large chromosomal translocations and altered genes related to blood cell progenitors cause AML [19]. As Treg cells in AML have the same key markers in other kinds of cancer, we first highlighted and reviewed the general Treg markers, and concentrated on AML to find the basic differences of AML with other kinds of cancers. In order to figure out how Treg cells influence AML disease, we also reviewed clinical database and the latest clinical trials started or completed on Treg key markers with special focus on AML. This review discusses different types of Treg cells, their therapeutic targets in cancer immunotherapy, and their antitumor impact in clinical studies.

2. CELL SIGNALING OF TREGS

2.1. Relationship between Tregs and Tumor Immunity

Treg cells belong to CD4(+) T lymphocyte subgroup express FoxP3, IL2 receptor (CD25) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) [20]. It must be emphasized that Treg cells, play a role in maintaining immune homeostasis and peripheral tolerance to prevent allergy and autoimmune disease. However, Treg-induced immune homeostasis can significantly limit the efficacy of antitumor immunity

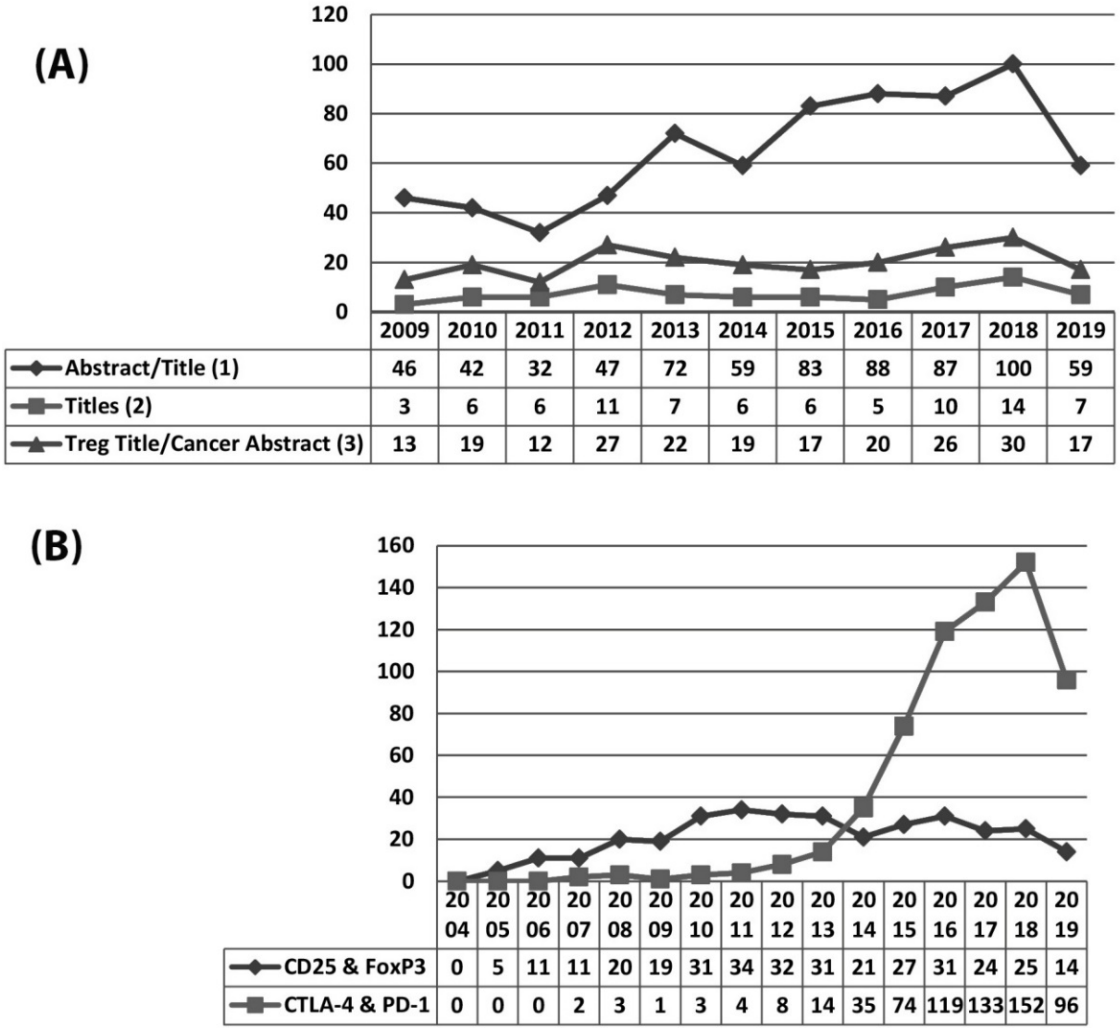


Fig. (1). Comparing publications on the role of regulatory T cell in cancer immunotherapy. (A) Results of search based on PubMed database on two keywords: Regulatory T cells and cancer. The chart compares the results adjusted as: (1) both keywords applied in the title or the abstract of publication, (2) both keywords used in the title of publication, and (3) regulatory T cells and cancer in the title or abstract of publication. This chart shows the number of articles published on the topics after 2012 which are twice compared with the previous years; **(B)** The number of publications based on the key markers of Treg cells. As CD25 and FoxP3 are two crucial markers of Treg cells, the authors first considered those articles in which these two keywords appeared in the title or abstract of the publication. There has been a steady trend published works over the last ten years on this topic. However, more works has been observed to be published after 2010. Interestingly, the number of publications based on two markers frequently received attention in cancer immunotherapy, CTLA-4 and PD-1, which increased dramatically in the last five years, from 35 to 152 publications in 2014 and 2018, respectively. The results for 2019 are for the first six months of that year.

which is linked to the number and function of Treg cells [21]. Overexpression of Treg markers has been reported in breast, colon, rectum and stomach cancer [22–24].

Peripheral blood and local accumulation of tumors in patients with multiple types of tumors, such as liver cancer, suggest the number of Treg cells is negatively correlated with prognosis and survival. Percentages of Treg cells in ovarian cancer can be increased depending on the severity of cancer stage; specifically, higher Treg percentages correlate with poorer disease-free survival in several cancers [25,26]. Systemic strategies to starve Treg cells were used in previous clinical studies with limited success [27,28].

2.2. Molecular Mechanisms of Treg Suppression

Tumors can suppress immune cells, such as T cells, NK cells, and dendritic cells, and promote CD4(+) cells to Tregs through various signaling proteins, such as programmed death (PD)-1, phosphoinositide 3-kinase (PI3K), toll-like receptor (TLR)-8 and transforming growth factor (TGF)-β.

2.2.1. Treg-suppressive Function through Secretion of Inhibitory Factors

Treg cells are differentiated by immunosuppressive molecules, such as IL-10, TGF-β, and tumor necrosis factor (TNF)-α, which play a role in preventing the information contact between tumor-specific CD8(+) and CD4(+) T cells. Results of phase I and II clinical trials suggest that TNF-α antagonist, etanercept, or the antibody infliximab have some therapeutic activity against metastatic breast cancer [29]. Immuno-suppressive role of TGF-β leads to inhibition of antitumor immune function [29] (Fig. **2**). The most important example of immuno-enhancement therapy for tumors is the use of TGF-β that is integral to the maintenance and generation of immunosuppressive Treg cells [30]. Treg cells localized in TME inhibit dendritic cells *via* IL-10 and TGF-β [31,32]. Compartmentalized Treg plasticity appears to be a key factor in ensuring an optimal environment for cancer development in intestines. The inhibitory function of intestinal Treg critically depends on IL-10, especially under microbial conditions [33].

2.2.2. Functional Inhibitors of Treg Surface

In the recent years, studies have reported that Tregs in some cases express immune suppressing factors, such as programmed death 1 (PD-1) and CTLA-4 [34]. Inhibitors of PD-1 and CTLA-4 have been developed rapidly for the treatment of cancer patients. Related drugs approved by the FDA are aimed at blocking the immune suppressive factors and therefore, enhance T cell activity [35]. Antibodies against CTLA-4, such as ipilimumab, inhibit Treg function and they have been proven to increase survival rate by 20 percent. Ipilimumab (monoclonal anti-CTLA-4 antibody) in particular was subsequently approved by the FDA for clinical use [36,37]. However, in a phase II trial involving patients with advanced pancreatic cancer who were administered with ipilimumab, one patient out of 27 showed a delayed response to it [29].

The glucocorticoid-induced TNF receptor related protein (GITR) is a marker of Treg cells induced by glucocorticoids. Dexamethasone, for example, induces membrane receptors found in T-cells, such as CD40, CD27, 4-1BB (CD137), OX40 (CD134), and GITR or TNFRSF18, which are mainly expressed in the thymus and peripheral CD4(+)CD25(+) T cells, macrophages and dendritic cells. It should be noted that , GITR expression increases after cell activation [38]. Shimizu *et al*. (2002) used flow cytometry to detect small GITR in BALB/c mice experiment [38]. The distribution of GITR in mice showed high distribution of Treg cells in the spleen and lymph node, and CD4(+)CD25(+) cells in thymus. Low expression of GITR was also found in CD4(+)CD25(-) cells, CD8(+) cells and CD4(-)CD8(-) double negative cells in the thymus. However, almost no expression of GITR was observed in CD4(+) CD8(+) double positive cells [39]. Different expressions of GITR in thymus cells suggested that it may be involved in the development of Treg cells in the thymus. It was also found that CD4(+)CD25(-) T cells of the peripheral lymphoid organs, CD8(+)T cells, B220(+) cells and macrophages were low in GITR expression. The GITR is high in CD4(+)CD25(+)Treg cells located in the thymus and peripheral lymphoid organs, while other types of cells could be well differentiated [40,41].

Blocking GITR or its ligands Glycoprotein A repetitions predominant (GARP) will inhibit the immunosuppressive function of Treg cells. GARP has been widely considered as a marker of Treg cell activation and it is involved in the microenvironment of immunosuppressive tumors. However, the expression and function of GRAP in cancer warrants further discussion and validation [42]. The inhibition of Treg cell function is an effective anti-tumor treatment strategy. The GARP is located on the surface of activated Treg cells. For instance, compared with benign thyroid diseases, GARP expression in papillary thyroid carcinoma (PTC) is significantly increased. This increase is positively correlated with FoxP3 expression, which is very important for the development of Treg cells [42]. However, there is no significant correlation between increased GARP expression and lymph node metastasis in PTC. Therefore, GARP has been reported as a potential new anticancer target [43].

2.2.3. Treg Cell Secret TGF-β, Adenosine, and Prostaglandin E2 in TME

TGF-β inhibits activation, maturation, and differentiation of immune cells, such as CTLs, NK cells, and DCs. A combined expression of IL- 12 and decorin (DCN) restores antitumor immune function by preventing TGF-β binding to its receptor [44]. Gemcitabine is also another TGF-β inhibitor. Therefore, immunotherapy combined with gemcitabine may be effective in treating pancreatic cancer patients [45]. TGF-β mediates cell differentiation and cell proliferation through TGF-β and BMP receptors which initiate Smad signaling (Fig. **3**).

Tumors secrete adenosine to escape from antitumor immunity. As shown in Fig. (**4**), adenosine binds to adenosine A2A receptors which expresses on effector

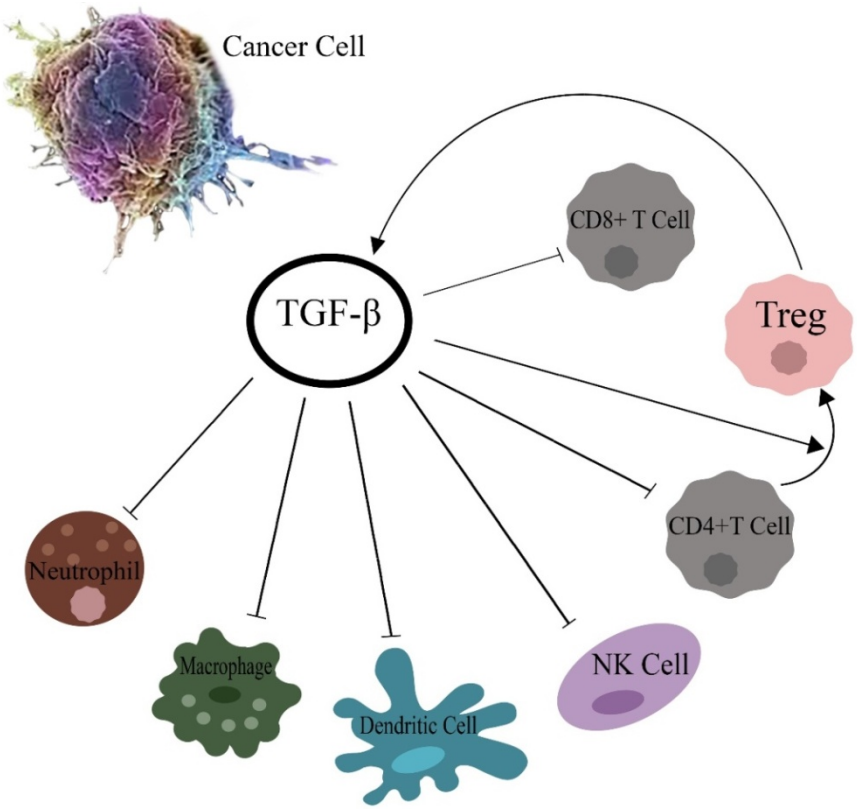


Fig. (2). TGF-β effects on different cells of the immune system. TGF-β suppresses all kinds of cells of the immune system, such as CD8(+) and CD4(+) T cells, NK cells, dendritic cells, macrophages, and neutrophils. In addition, TGF-β induces Treg cells and chemotaxis, and regulates immune checkpoint proteins, such as PD-1.

T-cells (Teff) and suppresses their antitumor functions *via* up-regulation of intra-cytoplasmic cAMP levels. Accumulation of adenosine-producing Treg cells in the TME shows that adenosine is released by tumor suppressive microvesicles (TMV) and the expression of TLRs on the tumor cell surface is tumor-related suppression mechanisms [46]. Adenosine is rapidly removed from the extracellular space by converted into inosine through adenosine deaminase (ADA) while entering the cell, whereby it is converted back into AMP by adenosine kinases. The absence of ADA on the surface of Treg cells leads to accumulation of pericellular adenosine that Treg cells produce, but do not degrade for mediating immune suppression [47,48].

CD39 is ectonucleotidase that hydrolyses ATP and ADP to AMP and suppresses inflammation. Stable expression of CD39 in Treg cells makes them a marker for CD4(+) Treg cells [49] (Fig. **4**). The CD73, another ectonucleotidase, is expressed in the cytoplasm of nTreg cells, but it is secreted on the cell membrane of iTreg cells [49]. The CD26, an extracellular peptidase, is also used as a good molecular marker for negative selection of Treg cells [50], and it is expressed in conventional cells (Tconv). Salgado *et al*. (2012) found that CD26(-)CD4(+) phenotype can be used for Treg cell isolation. CD39(+)CD26(-)Treg cells are capable of withstanding the strong inhibitory effect of adenosine [51].

CD39 and CD73 appear on the surface of nTreg and Tr1 cells [49,52]. They are constitutively expressed in human FoxP3(+) Treg cells and in mice experiment involving CD4(+)CD25(+) T cells and hydrolyze extracellular ATP or ADP into AMP. They eventually produce immunosuppressive adenosine [53,54]. High levels of IL-2 are required for the growth of Treg and effector T cells. Effector T cells compete and consume IL-2 resulting in the lack of IL-2 in the microenvironment, which in turn, leads to inhibition of effector T cells and even apoptosis. Earlier studies have reported depletion strategies targeting the IL2 pathway using antibodies or small molecules resulted in off-target effects, such as depletion of effector T cells or loss of dendritic cells, and incomplete depletion of Treg cells [53]. The ability of γδ T cells to modulate FoxP3 T cell response depends on the adenosine. There are multiple adenosine receptors, but the A2A receptor is more important. The inhibition of Treg cells can be com-

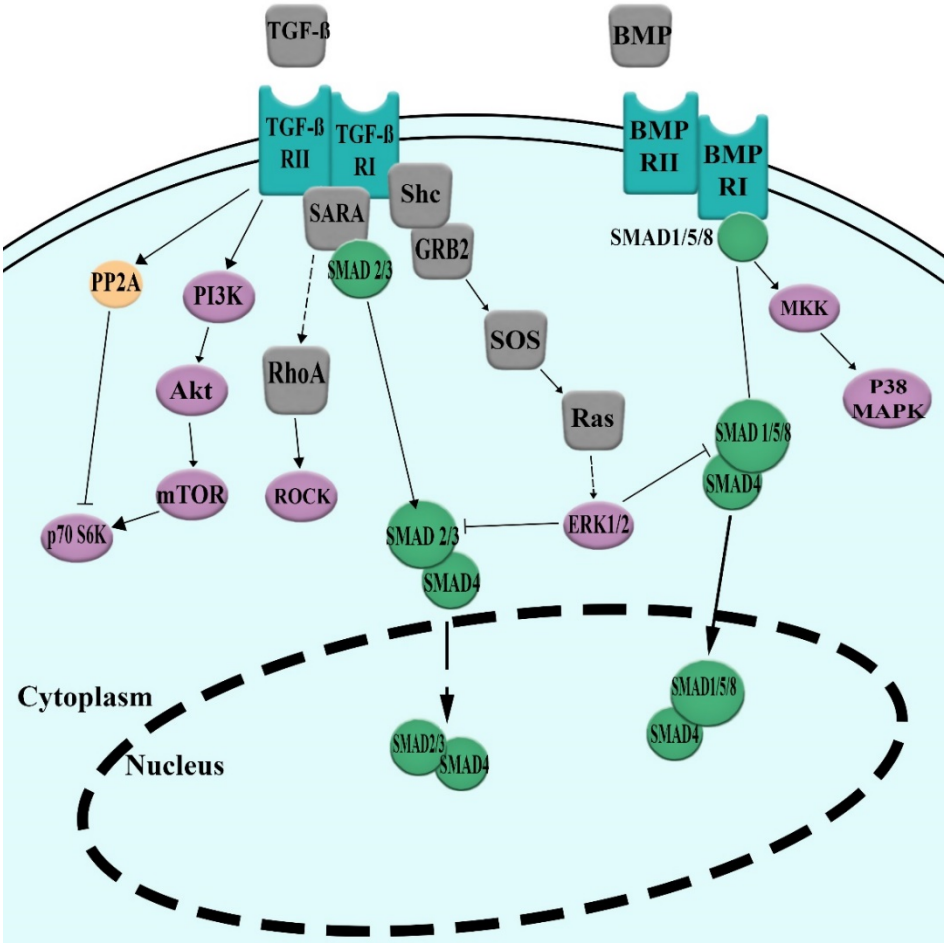


Fig. (3). TGF-β signaling pathways. TGF-β receptor combination can be divided into TGF-β and BMP which activate SMAD pathways. Smad 2/3 and Smad 1/5/8 are activated by TGF-β and BMP receptor, respectively. Phosphorylation of Smad 2 and Smad 3 by TGF-βRI triggers TGF-β signaling. SARA facilitates the interaction between TGF-βRI and Smad complex. TGF-β also activates Smad-independent PI3K and MAPK pathways. Smad pathways are important for cell development and differentiation, whereas PI3K and MAPK pathways are involved in cell proliferation. Therefore, TGF-β controls a balance between cell differentiation and proliferation leading to Treg development from CD4(+) T cells. TGF-β signaling is negatively regulated by ERK1/2; however, PP2A inhibits PI3K pathway. **PP2A:** Protein phosphatase 2; **PI3K:** Phosphoinositide 3-kinase; **SARA:** Smad Anchor for Receptor Activation; **GRB2:** Growth factor receptor-bound protein 2; **ERK1/2:** Extracellular signal-regulated kinase 1/2; **P38 MAPK:** Mitogen activated protein kinase; **MKK:** MAP kinase kinase kinase or MAP3K; **BMP:** Bone morphogenetic protein; **RI:** Receptor subunit I; **RII:** Receptor subunit II.

pletely abolished by blocking A2A receptor on the surface of γδ T cells [53].

3. INTERACTIONS OF TREGS AND OTHER IMMUNE CELLS

RAR-related orphan receptor gamma (RORγ) is the main transcription factor in Th17 cell differentiation, while FoxP3 is a specific transcription factor closely related to the regulatory function of Treg cells. Cytokines involved in the regulation of Th17 cells and Treg cells are TGF-β, IL-6, IL-21 and lL-12. Cytokine IL-21 combined with TGF-β increases the expression of RORγ by activating STAT3 pathway. CD4(+) T cells produce IL-17 which promotes the differentiation of the original CD4(+) T cells into Th17 cells [55]. Recent studies have shown that IL-21 inhibits CD4(+) CD25(+)FoxP3(+) Treg cells. The combination of IL-21 and TGF-β inhibits the expression of FoxP3, a major regulator of Treg cells, thereby reducing the differentiation of CD4(+) T cells to attenuate Treg-mediated immunosuppression levels [56,57]. lL-21 mRNA level is significantly increased in Th17 indicating that these cells could highly express IL-21 after IL-6 induction. IL-21 also inhibits CD4(+)CD25(+)FoxP3(+) Treg cytokines [58]. The aryl hydrocarbon receptor (AHR), through its ligand 2,3,7, 8-tetrachlorodiphenyl, induces and inhibits the activation of functional Treg cells in experimental autoimmune encephalomyelitis, thereby

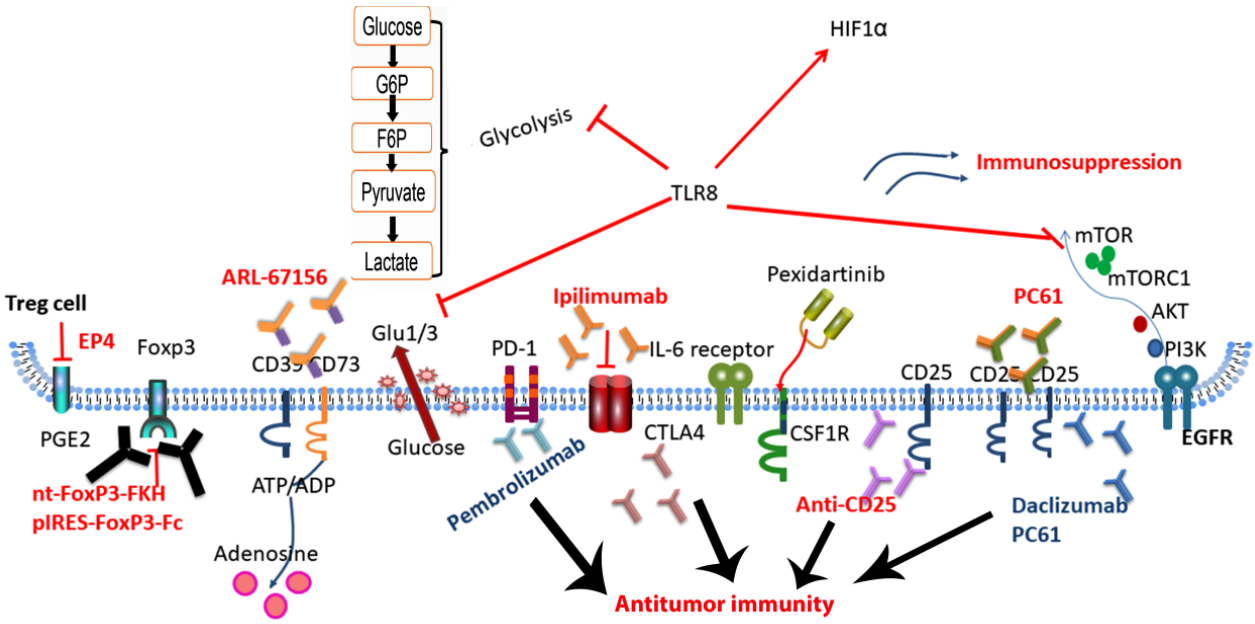


Fig. (4). Treg signaling pathways, Treg markers, and anticancer therapeutic targets. The above figure shows the decreased levels of glucose and glycolysis which results in Treg fragility. Specifically, TLR-8 inhibits glucose transfer using Glucose transporter 1/3 (Glu1/3) suppression. Drugs, such as cyclophosphamide, gemcitabine, and daclizumab (CD25 inhibitor), inhibit DNA synthesis and cell proliferation by targeting PI3K pathway and CD25, decrease Treg activity, and in turn, synergistically help cytotoxic T and NK cells to fight cancer cells. CTLA-4 antagonists are effective in activating antitumor immunity as well. CTLA4: Cytotoxic T lymphocyte antigen 4; Glu1/3: Glucose receptor 1/3; TLR8: Toll-like receptor 8; mTOR: mammalian Target of 2: Hexokinase 2; PFK1: Phosphofructokinase 1; FoxP3: Forkhead box P3.

specifically regulating the differentiation of Treg and TH17 cells [59,60] (Fig. **5**).

4. TREG CELLS IN AML IMMUNOTHERAPY AND CLINICAL STUDIES

Systemic Treg cells depletion is not desirable due to their critical role in maintaining immune homeostasis and preventing autoimmunity. Targeting Tregs specifically in the immunosuppressive TME may provide a more effective approach to limit the immunosuppressive environment to the tumor without inducing systemic adverse consequences [61]. Some researchers believe that Treg therapy removes normal immune effector cells, while clearing Treg cells. The idea of targeting Treg cells may be more effective to control the number and function of Treg cells [61,62].

Immune resistance induced by Treg cells is highly heterogeneous. Therefore, the normalization of immunotherapy depends on the development of antitumor immune response based on the genetic profile of tissues. The heterogeneity of the immune deficiency not only exists between different patients, but also in a single tumor, whereby pathological changes are observed in different regions [63,64]. The future challenge is to determine dominant pathways in a given patient to make the best decision for her/his treatment. It may be possible to find immunotherapy targets on pathways controlling Treg functions at the transcriptional level. Furthermore, the successes of the therapeutic strategies including DC-based vaccination used for Treg suppression in other kinds of cancer can be used in future studies of AML.

4.1. Anti-CD25 and IL-2 Therapy

Around 10-20 percent of AML cells express CD25 leading to poor prognosis of AML. It has been shown that CD25(-)CD34(+) AML cells could also change into CD25(+)CD34(+) AML cells. Therefore, CD25/IL-2 inhibitors may be a good therapeutic approach to overcome AML [65]. Targeting of CD25 restores antitumor immunity in a variety of pre-clinical models; it directly causes depletion of CD4(+)CD25(+) nTreg cells. As nTreg cells suppress antitumor immunity, anti-CD25 antibody has been used to deplete CD25(+) nTreg populations. However, anti-CD25 therapy is not Treg-specific and it also depletes CD25(+) effector T cells that drive antitumor immunity. Therefore, the efficacy of anti-CD25 therapy is restricted to a prophylactic setting [21]. IL-2 analogs deactivate the IL-2 receptor and inhibit Treg cell suppression. They are effectively designed against the alpha subunit (CD25) but are less efficacious against either the β or γ subunits of

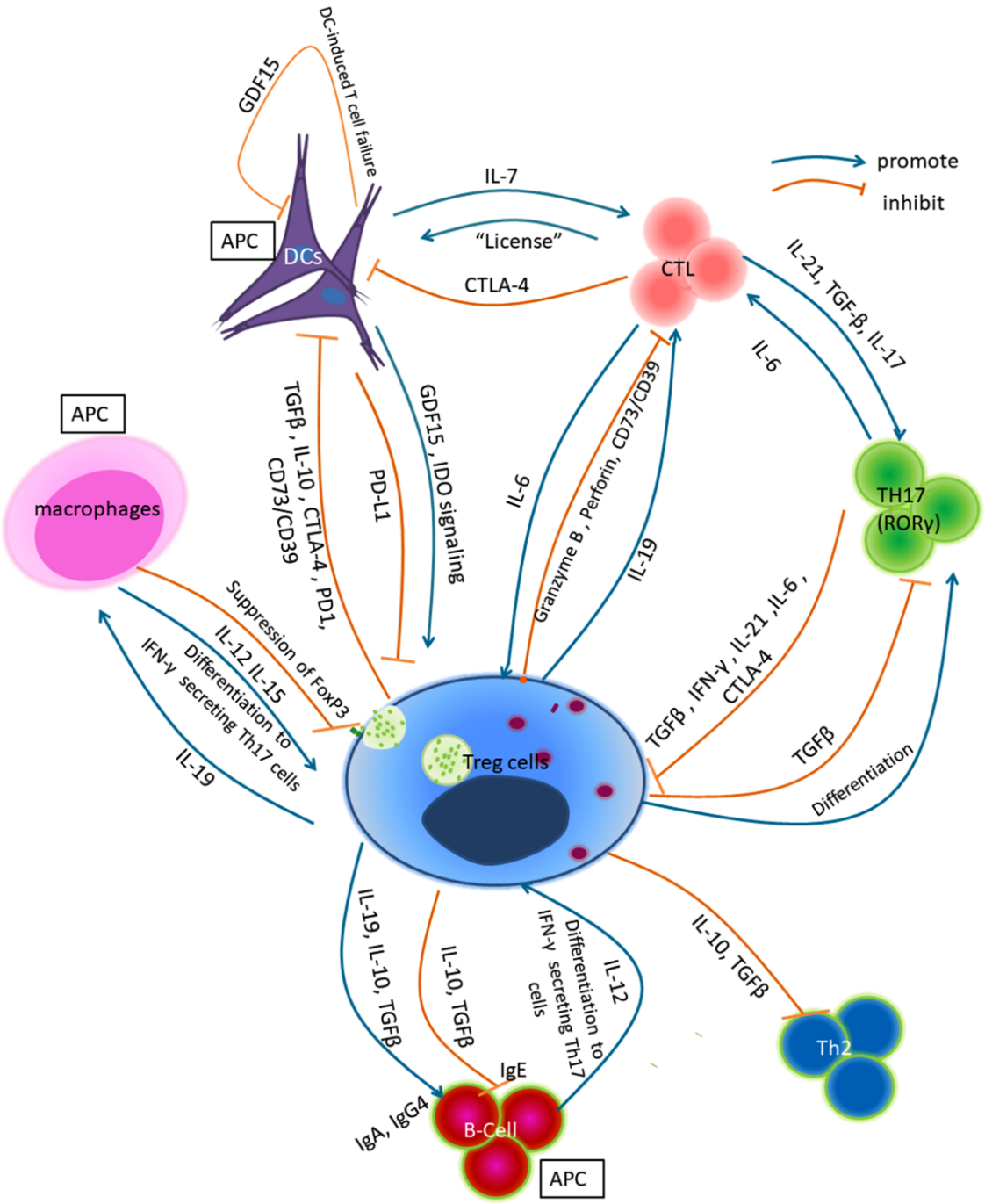


Fig. (5). Interactions of Tregs and other immune cells. Treg cells suppress different kinds of immune cells, such as dendritic cells (DCs), B cells, helper T cells, and CTLs by increasing amounts of cytokines (TGF-β, IL-6, IL-21, and IL-17). After IL-6 secretion, IL-21 is increased in Th17 cells. IL-21 inhibits CD4(+)CD25(+)FoxP3(+) Treg cells. TGF-β is produced by all immune cells and along with IL-6 plays a crucial role in immune cell homeostasis. The combination of IL-21 and TGF-β inhibits the expression of FoxP3, thereby reducing the differentiation of CD4(+) T cells to attenuate Treg-mediated immunosuppression. IL-21 also inhibits CD4(+)CD25(+)FoxP3(+) Treg cytokines. TGF-β in combination with IL-10 suppresses DCs. IL-6 controls the Treg/Th17 balance which plays an important role in autoimmune diseases. CD39 and CD73 are presented on the surface of nTreg and Tr1 Treg cells. They are constitutively expressed in FoxP3(+) Treg cells and hydrolyze extracellular ATP or ADP into AMP. They finally produce immunosuppressive adenosine. T cells including Treg cells also produce PD-1 and CTLA-4 which suppresses DCs and other T cells to turn the anticancer immunity off. **DC:** Dendritic cells; **NK:** Natural killer cells; **CTL:** Cytotoxic T Lymphocyte; **APC:** Antigen-presenting cell; **TGF-β:** Transforming growth factor beta; **TNF-α:** Tumor necrosis factor alpha; **GDF15:** growth differentiation factor 15.

Table 1. Key Treg markers for therapeutic purpose in AML and related clinical trials started. This table demonstrates different therapies which are being investigated for Treg markers in AML. Only clinical trials which are recruiting the volunteers (†) or those which were completed (*) have been collected from "clinicaltrials.gov" and shown in this Table.

Target	Drug(s)	Efficiency and Other Descriptions	Identifier
FLT3	Midostaurin Lestaurtinib Sorafenib Quizartinib NMS-03592088	Efficacy is under investigation. Anti-FLT3 monotherapy produces clinical responses which are usually incomplete and transient, and acquired resistance to anti-FLT3s has been reported. Anti-FLT3s in combination with other anticancer drugs are suggested.	NCT03512197† NCT03280030† NCT03686345† NCT03379727† NCT03591510† NCT03900949† NCT02428543† NCT02756962† NCT03922100†
CD25	ADCT-301 RFT5-SMPT-dgA Daclizumab	High-dose daclizumab is required to saturate IL-2R alpha (CD25) and a long-term monotherapy with anti-CD25 led to a sustained reduction of CD4(+) CD25(+) Treg cells. CD-25 inhibitor combined with TGF-β blockade is more effective in decreasing pancreatic tumor growth in mice. Anti-CD25 monotherapy is also able to decrease periphery and intra-tumor Tregs. Adverse effects: Daclizumab was stopped by its sponsor after reports of brain inflammation.	NCT02588092* NCT00025662* NCT00002681*
CD33/CD3 (Bispecific targeting)	JNJ-67371244	Adverse effects of JNJ-67371244 are under investigation [in Relapsed or Refractory Acute Myeloid Leukemia (AML) or Myelodysplastic Syndrome (MDS)]	NCT03915379†
IDO-1	Indoximod Epacadostat	Anti-IDO agents sensitize cancer cells to cytarabine and idarubicin, and can be used in combination with DNA targeting compounds or with any immune checkpoint inhibitors.	NCT02835729† NCT01822691*
COX-2	Choline magnesium trisalicylate Sodium salicylate FTS	Choline magnesium trisalicylate suppresses inflammation and affects NF-κB. It has been administered with cytarabine and idarubicin. FTS is a unique and potent Ras inhibitor.	NCT02144675* NCT00004245* NCT00867230*
CTLA-4	Ipilimumab	Adverse effects: Myocarditis, Neurologic, endocrine dysfunction, colitis, dermatitis, encephalitis, and hematologic and hepatic problems in melanoma, pulmonary, renal, and other cancers which demonstrated 21% fatality.	NCT03912064† NCT02846376† NCT02890329† NCT02397720† NCT00060372* NCT01757639*
PD-1	Nivolumab	Checkpoint inhibitors sensitize cancer cells to nucleic acid inhibitors, and can be used in combination with DNA targeting compounds such as azacitidine and cytarabine or with any immune checkpoint inhibitors.	NCT02846376† NCT02397720†
VEGFR/VEGF	Bevacizumab Dovitinib Sorafenib	Bevacizumab is an effective anti-angiogenesis drug targets VEGF and was used in combination with DNA synthesis inhibitors. Dovitinib and sorafenib are tyrosine kinase inhibitors (TKI) targets tyrosine kinases including VEGFR.	NCT00015951* NCT01831726* NCT01445080*
Angiopoietins 1 (Direct) VEGFR (indirect)	Trebananib	Effective anti-angiogenesis drug	NCT0155526*

(Table 1) Contd…

Target	Drug(s)	Efficiency and Other Descriptions	Identifier
PI3K	Idelalisib BKM120	Idelalisib affects drug metabolism by inhibition of CYP3A and P-glycoprotein. Idelalisib reduces respiration and oxygen consumption.	NCT02779283[†] NCT00710528[*] NCT01396499[*] NCT01833169[*]
CSF1R	JNJ-40346527 NMS-03592088	Evaluation of preliminary efficacy of JNJ-40346527 in participants with relapsed/refractory AML is under investigation. NMS-03592088 is a multifunctional compound which can also target FLT3.	NCT03557970[†] NCT03922100[†]

the IL-2 receptor. CD25 inhibitors are pharmacological Treg inhibitors in cancer immunotherapy [8,66]. AML is an incurable and weak immunogenic kind of cancer and treatment strategies are rare for patients suffering from AML. CD25 has the potential to be considered as a target in AML. STAT signaling pathway through activation of PIM kinase plays the main role in CD25 expression. Accordingly, PIM inhibitors are suggested as another therapeutic option in AML as well as CD25 inhibitors [67]. In addition, CD25 expression in AML predicts resistance of AML cells to chemotherapy, a feature observed in CD25(+)CD34(+) CD38(-) AML cells [68]. CD25 expression is also a prognosticate biomarker for AML as an adverse effect factor for overall survival [69] and has been associated with shorter overall survival [70].

IL-2 and its receptor, CD25, have great importance in AML because they show the activity of Tregs in TME of AML. Therefore, a few clinical trials started to find better therapy against these proteins. ADCT-301 and daclizumab, as anti-CD25 have been already studied in AML. However, the toxicity of daclizumab in some cases has been shown and the toxicity and efficacy of anti-CD25 should be taken into account (identifier: NCT02588092)(Table **1**). A phase II study assessed the safety and efficacy of graft-versus-host disease (GVHD) from allogeneic stem cell transplantation. Although T cell depletion is the most effective way to prevent GVHD, it also leads to adverse reactions, such as recurrence, transplant rejection and increased incidence of infection after transplantation. This study used a specific immunotoxin against the IL-2 receptor which was RFT5-SMPT-dgA to perform selective removal of alloreactive T cells from donor lymphocyte pools *in vitro*. The associated mortality and overall survival were measured 100 days after infusion (identifier: NCT00025662). Designing bispecific antibodies against CD3, a protein coupled with TCR, and CD33, a tumor-associated antigen, is also a quick method to suppress TRCR signaling of AML cells. JNJ-67571244 is an anti-CD3/CD33 which is under investigation in patients with relapsed or refractory AML. The study is divided into the screening phase, a treatment phase, and a post-treatment follow-up phase to determine the toxic effects of JNJ-67571244 therapy (identifier: NCT03915379).

4.2. Anti-IDO Therapy

In addition to FoxP3 and CD25, which suppressed anticancer immunity, indoleamine 2, 3 dioxygenase (IDO) is produced by AML cells and plays the same role in AML. IDO is also able to promote CD4(+)CD25(-) helper T cells to Treg cells. FoxP3 expression is correlated to IDO in patients with cytologically normal AML. Similar to CD25 expression, IDO production is also associated with poor prognosis in AML [71]. The overexpression of growth differentiation factor 15 (GDF15) in DCs increases immunosuppressive molecules and causes DC-induced T cell failure, and it promotes Treg cell generation through IDO signaling [72,73]. Overexpression of GDF15 in DCs significantly up-regulates immunosuppressive genes IDO1, IDO2, and inhibitory molecules PD-L1 and galectin-9. However, overexpressed GDF15 reduces Rel A and Rel B, and inactivates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [73]. More importantly, the expression of Akt, a crucial oncogenic signaling protein playing its role as the upstream target of PI3K [74], increases in GDF15-overexpressing DCs. However, both p38MAPK and ERK pathways appear to be unaffected. Interestingly, GDF15 inhibits NF-κB pathway resulting in the arrest of DC maturation, reduction in pro-inflammatory cytokine production, and suppression of DC immune function [73].

Treg cells suppress the function of dendritic cells and, in turn, reduce immunity response against tumors. Specifically, the CTLA-4 expressed by Treg cells inhibits CD80 and/or CD86 on the surface of dendritic cells [53]. The high expression of Treg CTLA-4, with the antigen presenting cells (APC) of CD80/86, induces

IDO expression which, in turn, leads to tryptophan degradation [71]. The IDO affects proliferation of T cells due to the absence of tryptophan, and this in turn, inhibits protein synthesis in T cells. Furthermore, tryptophan degradation not only has a cytotoxic effect on CD3(+) T, B and NK cells, it also kills these lymphoid cells. In addition, it has been reported that myeloid-derived suppressor cells (MDSCs) in breast cancer suppress T-cell functions through STAT3-dependent IDO upregulation [75].

Anti-IDO therapy is another key therapy against AML which targets IDO, another marker of AML cells. The primary objective of one trial study was to evaluate the therapeutic effect of INCB024360, an anti-IDO, in patients with myelodysplastic syndrome (MDS) and explore the long-term prognosis of patients taking INCB024360. The Overall Response Rate (ORR), Mean Time to AML, Median Overall Survival (OS), and adverse event incidence as the criteria for evaluating safety and efficacy of INCB024360 have been determined (identifier: NCT01822691). Another anti-IDO drug, indoximod, has been studied for solid tumor including breast cancer and there were no crucial adverse effects [76]. To study the limiting toxicity (RLT) regimen and recommended phase 2 dose (RP2D) for the new diagnosis of AML in the first-stage clinical treatment of indoximod, a clinical trial is started (identifier: NCT02835729). Indoximod has not been studied in AML yet and IDO therapy might be highly effective because it may cause Treg fragility in AML.

4.3. Anti-CTLA-4 Therapy

Another important receptor in immunotherapy is CTLA-4 determined as a crucial target in several cancers including AML. A phase I trial is investigating the role of ipilimumab which is an antibody that blocks CTLA-4 in the treatment of patients with persistent or progressive cancer after allogeneic hematopoietic cell transplantation (allo-HCT). Monoclonal antibodies can find the location of cancer cells, kill cancer cells without damaging the normal cells, or transfer anti-cancer substances to cancer cells. The dose of MDX-010 (ipilimumab) administered to 21 patients with persistent or progressive malignancy following allo-HCT will be assessed (identifier: NCT00060372). In another clinical trial, The incidence of dose limiting toxicities (DLT) and the percentage change in Tregs were tested in 42 subjects The purpose of this phase I trial was to evaluate the safety and optimal dosage of ipilimumab and its efficacy in the treatment of high-risk patients with AML who have recovered or no longer responded to treatment. The study found that the growth and spreadability of cancer cells may be interfered by ipilimumab. (identifier: NCT01757639). The adverse effects of patients with AML and other blood disorders are also under investigation in another trial. Ipilimumab enables CD25/Treg-deficient donor lymphocyte infusions (DLI) to improve the treatment of bone marrow disease (identifier: NCT03912064). The combinatorial effect of ipilimumab with nivolumab and azacitidine is also testing (identifier: NCT02397720).

Nucleus-transducible (nt)-FoxP3- Forkhead DNA binding domain (FKH) (nt-FoxP3-FKH) is nucleus-transducible form of various FoxP3 functional subdomains which restores NFAT-1 and in turn, inhibits the binding of endogenous FKH-containing proteins, such as FoxP3 to FKH DNA binding sequences without affecting the *via*bility and activation of T cells. The suppressive functions of TGF-β-induced iTreg cells and thymus-derived Treg cells were substantially blocked by nt-FoxP3-FKH, accompanied with down-regulation of CTLA-4 surface expression and IL-10 secretion of Treg cells. In addition, nt-FoxP3-FKH upregulated the expression of IL-2 and interferon (IFN)-γ in Treg cells [77] (Fig **6**).

4.4. Tyrosine Kinase Receptor Inhibitors

FMS-like tyrosine kinase 3 (FLT3) is a crucial therapeutic target in AML is a target of many drugs designed for AML including midostaurin. FLT3 is a membrane tyrosine kinase which activates cell signaling related to cell proliferation of AML cells (31466809). To evaluate the effects and efficacy of midostaurin in reducing the incidence of recurrence in CBF-AML patients, a phase II clinical trial has been started on 39 patients with or without FLT3 mutations. It has been hypothesized that midostaurin decreases recurrence by 28% (identifier: NCT03686345). The studies of midostaurin in combination with conventional chemotherapy including daunorubicin and cytarabine in FLT3-mutated AML patients are also under investigation (identifiers: NCT03686345, NCT03591510, NCT03900949). In addition to mutations of FLT3, the mutations leading to overexpression of VEGFR, another tyrosine kinase, are associated with angiogenesis of AML. Vascular remodeling also promotes resistance of AML to chemotherapy (30417360). To determine the efficacy and safety of treatment with dovitinib (TKI258), a multi-kinase inhibitor and anti-angiogenic drug, in 80 patients with a diagnosis of hematological malignancies that have been pre-identified with mutations of FGFR, PDGFR, VEGF, cKIT, FLT3,

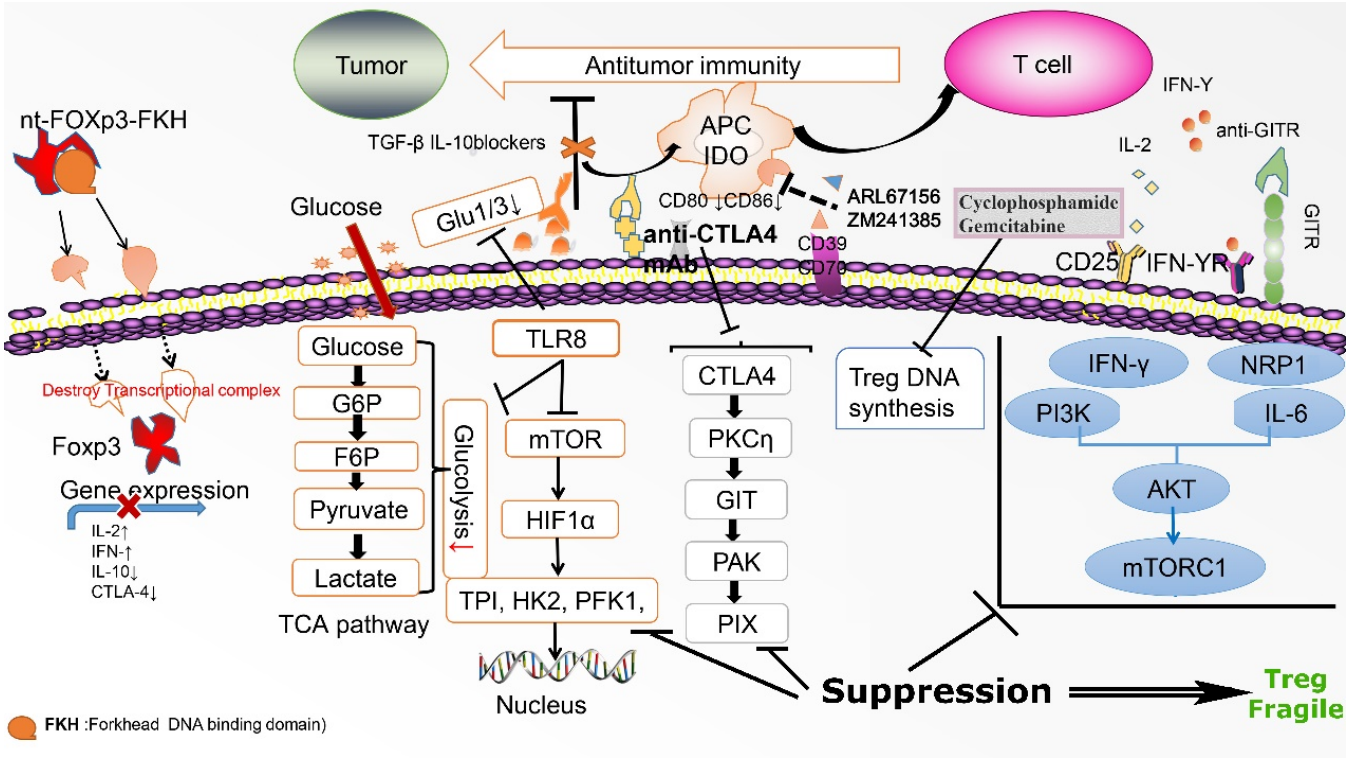


Fig. (6). Treg fragile and suppression of key signaling pathways. IFN-γ secretion leads to Treg fragility and reduction in Treg activity. IL-2 activates PI3K/Akt/mTOR pathway *via* CD25. To suppress Treg activity, DNA synthesis, PI3K/Akt/mTOR pathway, and glucose metabolism should be reduced by cytokines (*e.g.* IFN-γ) or chemicals, such as gemcitabine, and monoclonal antibodies such as anti-CD25 and anti-CTLA-4. FoxP3, another key marker of Treg, is another target in anti-Treg immunotherapy. In addition to Treg fragile, in order to activate anticancer immunotherapy, other immune cells, such as NK, neutrophil, CD8(+) T, and B cells should be activated. Therefore, TGF-β, which inhibits immune cells, should be suppressed by TGF-β blockers. TGF-β blockers are suggested as combination with chemotherapy.

CSFR1, Trk and RET but their disease progressed on or after standard treatment, a signal seeking study was done (identifier: NCT01831726).

4.5. Anti-PI3K Therapy

The PI3K/Akt pathway may participate in the regulation of IL-6 expression and secretion. Treating CD4(+) T cells with IL-6 and co-culturing the cells with pancreatic cancer cells have led to the identification of tumor-derived IL-6 to promote FoxP3 expression and Treg cell differentiation [78]. Treg cell stability is defined as sustained FoxP3 expression, hypomethylation of the conserved non-coding sequence 2 (CNS2) locus, and maintenance of inhibitory function. However, the prevalence and impact of Treg cell instability remains controversial [79]. In addition to the epigenetic changes at the FoxP3 locus, FoxP3 expression loss is a hallmark of unstable Treg cells. Maintaining FoxP3 expression involves many factors including IL2/STAT5 and Foxo1/3a. The FoxP3 induction and subsequent Treg development can also be prevented by sustained TCR stimulation, leading to constitutive activation of the PI3K/Akt/mTOR pathway [80,81]. vation of the PI3K/Akt/mTOR pathway [80,81]. AML cells express inducible T-cell costimulator ligand (ICOSL) leading to a high expression of CD25 and FoxP3 and in turn, suppress anticancer immunity. ICOS(+) Treg cells have more stronger ability to secret immunosuppressive IL-10 by which proliferation of AML is activated through PI3K/Akt, p38MAPK, and STAT signaling pathways. In fact, ICOS(+)Treg cells have two opposite functions to support AML proliferation. They play as AML activators and Immunosuppressor [82]. In addition to secretion of TGF-β and IL-10, Tregs accumulated in the peripheral circulation of AML patients suppress immunity by cell-cell contacts leading to resistance of chemotherapy [83].

Furthermore, in 23% of AML samples, sensitivity to inhibitors of colony-stimulating factor 1 receptor (CSF1R) has been reported [84]. CSF1R is a tyrosine kinase receptor associated to proliferation and differentiation of myeloid-lineage cells. A phase II trial examined the efficacy of inhibitor of both CSF1R and FMS kinase, JNJ-40346527, in 100 patients with relapsed or untreated AML. JNJ-40346527 may block the growth

of cancer cells by blocking some TME signals required for cell growth (identifier: NCT03557970). Meanwhile, recent evidence has shown that genetic inactivation of PI3Kδ in CD4(+)FoxP3(+) Treg cells sensitizes immune-resistance solid tumors to CSF1R (+) TAM depletion and, in turn, it enhances the effect of CSF1R blockade. The PI3Kδ inhibitor, idelalisib (Zydelig, Gilead), is highly effective for the treatment of chronic lymphocytic leukemia and AML, and its main effect is to block the interactions between leukemia cells and stromal cells in their niche [85]. The DLT, pharmacokinetic parameters, pharmacodynamic effects and clinical response rate following idelalisib treatment in patients with hematologic malignancies were evaluated. In 192 patients with B-cell non-hodgkin lymphoma (NHL) and AML were selected, and the study was conducted with progressively increasing doses followed by oral administration of idelalisib (identifier: NCT00710528). BKM120, another PI3K inhibitor, was studied in 16 patients with AML. This Phase I clinical study was aimed at finding the highest tolerable dose and the safety of BKM120 in patients with relapsed AML or did not respond to treatment (identifier: NCT01396499); however, the effectiveness of BKM120 was elucidated in PI3K-activated AML patients and overall the adverse effects were then clearly reported (identifier: NCT01833169).

4.6. Pros, Cons, and Adverse Effects of Anti-Treg Immunotherapy

Treg cells increased in peripheral blood have increased number of CTLA-4 which causes suppression of T cells and relapse of AML [86]. Treg cells reduce the efficacy of immunotherapy. Modification of AML with CD80 and IL-2 by means of lentiviral vectors which improved the quantity of cytotoxic T cells and therefore, the efficacy of immunotherapy [87]. B7-H1 upregulation, a homolog for CD80/CD86 can also suppress antileukemia immunity [88]. IDO(+) AML cells increase the number of CD4(+) CD25(+) Treg cells leading to CTLA-4 and FoxP3 expression. CD4(+) CD25(+) T cells cocultured with IDO+ AML cells are not able to produce IL-2, do not proliferate, and instead, suppress other T cells. The important behavior of AML cells is to induce T cell tolerance *via* changing the CD4(+) CD25(-) T cells into CD4(+) CD25(+) T cells through IDO-dependent mechanism [89].

Despite the clinical success of anti-CTLA-4 mAb, treatment with anti-CTLA-4 mAbs often leads to the development of immune-related adverse effects (irAE) that predominantly affect the skin and gastrointestinal tract [90]. Targeting CTLA-4 would be an effective therapy against cancer if the irAE is under control. The irAE causes a reduction in ratios of Treg to effector T cells during a systemic T cell activation [91]. It has been pointed out that circulating PD-1 Tregs of melanoma patients in stage III is reduced after the initiation of adjuvant treatment with the PD-1 inhibitor, nivolumab; however, CTLA-4 Tregs are increased as a response to nivolumab adjuvant therapy [92]. In order to control irAE, maintaining immune homeostasis after immune checkpoint blockers treatment is vital. Preclinical models of irAE have shown a negative correlation between Treg numbers and irAE [34]. In most cases, the T lymphocytes hyperactivation is induced by immune checkpoint blockers and in turn, has a specific response against tumor antigens. It has been discovered that antitumor activity in tumor tissues leads to side effects in normal tissues called "off-target toxicity". The CD8(+) cytotoxic T lymphocytes-mediated cell lysis releases neoantigens, tumor antigens and autoantigens from normal tissues. Off-target toxicity reduces immune tolerance, which is exacerbated by inhibition of Tregs [18]. Anti-GITR, TRX518, reduces circulating and intratumoral Tregs, and it is very effective when combined with PD-1 inhibitors in advanced refractory tumors [93].

Myositis is a severe irAE with high mortality rate associated with immune checkpoint inhibitors. Myocarditis occurs at an early stage of monotherapy with immune checkpoint inhibitors, as well as with combinatorial therapy. Accordingly, systemic cardiac screening is suggested during treatment [94]. A total of 126 cases of pembrolizumab and nivolumab as monotherapy anti-PD-1 as well as 26 cases of nivolumab (anti-PD-1) in combination with ipilimumab (anti-CTLA-4) have been analyzed in melanoma, pulmonary, renal, and other cancers which produced 21% fatality [95]. Neurologic irAEs also affected one percent of patients treated with anti-immune checkpoint monotherapy and between two and three percent of patients treated with anti-immune checkpoint combinatorial therapy [96]. Another study that analyzed anti-immune checkpoint therapy showed endocrine dysfunction, colitis, dermatitis, encephalitis, and hematologic and hepatic problems in patients with different kinds of cancer, such as melanoma, lung, and renal cancers [97].

Pharmacokinetics and pharmacodynamics of daclizumab (anti-CD25) were examined in 34 patients with T cell leukemia/lymphoma. It was found that a high-dose daclizumab was required to saturate IL-2R alpha (CD25) and a long-term maintenance monotherapy

with anti-CD25 led to a sustained reduction of CD4(+) CD25(+) Treg cells [98]. In addition, it has been shown that IL-12 in combination with PC61 (anti-CD25) can lower the dose of cytokine required for the best therapeutic function. The IL-12-related toxicity reduces anti-CD25 efficacy [99].

Furthermore, CD25 inhibitor combined with TGF-β blockade was more effective on pancreatic tumor growth in mice. As anti-CD25 monotherapy is also able to decrease periphery and intra-tumor Tregs, anti-CD25 and anti-TGF-β combinatorial therapy synergistically decreases periphery and intra-tumor FoxP3(+) Tregs, and in contrast, increases intra-tumor CD8(+) TILs compared with anti-CD25 and anti-TGF-β monotherapies [100]. Combinatorial anti-TGF-β therapy is also suggested in combination with DC vaccines and low dose gemcitabine which increases Teff:Treg ratio [45,101]. As gemcitabine results in bone marrow suppression, cardiomyopathy, and liver and kidney problems, its low dose in combination with anti-IDO and anti-TGF-β is suggested [102,103]. Cardiomyopathy of cytotoxic agents may result from inhibition of PI3K pathway. Arrhythmia is one of the major adverse effects of anticancer drugs which warrants further study [104]. Anti-IDO agents sensitize cancer cells to gemcitabine and they can be used in combination with gemcitabine or immune checkpoint inhibitors that reduce resistance to PD-1/ PD-L1 inhibitors [105,106]. Anti-GARP therapy is another way to suppress TGF-β pathway, but its strategy is not adequate for antitumor treatment [107].

4.7. Key Treg Markers in AML Compared to Other Kinds of Cancer

Immunological principles based on Treg cell self-stabilization and tumors evading immune mechanism have led to more effective immune-normalization therapies. This optimal combination therapy is more effective in combating cancer cells. In order to create the next generation immunotherapy, a better understanding of function and regulation of Treg in antitumor immunity, combined with the discovery of new immune targets, are vital for enhanced clinical efficacy with reduced adverse reactions. The development of immune-normalization therapy depends on identifying specific defects in the antitumor immune responses. Determining the predominant pathway involved in tumor escape is crucial. However, individual patients may show different molecular profiles which require different therapeutic strategies.

To compare and predict the importance of Treg normalization in cancer immunotherapy for CTLA-4, PD-1, FoxP3, and CD25, which are strongly targeted by many monoclonal antibodies in cancer immunotherapy, data were obtained from Cancer Genome Atlas (TCGA) for ten of the most frequent kinds of cancer (Breast cancer, Colon adenocarcinoma, Esophageal cancer, Lung adenocarcinoma, Acute myeloid leukemia, Liver hepatocellular carcinoma, Skin cutaneous melanoma, Prostate adenocarcinoma, Stomach adenocarcinoma, and Pancreatic adenocarcinoma) (Fig. **7**). Using the results, we prognosticated that PD-1 inhibitors are risky and their adverse effects should be taken into account when they are administered to treat AML, lung adenocarcinoma, and prostate adenocarcinoma. It was further discovered that immunotherapy of skin cutaneous melanoma appears to be highly effective because CTLA-4, CD25, and PD-1 are significantly overexpressed in different stages (Fig. **8**) [112]. In contrast, Treg molecular markers, FoxP3 and CD25, have stronger expression in almost all kinds of cancer analyzed here. However, CD25 inhibitors are more effective than FoxP3 inhibitors in predicting patient survival (Fig. **9**).

It has been shown that modulation of inhibitory TME made by Tregs may enhance NK cell clinical activity and produce encouraging results in the treatment of refractory AML [113]. CD4(+)CD25(+)FoxP3(+) Tregs accumulate in bone marrow microenvironment in AML. The AML cells expressed ICOSL which can provide co-stimulation through ICOS for conversion and expansion of Tregs sustaining high FoxP3 and CD25 expression, as well as a suppressive function. CD4(+)CD25(+)ICOS(+) T cells possessed stronger ability to secrete IL-10 than CD4(+)CD25(+)ICOS(-) T cells therefore led to proliferation of AML cells through activation of Akt, ERK1/2, p38, and STAT-3 signalling pathways [82]. In addition, the circulating frequencies of CD4(+)CD25(+) Tregs and CD4(+) CD25(high) Tregs in AML patients were elevated compared with those in healthy controls and complete remission patients [114]. The CD25 and TGF-β combination blockade had a higher tumor growth inhibitor value. This combined therapy significantly decreased periphery and intra-tumor FoxP3(+) Tregs, while increasing intra-tumor CD8(+) TILs levels [100]. Using TCGA, we found that CTLA-4 and CD25 are significantly overexpressed in AML compared to healthy people; however, other kinds of cancer such as breast cancer, lung adenocarcinoma, and prostate adenocarcinoma did not show overexpression in CTLA-4 and

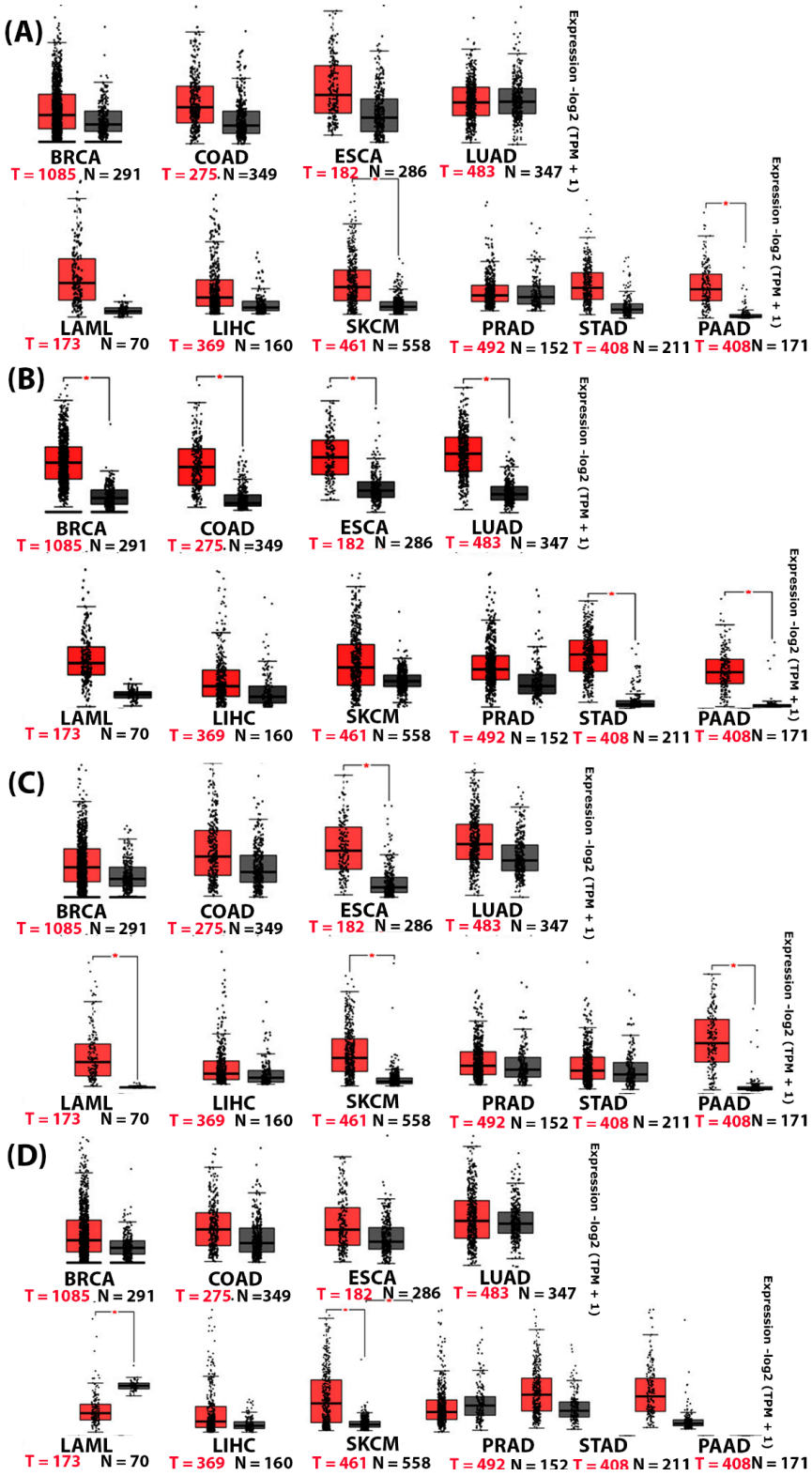


Fig. (7). Comparing expression levels of (A) CTLA-4, (B) FoxP3, (C) CD25, and (D) PD1 between normal people and cancer patients (specifically in ten common cancers). (A) CTLA4 is significantly overexpressed in ESCA, LAML, STAD, and PAAD. There is no differential expression of CTLA-4 in LUAD and PRAD. Anti-CTLA-4 agents maybe one of the best therapeutic options with less harmful effects on patients suffering from LAML, STAD, and PAAD. **(B)** FoxP3 is strongly expressed in all types of cancer. **(C)** CD25 (IL2RA) is significantly expressed in ESCA, LAML, SKCM, and PAAD. CD25 inhibitors, such as daclizumab, are one of the best therapeutic options with less harmful effects on patients suffering from LAML, SKCM, and PAAD. **(D)** PD-1 is overexpressed in SKCM, PAAD, STAD, BRCA, and COAD. It is observed that PD-1 is also highly expressed in healthy people. Normal people show higher expression of PD-1 compared to corresponding cancer cases.

Therefore, it is clear that PD-1 inhibitors, such as pembrolizumab, may have adverse effects on, for example, LAML cases. Based on the above results, we can prognosticate the effects of each drug category in their target expression between normal people and cancer patients. Clinical data analysis helps us to predict the adverse effects as well. **BRCA:** Breast cancer; **COAD:** Colon adenocarcinoma; **ESCA:** Esophageal cancer; **LUAD:** Lung adenocarcinoma; **LAML:** Acute myeloid leukemia; **LIHC:** Liver hepatocellular carcinoma; **SKCM:** Skin cutaneous melanoma; **PRAD:** Prostate adenocarcinoma; **STAD:** Stomach adenocarcinoma; **PAAD:** Pancreatic adenocarcinoma. T and N refer to cancer patients and normal people respectively. Transcripts per Million (TPM) is a normalization method for RNA-seq. Data collected from the Cancer Genome Atlas.

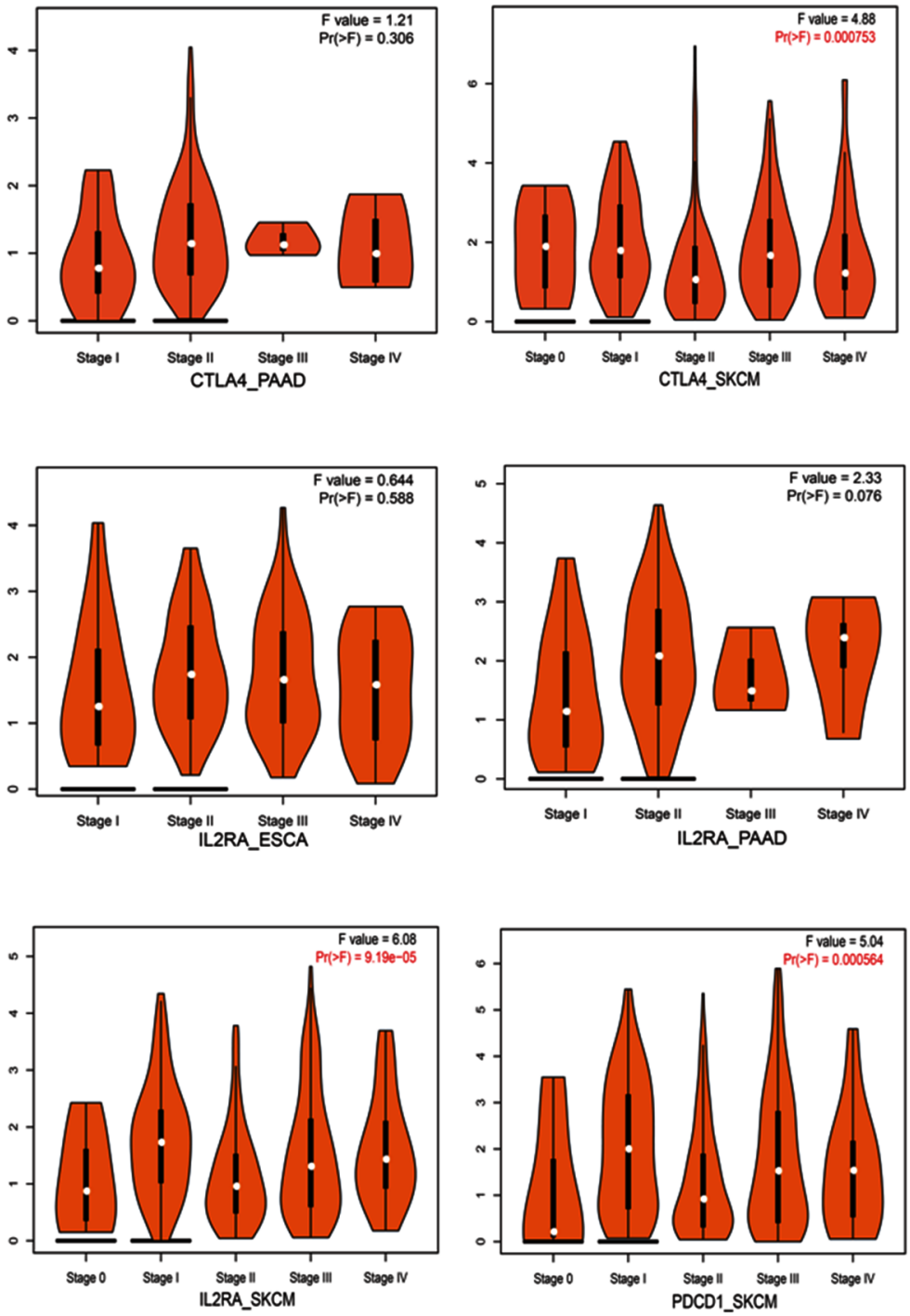
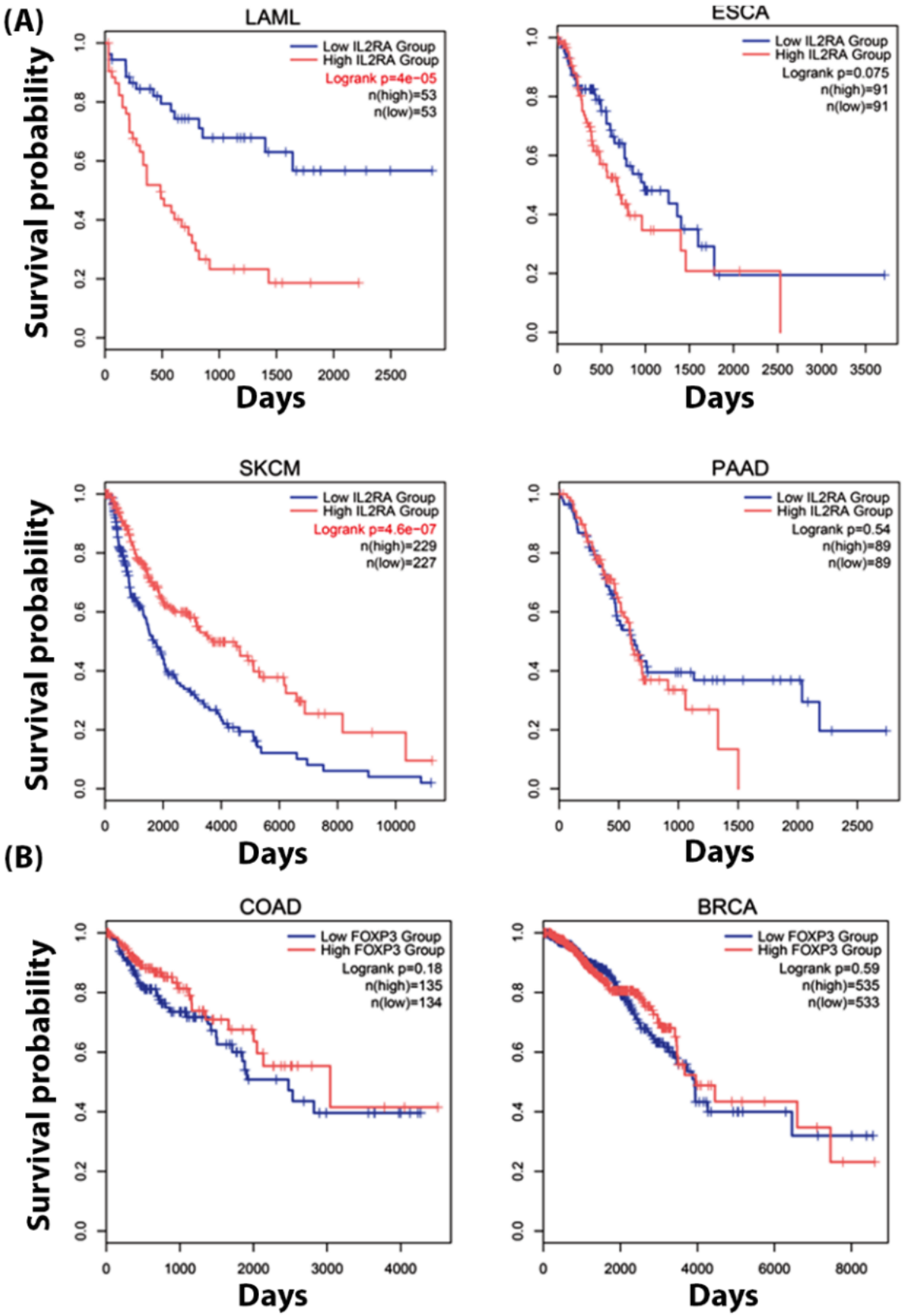


Fig. (8). The expression of CTLA-4, CD25 (IL2RA), and PD-1 (PDCD1) genes in different stages of skin cutaneous melanoma (SKCM) and pancreatic adenocarcinoma (PAAD). Using a box plot, we show significant expression of IL2RA, CTLA-4, and PD-1 in SKCM which make this malignant melanoma a good target for immunotherapy.


(A)
LAML
Low IL2RA Group
High IL2RA Group
Logrank p=4e−05
n(high)=53
n(low)=53
Survival probability
Days
ESCA
Low IL2RA Group
High IL2RA Group
Logrank p=0.075
n(high)=91
n(low)=91
Days
SKCM
Low IL2RA Group
High IL2RA Group
Logrank p=4.6e−07
n(high)=229
n(low)=227
Survival probability
Days
PAAD
Low IL2RA Group
High IL2RA Group
Logrank p=0.54
n(high)=89
n(low)=89
Days
(B)
COAD
Low FOXP3 Group
High FOXP3 Group
Logrank p=0.18
n(high)=135
n(low)=134
Survival probability
Days
BRCA
Low FOXP3 Group
High FOXP3 Group
Logrank p=0.59
n(high)=535
n(low)=533
Days

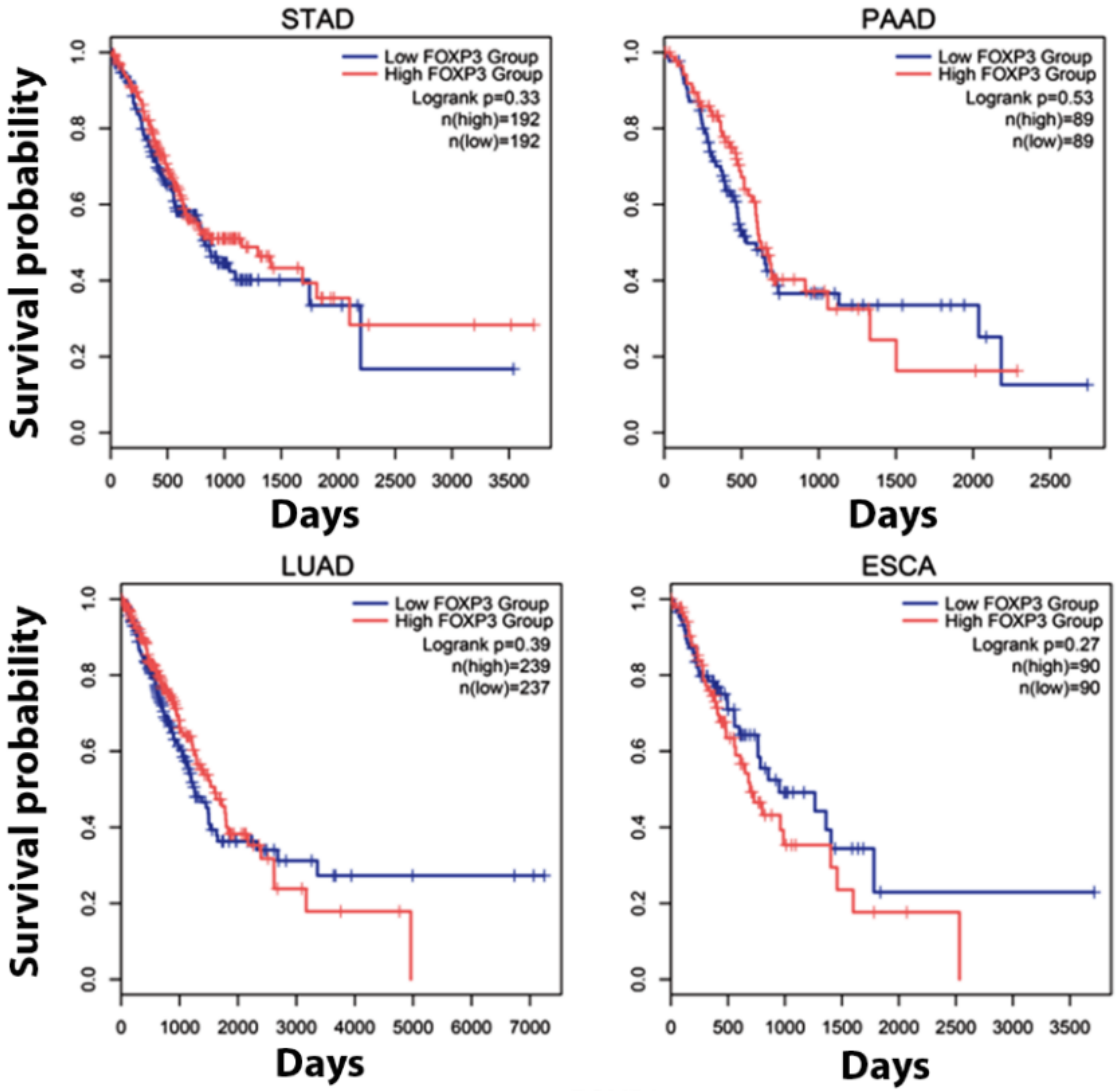


Fig. (9). Survival analysis based on the expression status of (A) CD25 (IL2RA) and (B) FoxP3 genes and the Kaplan-Meier curves. Survival analysis of LAML and SKCM shows a significant difference between low CD25 expression (blue line) and high CD25 expression (red line) groups. This result clearly shows the survival rate in LAML increased by 40% where there is low expression of CD25. Therefore, CD25 inhibitors seem to be highly effective in LAML. In contrast, FoxP3 survival analysis shows no significant difference between two groups (Low expression and high expression) in different kinds of cancer. **BRCA:** Breast cancer; **COAD:** Colon adenocarcinoma, **ESCA:** Esophageal cancer; **LUAD:** Lung adenocarcinoma; **LAML:** Acute myeloid leukemia; **SKCM:** Skin cutaneous melanoma; **STAD:** Stomach adenocarcinoma; **PAAD:** Pancreatic adenocarcinoma. The *log rank test* calculates the chi-square (X^2) for each event time for each group and sums up the results.

CD-25. CTLA-4 is an immune checkpoint protein and CD25 is Treg marker. In contrast to AML, the use of anti-CTLA-4s and anti-CD25s may not be effective treatments against several kinds of cancer.

CONCLUDING REMARKS

One of the major obstacles to successful tumor immunotherapy is tumor-associated Treg cells which are resistant to physiological deactivation. This excessive resistance to destabilization can be mediated through several known factors in the TME. Tumors have some environmental factors that cause phenotypic changes in Treg cells leading to functional instability. It has also been reported cryo-thermal therapy shifts the tumor chronic microenvironment from Th2 immunosuppressive and pro-tumorigenic to Th1 immunostimulatory and tumoricidal state [115]. Immune normalization is the correction of defective Treg cells in the immune mechanism and restoring it to natural levels of immunity. IL-2 still remains a crucial target in the activation of antitumor immunity [116]. However, immunotoxicity should be taken into account. The depletion of hu-

man T cell subsets during the IL-2 therapy decreases toxicity. Treg cells play a role in the maintenance of immune tolerance, and therefore IL-2 toxicity is induced by selective depletion or inhibition of Treg cells after IL-2 therapy [117].

Tumor DNA damages interact with the immune system, and it has been shown the tumor DNA repair has an important role in driving response to immune checkpoint blockade [118]. Therefore, patient DNA plays a key role when the DC vaccine or adoptive treatment is given to the patient [119]. However, in most cases, the strategies developed based on antitumor immunosuppression may only serve as regular activators. The immune system does increase in response to tumors, but it also pushes the TME to super-physiological levels, increasing the risk of irAEs [120]. In conclusion, Treg-based immunotherapy may lead to autoimmune diseases. Therefore, for effective and safe outcome, it is suggested that this therapy is combined with chemotherapy. However, it has to be noted that chemotherapy has its own adverse effects and may result in kidney and heart problems (though this may need further research confirmation). This review shows Treg cells are strongly increased in AML, and the analysis of key markers shows that Treg-based immunotherapy is not effective for all kinds of cancer.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This research was supported by National Natural Science Foundation of China (Grant nos. 81503093, 81602166, and 81672444) and by Joint Funds of Southwest Medical University & Luzhou (2016LZXNYD-T01, 2017LZXNYD-Z05 and 2017LZXNYD-J09).

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