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Regulatory T cells in cancer patients and their roles in cancer development/progression

Abstract

The immune system can protect body against malignant cell formation and cancer development. However, in some cases malignant cells survive and a tumor develops leading to cancer. The immunosuppressive function of some subsets of immune cells is involved in evading the tumor cells from immunosurveillance. In the past decade, elevated levels of regulatory T cells (Tregs) were reported in the peripheral blood and tumor tissue of cancer patients. In most cancer patients, increased levels of Tregs were correlated with poor prognosis. In contrast, increased frequencies of Tregs were associated with favorable prognosis in some cancer patients. So far, the precise roles of Tregs in carcinogenesis and cancer progression have not been addressed. Better understanding of the immunobiology of Tregs is beneficial for elucidation of their roles in cancers and innovation of more effective cancer therapy modalities. In this review, we present extensive information about the immunophenotype and functions of Tregs, mechanisms that are implicated in the recruitment, differentiation, and/or expansion ofthese cells at tumor sites, as well as their frequencies and roles in cancer patients. We also assessed their predictive value in cancer patients.

Keywords: regulatory T cells, immunophenotype, function, frequency, cancer, disease progression, prognosis

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Abbreviations: MDSCs, myeloid derived suppressor cells; Th, T helper; NKT, natural killer T; IL, interleukin; Treg, regulatory t cell; Tr1, type 1 regulatory T; TGF-β, transforming growth factor-β; TCR, T cell receptor; CTLA-4, cytotoxic t lymphocyte associated antigen-4; GITR, glucocorticoid induced TNF receptor family-related protein; Foxp3, Forkhead box protein P3; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, x-linked; LAG-3, lymphocyte activation gene-3; PD-1, programmed death 1; PD-L, PD-Ligand; HLA, human leukocyte antigen; nTreg, Naturally Occurring Treg; iTreg, peripherally induced Treg; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon gamma; APC, antigen presenting cell; GITRL, GITR ligand; Bcl-2, B-cell lymphoma 2; Bim, Bcl-2 interacting mediator of death; CCR, CC-Chemokine receptor; CXCR, CXC-chemokine receptor; CCL, CC ligand; CXCL, CXC ligand; VEGF, vascular endothelial growth factor; RANK, receptor activator of NFκB; RANKL, RANK ligand; ICOSL, ICOS ligand; ICOS, inducible costimulator; PGE2, prostaglandin E2; TLR, toll-like receptor; ILT3, immunoglobulin-like transcript 3; ILT4, immunoglobulin-like transcript 4; NY-ESO-1, new york esophageal squamous-cell carcinoma-1; AICD, activation induced cell death; ACAD, activated cell autonomous death; FasL, fas ligand; c-FLIP, cellular FLICE (FADD-Like IL-1β-Converting Enzyme)-Inhibitory Protein; GARP, glycoprotein a repetitions predominant; TIM, T Cell Immunoglobulin Mucin 3; Klrg1+, Killer Cell Lectin-Like Receptor G1; TNFR, tumor necrosis factor superfamily receptor; NK, natural killer; M-CSF, macrophage colony-stimulating factor; LAGE-1, L antigen family member 1; ARTC1, antigen recognized by treg cells 1; CEA, carcinoembryonic antigen
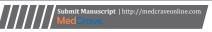
Introduction

In the late 1950s, Burnet for the first time introduced the concept of "tumor immunosurveillance", as a new role for the immune system in the body, in which the immune system has the ability to prevent tumor or cancer development through recognition and elimination of malignant transformed self-cells.[1] However, malignant cells can be evolved to survive in the host by recruiting various mechanisms. Indeed, "tumor immunoediting" during tumor development can hamper antitumor immune responses and facilitate tumor growth.[2] Tumor cells can escape immunosurveillance by multiple mechanisms including secretion of immunosuppressive molecules, low immunogenicity of tumor antigens, failure to process and present tumor antigen, lack of expression of costimulatory molecules which are necessary for T cell activation, covering or blocking of tumor antigens on the surface of tumor cells, expression of apoptosis-inducing molecules as well as induction of immunosuppressor cells.

Several immune and non-immune cells with immunosuppressive activity or tumor promoting effects may exist in the tumor sites, including monocytes/macrophages, neutrophils, dendritic cells, mast cells, eosinophils, MDSCs, Th2 cells, regulatory T cells, NKT cells, B cells, and fibroblasts. Tumor cells, endothelial cells, and other cells are also implicated in the tumor growth. In this paper, we reviewed the phenotype, function, and frequency of regulatory/suppressor T cells, as a major cell type involved in the regulation of immune responses, in tumor/cancer setting as well as their roles and predictive value in cancer patients.

Suppressor/Regulatory T Cells

In the early 1970s, Gershon and Kondro described "suppressor cells" that suppress T cell responses in an antigen specific manner and transferring these cells to naive mice induced antigen specific tolerance.[3] A few years later, T cells with immunosuppressive activity were reported in tumor bearing mice.[4,5] These studies in murine tumor models showed that T cell responses can be elicited in immunogenic tumors but tumors grow due to the development of tumor-induced suppressor CD4+ T cells. Suppression of cytotoxic antitumor immune responses by human autologous melanoma-induced T cell clones was also observed.[6] However, lack of a marker for recognition of suppressor T cells hindered more research in the field of suppressor

T cells for two decades. In 1995, Sakaguchi and colleagues observed that lack of a subpopulation of CD4^{+} T cells coexpressing CD25, IL-2 receptor alpha-chain, leads to autoimmune disorders in mice.[7] Afterwards, CD25 was used as a diagnostic marker for T cells with immunoregulatory properties, and CD4+CD25^{+} T cells, termed as regulatory T cells, were became the interest of many immunological studies. These studies suggested a crucial role for regulatory T cells in maintaining the immunological tolerance to self and non-self antigens.[8] Accordingly, regulatory T cells were demonstrated to be essential for control of immune responses against microbes, allergens, allogeneic transplants, and the fetus during pregnancy. Simultaneous studies also indicated implication of regulatory T cells in cancer.

Several phenotypically and functionally distinct subsets of regulatory T cells have been described; they belong to both CD4^{+} and CD8^{+} T cell subpopulations. CD4^{+} regulatory T cells are classified into three subsets, including CD4+CD25^{+} regulatory T cells (Tregs), IL-10-producing Tr1 cells, and Th3 cells. CD4+CD25-Foxp3-CD69^{+} T cells were reported as a new subset of regulatory T cells which inhibited the proliferation of CD4^{+} T cells via cell membrasne-TGF-β1. Various subsets have also been reported for CD8^{+} regulatory T cells, including CD8+CD25^{+} Tregs, CD8+CD28- Tregs, and IL-10 producing CD8^{+} T cells, but they are less characterized. Human CD8+CD25^{+} Tregs are appeared to share phenotypic and functional features with CD4+CD25^{+} Tregs. In addition, CD8+CD122^{+} Tregs were reported to have essential roles in the maintenance of T cell homeostasis. Also, γδ T cells have immunoreguatlory function. A small fraction of human peripheral blood and tumor infiltrating γδ T cells express FOXP3. In addition, stimulation of mouse splenocytes with anti-TCRγδ in the presence of TGF-β has led to appearance of CD25+Foxp3^{+} γδ T cells. TGF-β-induced CD25+Foxp3^{+} γδ T cells had increased TGF-β and GITR expression and mediated a potent immunosuppressive effect on anti-CD3-stimulated T cell activation and proliferation.[9] In the tumor setting, CD4+CD25^{+} Tregs are most studied as they have been frequently reported in various animal tumor models and cancer patients. Tr1 cells and CD8^{+} Tregs have also been reported in some tumor studies.

Immunophenotypical Features of Tregs

CD25 is being used as a marker for Tregs, especially in mice that are held under pathogen-free conditions. In human, this molecule is also expressed on recently activated effector T cells. One charactristics of these cells is expression of CD4+CD25+ phenotype. Accordingly, the suppressive activity of human CD4+CD25^{+} T cells was observed only in a fraction that expressed + levels of CD25 (CD25+).[10] CD4+CD25^{+} Tregs constitutively express CTLA-4 (CD152). However, CTLA-4 is also expressed on activated CD4^{+} and CD8^{+} T cells, which delivers a negative signal leading to downregulation of T cell activation. GITR (CD357) is another cell surface molecule that is expressed on Tregs. Tregs can express + levels of GITR. It should be noted that, expression of GITR is not limited to Tregs, as expression of this molecule is upregulated in conventional T cells upon activation. After defining that the IPEX syndrome in human and Scurfy phenotype in mice are caused by mutations of FOXP3 resulting in the loss of Tregs; Foxp3 was served as the most specific marker for Tregs. Foxp3 expression in mouse has been thought to be restricted to CD4^{+} Tregs and little or no expression in CD8^{+} T cells or other cell population, but FOXP3 expression in human is not restricted to CD4^{+} Tregs as its expression has been detected in many subsets of T cells including recently-activated T cells, however, its expression level is +er in Tregs than that in effector T cells. Recently, expression of Foxp3 was also detected in mouse conventional T cells.[11] Stimulation of naïve T

cells can lead to induction of FoxP3 and acquisition of Treg activity in human T cells.[12] Expression of Foxp3 was also detected in some epithelial cells.[13] Another feature of Tregs is expression of low levels or lack of CD127, which is IL-7 receptor. However, CD127 expression is downregulated on all human T cells after activation; but it is re-expressed on the majority of effector and memory T cells. IL-7 receptor signaling was, recently, found to be involved in the development and peripheral homeostasis of CD4+CD25+Foxp3^{+} Tregs. Human CD25+ Tregs are CD45RA-CD45RO+ and mouse CD4+CD25^{+} Tregs are CD45RBlow.[10] CD4+CD25^{+} Tregs express other cell surface molecules associated with activation and migration of T cells such as L-selectin (CD62 ligand), CD71 (transferrin receptor), and OX40 (CD134). CD28/B7 costimulation is essential for the homeostasis of the CD4+CD25^{+} Tregs. LAG-3 (CD223), a CD4 homolog that binds MHC class II, is another cell surface molecule on Tregs.[14] Expression of + levels of PD-1 receptor (CD279) and its ligand (PD-L) 1 (B7-H1; CD274), Fas (CD95), and folate receptor has been detected in Tregs. Human CD4+CD25+FOXP3^{+} Tregs also express CD39 (nucleoside triphosphate diphosphohydrolase) as well as CD73 (ecto-5′-nucleotidase), and produce immunosuppressive adenosine. Expression of HLA-DR was observed on *in vitro* human stimulated CD4+CD25+ Tregs.[10] The majority of these molecules are usually expressed on the surface of activated lymphocytes, including Tregs with suppressive function.

CD4+CD25^{+} Tregs have been classified into two subsets: thymic derived or naturally occurring Tregs (nTregs) and adaptive or peripherally induced Tregs (iTregs). Expression of Helios, a member of the Ikaros transcription factor family, has been detected in all of the thymic derived Tregs but only in 70% of peripheral Tregs.[15] *In vitro* activation of T cells derived from TCR-transgenic *Rag^{-/-}* mice (thus no endogenious Tregs) by polyclonal or antigen specific stimulation led to generation of iTregs that expressed Helios. Transient expression of Helios on activated human and mouse conventional T cells and Tregs was also reported.[16] Recently, it was suggested that expression of Helios might be upregualted in peripherally induced Tregs after activation by dendritic cells.[17] Therefore, expression of Helios can be defined as a marker for T cell activation and proliferation. Neuropilin-1, a type 1 transmembrane protein, has also been reported to be a cell surface marker of Tregs. The expression of neuropilin-1 is under the control of Foxp3 as ectopic expression of Foxp3 in naïve T cells led to induction of neuropilin-1 expression. Expression of neuropilin-1 has been suggested to be useful in distinguishing nTregs and iTregs base on the observation that iTregs expressed low levels of neuropilin-1 compared to nTregs, both *in vitro* and *in vivo*.[18,19] Expression of neuropilin-1 was low on mucosa-generated iTregs under non-inflammatory conditions, while it was expressed at + levels on thymus-derived nTregs. However, upregulation of neuropilin-1 was observed in iTregs under inflammatory conditions.[18]

Functional Characteristics of Tregs

Initial *in vitro* studies showed that CD4+CD25^{+} Tregs are unable to secrete IL-2 and IFN-γ and suppress proliferation and cytokine secretion (IL-2, IFN-γ, and IL-13) of cocultured CD4+CD25- T cells both in mouse, and human. CD4+CD25^{+} T cells from human peripheral blood produced +er levels of IL-4 but similar amounts of IL-10 produced by CD4+CD25- T cells.[20] In another study, peripheral blood CD4+CD25^{+} T cells secreted IL-10 upon stimulation with allogeneic, but not syngeneic, mature dendritic cells, however, the suppressive activity was independent of IL-10.[21] Other study reported that blood CD4+CD25+ cells did not secrete IL-10 upon stimulation in vitro, while CD4+CD25- T cells did.[10] In these studies, human Treg

mediated suppression was cell-to-cell contact dependent and the suppressive activity was lost by the addition of exogenous IL-2 (and IL-15) or anti-CD28 costimulation. Similarly, providing anti-CD28 costimulation or exogenous IL-2 in conjunction with TCR stimulation inhibited *in vitro* suppressive activity of mouse CD4⁺CD25⁺ Tregs. Further investigations showed that direct cell contact, particularly, binding of cell surface molecules such as CTLA-4 on suppressor T cells to CD80 and CD86 molecules on effector T cells is involved in Treg suppressive activity. Mouse CTLA-4⁺ Tregs induced IDO expression in APCs through CTLA-4. Interactions between GITR and GITRL led to expansion of Tregs, Tr1 cells, as well as effector CD4⁺ T cells, both *in vitro* and *in vivo*. GITRL expressed on APCs was important for optimal immunosuppressive function of Tregs. Modulating the activation state and function of APCs is also important in Treg mediated immunosuppression. Perforin and granzyme A from human Tregs induce apoptosis and cytolysis in conventional T cells and APCs. Immunosuppressive activity of mouse Tregs is mediated through a granzyme B-dependent but perforin-independent mechanism. IL-35 can act as an autocrine Tregs growth factor. It has been recently reported that IL-35 induces a distinct population of regulatory T cells that mediate suppression via IL-35 but not IL-10 or TGF-β.[22] Overall, findings of many studies show that Tregs can suppress immune responses of effector T cells as well as other immune cells through direct cell-cell contact dependent mechanisms and release of various soluble factors.

Tregs are believed to be anergic cells; however, they can proliferate, particularly in lymphopenic host. Mouse CD4⁺CD25⁺ cells failed to proliferate *in vitro* after TCR stimulation alone, but they proliferated well by adding exogenous IL-2 or TCR stimulation. A modest proliferation of human CD4⁺CD25⁺ Tregs was also observed by providing either costimulation with CD28 cross-linking or addition of IL-2 to a maximal anti-CD3 stimulus.[10] In another study, human CD4⁺CD25⁺ Tregs suppressed naive and memory T cell proliferation and expanded without loss of function *in vitro*.[23] CD4⁺CD25⁺ Treg clones produced TGF-β, but not IL-10, which shows they were distinct from Tr1 cells. Further studies showed that Tregs actually cycle more actively than conventional T cells spontaneously or in response to specific antigen.[12] In *Rag1⁻/⁻* hosts reconstituted with conventional T cells, Tregs induced from conventional T cells and expressed lower Bcl-2 and +er Bim/Bcl-2 ratio than conventional T cells, suggesting iTregs and conventional T cells differ in their sensitivity to apoptotic stimuli.

Tregs in Animal Tumor Models

More than three decades ago, presence of T cells with immunosuppressive activity was demonstrated in tumor bearing mice and suppressive activity of these cells was related to the progressive growth of immunogenic tumors in immunocompetent mice.[4,5] In 1997, IL-10 producing CD8⁺ T cells with immunosuppressive activity were found in the tumor draining lymph nodes of fibrosarcoma tumor bearing mice.[24] In 1999, *in vivo* depletion of CD4⁺CD25⁺ T cells by administration of anti-CD25 monoclonal antibody prior to tumor inoculation was led to protection of mice against tumor challenge.[25] Administration of combination of anti-CTLA-4 and anti-CD25 monoclonal antibodies synergistically induced antitumor immunity. Later studies showed that CD4⁺CD25⁺ T are involved in suppression of antitumor immunity in various tumor models. Depletion of CD4⁺CD25⁺ Tregs augmented the generation of specific immune T cells in tumor draining lymph nodes. In a rat colon carcinoma model, the volume of tolerogenic tumors was correlated with an expansion of CD4⁺CD25⁺ Tregs in lymphoid tissues. These Tregs delayed *in vivo* rejection of immunogenic tumors and suppressed *in vitro* T cell responses against immunogenic tumor cells. Administration of cyclophosphamide led to depletion of Tregs and delay in tolerogenic tumor growth and enhanced the curative effects of immunotherapy. In murine fibrosarcoma (Ld-expressing Ag104), the majority of tumor infiltrating lymphocytes at the late stage of tumor growth was appeared to be CD4⁺CD25⁺ T cells. Intra-tumoral depletion of CD4⁺ T cells led to expansion of IFN-γ secretion of CD8⁺ T cells at tumor sites and rejection of late-stage tumors. Depletion of CD4⁺ T cells induced concomitant antitumor immunity in mice bearing a poorly immunogenic melanoma. Concomitant immunity also induced by administration of cyclophosphamide or agonistic anti-GITR antibody. CD4⁺CD25⁺ T cells suppressed concomitant antitumor immunity generated by adoptively transferred CD4⁺ and CD8⁺ T cells in *Rag1⁻/⁻* mice bearing progressive melanoma tumors. Antigen specific-CD4⁺CD25⁺ Tregs were shown to abrogate tumor rejection induced by adoptively transferred antigen-specific CD8⁺ T cells in a mouse transgenic colon carcinoma model.[26] Increased number of CD4⁺CD25⁺ T cells in the tumor draining lymph nodes and enhanced production of inhibitory cytokines during tumor progression has been reported in a transgenic mouse model of prostate tumor.[27] In a murine colon carcinoma model, the ratio of CD4⁺CD25⁺ T cell/CD4⁺ T cell was increased in the spleen of tumor-bearing mice but not in the peripheral blood. It was shown that both Tregs and antitumor effector T cells are primed in the same draining lymph nodes during tumor progression in fibrosarcoma bearing mice. Tregs in tumor draining lymph nodes were CD4⁺CD25⁺Foxp3⁺CD62L+ and expressed CTLA-4, GITR, LAG-3, LFA-1, and VLA-4. CD86 was also detected in a subpopulation of Tregs, which was involved in the suppressive activity of Tregs. Growth of weakly immunogenic fibrosarcoma tumor cells in the flank of mice led to increased Treg number in the tumor but not systemically. Immunization with tumor-associated self-antigens leads to development of +ly active CD4⁺CD25⁺Foxp3⁺Tregs, which is contributed to susceptibility of mice to tumor challenge.

Tregs in Cancer Patients

In 2001, CD4⁺CD25⁺ Tregs were reported in both the thymus and peripheral blood of healthy humans.[10,20,21] At the same time, CD4⁺CD25⁺ Tregs were observed in the circulation and within the tumor infiltrating lymphocytes of patients with early-stage non-small cell lung cancer or ovarian cancer.[28] Increased levels of CD4⁺CD25⁺ Tregs were also reported in the peripheral blood, tumor draining lymph nodes, and tumor tissues of patients with pancreas or breast adenocarcinoma. Increased populations of CD4⁺CD25⁺ Tregs were detected in the peripheral blood and tumor infiltrating lymphocytes of patients with gastric and esophageal cancers, and gastrointestinal malignancies. CD4⁺CD25⁺ Tregs were also reported in the circulation of melanoma patients immunized with melanoma antigens. In a later study, expression of FOXP3 was also examined and increased levels of CD4⁺CD25+Foxp3⁺ Tregs were observed in the lymph nodes of metastatic melanoma patients.[29] In ovarian cancer patients, CD4⁺CD25⁺FOXP3⁺ Tregs were found in the peripheral blood, malignant ascites, tumor tissue, and tumor draining lymph nodes.[30] Elevated levels of CD4⁺CD25⁺ Tregs were also reported in tumor draining lymph nodes of patients with cervical and endometrial cancer. Thereafter, CD4⁺CD25⁺ Tregs were reported in other types of cancers, including hepatocellular carcinoma, head and neck cancer, prostate cancer, colorectal cancer, renal cell carcinoma, Ewing sarcoma, and Merkel cell carcinoma. Increased levels of Tregs in Hodgkin lymphoma, chronic lymphocytic leukemia, B cell non-Hodgkin lymphoma, acute myeloid leukemia, acute myelogenous leukemia, and multiple myeloma have been reported in many studies.

Other Immunoregulatory T Cells in cancer patients

Tr1 cells

Immunosuppressive CD4+IL-10+ Tr1 cells and CD4+CD25+ T cells were observed at elevated proportions in lymph nodes of Hodgkins lymphoma patients. Tr1 cells were reported in head and neck squamous cell carcinoma patients, and their frequency was + in tumor infiltrating lymphocytes but not in the circulation.[31] Tr1 cells were also reported in the circulation of ovarian cancer patients after adoptive transfer of *in vitro* cultured T cells. However, in most of these studies, the +er levels of Tr1 cells in cancer patients compare to that of healthy individuals were after *in vitro* stimulation with or without tumor antigens plus Tr1-enhancing cytokines of patient's peripheral blood mononuclear cells. Nonetheless, Tr1 cells are appeared to be important in moderating antitumor immune responses in cancer patients, as in a melanoma tumor model, in which IL-10 expressed at tumor sites induced generation of immunosuppressive CD4+ T cells leading to systemic breakdown of antitumor immunity.

CD8+ Tregs

CD8+ Tregs were reported in patients with ovarian carcinoma, prostate cancer, colorectal cancer, malignant melanoma, hepatocellular carcinoma, and Merkel cell cancer. In ovarian cancer patients, accumulation of CD8+ Tregs was observed in ascites, draining LNs and peripheral blood. CD8+CD28- Tregs as well as CD4+CD25+ Tregs were reported in the peripheral blood of patients with lung cancer and pleural mesothelioma. Tumor infiltrating CD8+ Tregs are also observed in other cancers.

Trafficking/Migration of Tregs

Tregs may exist at the tumor sites due to trafficking, differentiation from naive or activated T cells, expansion, and/or preferential survival in the tumor microenvironment. Interactions between a number of chemokines/chemokine receptors and integrins/integrin receptors are involved in the trafficking of Tregs. Tregs were reported to express a variety of chemokine receptors such as CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CCR10, CXCR3, CXCR4, and CXCR5. In tumor studies, different chemokine receptors were found to be implicated in infiltration of Tregs into tumors. In ovarian cancer patients, Curiel and coworkers (2004) demonstrated that the chemokine CCL22, secreted by ovarian cancer cells and tumor associated macrophages, mediates migration of CCR4-expressing Tregs from the draining LNs toward the CCL22-rich tumor microenvironment.[30] Afterwards, recruitment of CCR4-expressing Tregs to tumors was reported in patients with Hodgkin lymphoma, and breast cancer. Increased frequencies of tumor infiltrating CCR4+ Tregs were found in patients with oral squamous cell carcinoma, and colon adenocarcinoma. Trafficking of CCR4+ Tregs toward tumor sites was also reported in other cancers, such as prostate, B cell non-Hodgkin lymphoma, breast cancer lung metastasis, and malignant pleural effusion. Specific recruitment of CCR4+ Tregs into the cerebrospinal fluid under the influence of CCL17 and CCL22 was also observed in lymphomatous and carcinomatous meningitis. Increase levels of CD4+CD25+ Tregs were reported in the bone marrow microenvironment in prostate cancer patients with bone metastasis. These Tregs expressed + levels of CXCR4. In a murine tumor model of pancreatic cancer, homing of Tregs into tumor was CCR5-dependent. CXCR3+ Tregs selectively accumulated in human ovarian carcinomas. Tumor infiltrating Tregs also express other chemokine receptors, for example CXCR4 in cervical cancer, CCR10 in ovarian cancer, CCR6 in mouse colorectal tumor, and CCR5 in

mouse skin tumor. In contrast, in one study in late stage ovarian cancer patients, tumor infiltrating Tregs expressed markedly low levels of CCR4 relative to circulating Tregs.[32]

In the tumor site, various chemokines were reported that could attract Tregs toward tumors. In multiple studies, CCL17, and CCL22 were reported to be involved in the migration of CCR4-expressing Tregs toward tumor tissue. CCL17 and CCL22 within the tumor microenvironment were associated with infiltration of Tregs into tumor tissue in gastric cancer, and esophageal squamous cell carcinoma. In the tumor microenvironments, different cells were major sources of CCL17 and CCL22, including tumor cells, tumor associated macrophages, tumor associated neutrophils, immature myeloid cells, and tolerogenic dendritic cells. Tumor associated neutrophils produced markedly +er levels of CCL17 in comparison with splenic or peripheral blood neutrophils and recruited Tregs to the tumor site in mouse and human.[33] CXCL12 expressed in the tumor tissue was correlated with infiltration of CXCR4-expressing FoxP3+ Tregs in cervical cancer. The levels of CXCL12 were +er in bone marrow fluid of prostate cancer patients with bone marrow metastasis than normal donors and CXCR4/CXCL12 signaling pathway was involved in the trafficking of Tregs to the bone marrow [34]. Hypoxia-induced secretion of CCL28 by ovarian tumor cells resulted in recruitment of CCR10-expressing Foxp3+ Tregs to the tissue. Increased production of CCL20 by tumor-associated macrophages was involved in recruitment of CCR6-expressing Tregs to mouse colorectal tumor tissue. Tumor infiltrated CCR5+ Tregs were appeared to be recruited to mouse skin tumors through CCL3, CCL4, and CCL5 produced by tumor infiltrating monocytic MDSCs. Tumor derived CCL5 recruited Tregs to tumors and enhanced TGF-β-mediated killing of CD8+ T cells in colon cancer. Ligands for CCR5 and CXCR3 were expressed in CD25-+ breast tumors. Other factors are also participated in trafficking of Tregs into tumors. VEGF derived from tumor was involved in recruiting of Tregs to melanoma tumor in mice and neuropilin-1 expressed on CD4+Foxp3+ Tregs was important in this process as its deficiency on Tregs impaired melanoma growth in mice.[35] These findings show that tumors can attract Tregs by secretion of various chemokine as well as other factors. In addition, + endothelial venules have been detected in some human breast tumors. Recently, it was shown that induction of MHC class II molecule expression in the vascular endothelium by IFN-γ and recognition of self antigens expressed by endothelium cells is involved in the trafficking of Tregs to target tissue, which may also be involved in the tumor tissue.

Tregs Proliferation

Observation of a rapid *in vivo* turnover rate in human CD4+CD25+Foxp3+ Tregs, and preservation of telomere length in CD4+CD25+ Tregs expanded *in vivo* point out an active proliferation of Tregs. There are also some evidences that may indicate proliferations of Tregs occur in cancers. FOXP3+GITR+ Tregs from head and neck squamous cell carcinoma patients were significantly more sensitive to apoptosis than non-Treg cells, which may indicate a rapid turnover in the peripheral circulation.[36] CD4+CD25+ Tregs from acute myeloid leukemia patients were less resistant to apoptosis but showed +er proliferation in compare to that of healthy individuals. CD4+CD25+ Tregs with +er apoptotic and proliferating status were detected in breast cancer patients. In addition, induction or expansion of Tregs was reported in response to some tumor vaccines both in mice, and cancer patients. Peripheral expansion of naive CD4+CD25+FOXP3+ Tregs was reported in patients with multiple myeloma. Ki-67, a nuclear protein expressed by proliferating cells, has been detected in tumor infiltrating Tregs. CD4+CD25+ Tregs

in the bone marrow microenvironment of prostate cancer patients exhibited active cell cycling as they expressed + levels of Ki-67 as well as multiple cell cycling genes. Moreover, tumor associated bone marrow dendritic cells promoted expansion of Tregs through RANK/RANKL signaling in vitro. Analysis of TCR repertoire in tumor infiltrating Tregs and effector T cells showed that each cell population has a distinct and skewed repertoire and TCR repertoire of Tregs was skewed toward some public sequences indicative of clonal expansion. These findings suggest that proliferation of Tregs may contribute to the increased frequencies of Tregs in cancer patients, although more investigations are required for demonstrating that cancers provoke proliferation of Tregs.

Tregs Differentiation from Naive or Activated T Cells

Tregs may differentiate from naive or activated T cells in the tumor microenvironment, tumor draining lymph nodes or other sites. Tumor-induced expansion of CD4$^+$CD25$^+$ Tregs in thymectomized, and anti-CD25-treated tumor bearing mice indicates that Tregs have been converted from CD25- T cells. Induction of conventional CD4$^+$ T cells conversion into Tregs by follicular lymphoma B cells has been contributed to accumulation of Tregs at the tumor tissue. In contrast, Hindley and colleagues by analysis of the TCR repertoires in tumor infiltrating conventional T cells and Tregs, concluded that there is no evidence for conversion of Tregs from conventional T cells in carcinogen-induced tumors because a significant overlap between TCR repertoires of the two cell population was not detected. Skewed TCR repertoire toward public sequences and distinct from TCR repertoire in CD4$^+$CD25- T cells was also observed in tumor infiltrating CD4$^+$Foxp3$^+$ Tregs in mouse melanoma. It should be noted that limited analysis of TCR repertoires might not show the differentiation of T cells from conventional T cells within tumors. Indeed, there are numerous evidences showing that tumor cells and tumor stromal cells can induce differentiation of Tregs. Induction of CD4$^+$CD25$^+$FOXP3$^+$ Tregs and IL-10$^+$ Tr1 cells by CD14$^+$HLA-DR-MDSCs from patients with hepatocellular carcinoma was observed.[37] Dendritic cells were frequently reported in the tumor sites, which can induce Tregs. Induction of tumor-specific Tr1 cells, and FOXP3$^+$ Tregs was reported to be mediated by mature dendritic cells. Tumor cells were reported to induce expression of TGF-β in immature myeloid dendritic cells and subsequent proliferation of CD4$^+$CD25$^+$ Tregs within tumor draining lymph nodes in mice with melanoma and rats bearing colon tumors. Plasmacytoid dendritic cells from ascites of ovarian cancer patients were able to induce CD8$^+$CD45RO$^+$CCR7$^+$IL-10$^+$ Tregs in vitro. Production of TGF-β and induction of CD4$^+$CD25$^+$Foxp3$^+$ Tregs by human dendritic cells under the influence of lung carcinoma cells was reported. Tumor associated plasmacytoid dendritic cells were able to induce differentiation of naïve T cells into IL-10 producing Tregs through ICOSL. Plasmacytoid dendritic cells from tumor draining lymph nodes activated Tregs via IDO in mouse melanoma. IDO-expressing leukemic dendritic cells impaired anti-leukemic immune responses by induction of Tregs in vitro. Plasmacytoid dendritic cell promoted immunosuppression in ovarian cancer patients, which was shown to be mediated by ICOS costimulation of FoxP3$^+$ Tregs. Monocyte-derived dendritic cells from breast cancer patients were believed to induce Tregs. Tumor associated macrophages can preferentially attract or induce Tregs and Th2 cells by secretion of chemokines, such as CCL17 and CCL22, and IL-10, respectively. CCL18 secreted by tumor-associated macrophages also recruit naïve T cells to the tumor microenvironment, which in turn can differentiate into Tregs. Other cell types in the tumor microenvironment can also participate in the differentiation of Tregs. Activation and induction of mouse allogeneic

Tregs by vascular endothelium has been shown, which may point to a possible role of tumor vascular endothelium in the induction of Tregs within the tumor.

Several factors have been shown to promote expansion or differentiation of Tregs, including IL-2, TGF-β, IDO, PGE2, and certain TLR ligands such as heat shock protein 60; a ligand for TLR2. Expression of some molecules such as TGF-β1 by tumor cells can be accountable for the increased levels of Tregs in tumor bearing hosts (our unpublished data). Tumor-derived TGF-β can induce CD4$^+$CD25$^+$ Treg differentiation from CD4$^+$CD25- T cells. Gene silencing of TGF-β1 in mouse B16 melanoma cells resulted in reduction of tumor associated Tregs and enhancement of dendritic cell vaccine-induced antitumor immunity (38). Various cells such as tumor cells, MDSCs, dendritic cells, Tregs, and Th3 cells can produce TGF-β. TGF-β is important in the development of iTregs after TCR-stimulation. TGF-β converts peripheral CD4$^+$CD25- naïve T cells to CD4$^+$CD25$^+$ Tregs through induction of Foxp3 expression and downregulation of Smad7. Indeed, tumor-derived TGF-β participates in tumor evasion of the immune system by various mechanisms, and converting CD4$^+$CD25- T cells into CD4$^+$CD25$^+$ Tregs is one of these mechanisms. Production of TGF-β and induction of CD4$^+$CD25$^+$Foxp3$^+$ Tregs by human dendritic cells under the influence of lung carcinoma cells have been reported. Tryptophan degradation by IDO is another immunosuppression mechanism of tumor cells. Modulation of tryptophan catabolism by human leukemic cells has been observed to convert CD4$^+$CD25- T cells into CD4$^+$CD25$^+$ Tregs. Tryptophan deviation induced inhibitory receptors ILT3 and ILT4 on dendritic cells and, in turn, these tolerogenic dendritic cells promote induction of CD4$^+$CD25$^+$Foxp3$^+$ Tregs. Upregulated expression of IDO1 in the tumor tissues was correlated with increased expression of FoxP3 in the tumor tissues in non-Hodgkin's lymphoma. IL-10- and TGF-β1-secreting CD4$^+$CD25+Foxp3$^+$ Tregs in the tumor microenvironment have also been suggested to induce conversion of naïve T cells into Tregs. The presence of IL-10 led to the development of Tr1 cells from conventional T cells during tumor growth in a B16 melanoma model. Elevated levels of IL-10 and Tregs were found in human papillomavirus-related cervical cancer. Inducion and expansion of Tr1 cells were reported in glioma and head and neck squamous cell carcinoma. Tumor COX-2/PGE2-dependent induction of FOXP3 expression and CD4$^+$CD25$^+$ Tregs activity was observed in lung cancer. In addition, tumor cells can actively release endosome-derived 50-100 nm exosomes, which in some instances have immunosuppressive functions. Exosomes carrying membrane-bound TGF-β were identified in cancer patients that skewed CD4$^+$ T cell responses in favor of Tregs.

Suboptimal antigen presentation and weak costimulation can convert conventional T cells into Tregs, which may also occur within tumors. Immunization with tumor associated self-antigens led to development of CD4$^+$CD25$^+$Foxp3$^+$ Tregs. Tumor antigen-specific Tregs were induced or expanded after tumor vaccination. Peptide-specific activation of CD8$^+$ Tregs was reported in tumors. Certainly, costimulatory molecules have an important role in the peripheral homeostasis of Tregs; for instance, it has been shown that CD28 was involved in the peripheral conversion of CD4$^+$CD25- T cells into CD4$^+$CD25$^+$ Tregs. Recently, PD-L1 has been shown to promote the induction and maintenance of iTregs as it enhanced and sustained Foxp3 expression and the suppressive function of iTregs [39]. PD-L1 is constitutively expressed on APCs, T cells, and some other cell types, suggesting that these cells can induce differentiation of Tregs through PD-1/PD-L1 interactions. Coinhibitory signaling through CTLA-4 and PD-L1 is required for the induction of Foxp3 expression in the

presence of TGF-β in T cells and generation of iTregs. In vitro, mouse splenic dendritic cells induced conversion of naïve antigen-specific CD4+ T cells into CD4+Foxp3+ Tregs in the presence of TGF-β. PD-L1 signaling is also required for the induction of Foxp3 expression in naïve CD4+ T cells *in vitro* or generation of tumor-induced iTregs in mice bearing melanoma tumor overexpressing chicken-OVA antigen. Overexpression of PD-L1 on some types of tumors has been reported and this overexpression is associated with poor prognosis in patients with hepatocellular carcinoma and ovarian cancer. PD-L1 is expressed by malignant T cells, dendritic cells within the tumor microenvironment, and peripheral blood monocytes in T cell non-Hodgikin's lymphoma patients and was contributed to promotion of T cell hyporesponsiveness and the induction of FoxP3+ Tregs; thus, tumors can induce Tregs through PD-1/PD-L1 interactions. On the other hand, conventional T cells upregulate PD-1 upon activation. This means activated T cells become susceptible for differentiation into Tregs by upregulation of PD-1. It should be noted that, expression of + levels of PD-1 and PD-L1 has been reported in Tregs. Elevated levels of PD-1 on Tregs may be indicative of their differentiated from activated T cells. Furthermore, expression of + levels of PD-L1 on Tregs confers the capability to induce differentiation of activated T cells into Tregs via PD-1/PD-L1 interactions. It has been shown that PD-L1 is induceable on activated intra-tumoral CD4+CD25+ Tregs isolated from B cell non-Hodgkin's lymphoma patients in vitro. In addition, PD-1 is constitutively expressed on intra-tumoral CD4+CD25- T cells in B cell non-Hodgkin's lymphoma patients. It has been reported that, PD-1 was also upregulate on T cells in malignant melanoma. Tumor infiltrating CD8+ T cells expressed + levels of PD-1, and notably; increased expression of PD-1 was correlated with an exhausted phenotype and impaired effector function in tumor antigen-specific CD8+ T cells. PD-1-dependent regulation of NY-ESO-1-specific CD8+ T cell expansion has been reported in melanoma cancer patients. Expression of + levels of PD-1 was also observed on tumor infiltrating Tregs. These findings suggest that activated T cells may differentiate into Tregs in the tumor site through PD-1/PD-L1 interactions.

Cell surface molecules expressed on tumor cells are also implicated in the induction of Tregs. CD200 (OX2), a transmembrane cell surface glycoprotein expressed by various cells, is involved in the induction of peripheral tolerance. Monoclonal antibodies to CD200 receptor enhanced inducion of CD4+CD25+ Tregs [40]. It has been reported that CD200 is overexpressed on acute myeloid leukemia blasts. In a recent study, increased levels of CD200 expression on acute myeloid leukemia blasts were correlated with increased frequency of Tregs. Moreover, blocking of CD200 using a humanized anti-CD200 antibody was able to reduce Treg frequency in patients with B cell chronic lymphocytic leukemia.[41]

Tregs Survival

There are conflicting data on the sensitivity of Tregs to apoptosis in cancer patients. Tregs from the peripheral blood of head and neck cancer patients and acute myeloid leukemia patients showed +er sensitivity to apoptosis. Tregs with +er apoptotic status were also reported from breast cancer patients. In contrast, Tregs from patients with metastatic epithelial cancer were more resistance to apoptosis-inducing stimuli than other lymphocyte subsets.

T cell apoptosis can be triggered by AICD or by ACAD. AICD is mediated by Fas/FasL interactions and ACAD is induced by cytokine deprivation. TCR-restimulation derives expression of FasL and subsequent T cell death. Tregs were reported to be +ly susceptible to FasL-mediated cell death but not to TCR-mediated

cell death, in contrast with effector T cells. Tregs expressed + levels of Fas and were susceptible to FasL-mediated apoptosis. TGF-β can play an important role in protection of T cells from FasL-mediated cell death. TGF-β inhibited FasL expression and subsequent AICD in conventional T cells. In addition to rescuing of conventional T cells from Fas-mediated cell death by inhibition of FasL expression during the shutdown-phase of an immune response, TGF-β could also exert prosurvival effect on T cells by induction of Bcl-xL after costimulation. In the tumor environment, Tregs might be resistant to apoptosis due to the anti-apoptotic effects of tumor-derive TGF-β and likely other factors. Moreover, FasL has been recently shown to be selectively expressed in the vasculature of human and mouse solid tumors, but not in normal vasculature. Tumor-derived VEGF-A, IL-10 and PGE2 induce expression of FasL in tumor endothelial cells, which could provoke death in effector CD8+ T cells, but Tregs are resistant to this death mechanism because of +er expression of c-FLIP in Tregs. Accordingly, FasL expression in tumor endothelium is associated with a predominant tumor infiltrating Tregs, while CD8+ T cell infiltration into these tumors is rare.

nTregs showed less sensitivity toward oxidative stress-induced cell death compared to conventional T cells from healthy individuals. As oxidative stress is appeared to be increased in tumors, this property of Tregs may provide an increased survival capacity within the tumor, which may accountable for the increased levels of Treg proportion in comparison with naive/activated conventional T cells at least in some types of cancer. Moreover, in a mouse model of brain glioma tumor, Tregs expressed + levels of heme-oxygenese-1 and +er expression of this enzyme was associated with increased survival of Tregs under hypoxic conditions. Thus, Tregs can use multiple mechanisms to survive in the tumor microenvironment.

Altogether, these findings show that the presences of Tregs at the tumor sites can be due to trafficking, de novo differentiation from conventional T cells, proliferation, and/or superior survival at the tumor microenvironment. Increased levels of Tregs have been observed in the peripheral blood of patients with ovarian cancer, lung cancer, pancreatic cancer, breast cancer, colorectal cancer, esophageal cancer, gastric cancer, head and neck cancer, hepatocellular cancer, renal cell cancer, melanoma, leukemia, chronic lymphocytic leukemia, lymphoma, and multiple myeloma. The + levels of Tregs in the peripheral blood may results in the increased levels of Tregs in the tumor, representing trafficking of Tregs from blood into tumors. Furthermore, expansion and differentiation of Tregs can occur either at tumor environment or lymphoid tissues, such as tumor draining lymph nodes. In accord with this, elevated levels of CD4+CD25+ Tregs were reported in tumor draining lymph nodes of patients with cervical and endometrial cancer, gastric cancer, colorectal cancer patients, breast cancer, and melanoma. Local microenvironment is important in the generation of iTregs. Suboptimal antigen presentation and/or weak costimulation may be responsible for induction of Tregs from conventional T cells within tumors. In addition, tumor cells and tumor stromal cells produce several factors such as TGF-β, IL-10, IDO, PGE2, VEGF, CD70, and galectin-1, which directly or indirectly induce differentiation and expansion of Tregs within the tumor or lymphoid tissues. Tregs may also have superior survival capacity than conventional T calls in the tumor microenvironment.

Immunophenotypical features of tregs in cancer patients

There are conflicting data on the immunophenotype of Tregs in cancer patients or mouse tumors. Several different cell surface markers have been investigated on Tregs in each study; accordingly

diverse phenotypes have been reported for Tregs in different studies. In addition, Tregs may be differently expressed these markers based on the type of cancer or stage of disease, and even there may be diversity in the immunophenotype of Tregs among patients with a same cancer. Nonetheless, these data are useful for our understanding of general phenotype of Tregs and also for assessing the activation status of Tregs as well as estimating function of Tregs.

It has been shown that CD4+CD25+ Tregs from patients with ovarian cancer or non-small cell lung cancer were CTLA4+ [28]. CD4+CD25+ T cells from the peripheral blood of patients with breast or pancreas cancer similar to CD4+CD25+ T cells from peripheral blood of normal donors expressed CTLA-4 and CD45RO on the cell surface. Tumor infiltrating Tregs were shown to express + levels of CTLA-4, PD-1, and CCR4. CD25+FOXP3+CTLA-4+CCR4+ Tregs were reported in colon adenocarcinoma patients. Foxp3+CTLA-4+GARP+ Tregs were reported in hepatocellular carcinoma patients. CD4+CD25+FOXP3+ Tregs from the peripheral blood and pleura effusion of patients with non-small cell lung cancer expressed + levels of CTLA-4 and GITR. CD4+CD25+ Tregs from renal cell carcinoma patients were GITR+CD45RO+. CD4+CD25+FOXP3+ from the peripheral blood of various cancers expressed GITR, intracellular CTLA-4, and CD45RA. In patients with head and neck squamous cell carcinoma, tumor infiltrating CD25+ Tregs were GITR+, IL-10+, and TGF-β+, while peripheral blood Tregs did not express GITR, IL-10, TGF-β, but expressed + levels of CD62L and CCR7. CD4+CD25+Foxp3+ T cells in the peripheral blood of patients with nasopharyngeal carcinoma, an Epstein-Barr virus-associated disease, overexpressed GITR. Tumor infiltrating T cells expressed + levels of CTLA-4 and GITR, but tumor infiltrating Tregs expressed the +est levels of these molecules. In hematologic malignancies; CD4+CD25+FOXP3+ Tregs were CTLA-4+GITR+ in B cell chronic lymphoid leukemia, CTLA4+ in B cell chronic lymphocytic leukemia ,CD45RAlowCD45RO+ and some were CTLA4+ in B cell non-Hodgkin lymphoma, GITR+CD62L+ Tregs in multiple myeloma, and CD4+CD25+CD127low Tregs in acute myeloid leukemia were reported. After *in vitro* expansion of tumor infiltrating T cells from prostate cancer patients, CD4+ T cell clones with suppressive function expressed CD25, FOXP3, GITR, CD122 (IL-2 receptor β chain), CCR4, and TLR8 and suppressive CD8+ T cell clones expressed CD25, FOXP3, CD122, and TLR8, but were negative for GITR and CCR4. Nonsuppressive CD4+ or CD8+ T cells clones did not express CD25 and FOXP3 molecules. LAG-3-expressing Tregs were detected in the peripheral blood and tumor sites of melanoma or colorectal cancer patients. Tregs from the peripheral blood of non-small cell lung cancer patients expressed + levels of TGF-β and CD39. Expression of CD39 was also detected on Tregs from patients with head and neck cancer, and tumor infiltrating CD8+ Tregs. Several other cell surface molecules have been reported in tumor infiltrating Tregs, such as ICOS on human melanoma-infiltrating CD4+CD25+Foxp3+ Tregs, TIM-3 in lung cancer patients, and various chemokine receptors as previously mentioned. Tumor infiltrating CD4+CD25+ Tregs from patient with primary or metastatic liver cancers expressed + levels of GITR, CTLA-4, ICOS, and HLA-DR compared with tumor-free liver Tregs or peripheral blood Tregs. Tim-3+ CD4 T cells isolated from patients with hepatocellular, cervical, colorectal, and ovarian carcinoma expressed +er levels of CD25, Foxp3, CTLA-4, and GITR than Tim-3- CD4 T cell counterparts. Most Tim3+ CD4 T cells isolated from the paired non-tumor tissues and peripheral blood did not express these molecules. Furthermore, tumor-derived Tim-3+ CD4 T cells, but not tumor-derived Tim-3- CD4 T cells, suppressed the proliferation of autologous CD8+ T cells in vitro. Most of molecules that are expressed in Tregs are also upregulated upon activation of conventional T cells. For instance, TIM-3 was expressed in activated human CD4+ T cells and regulated the expression of Th1 and Th17 cytokines. Also, expression of GARP was induced in human CD4+FOXP3+ Tregs upon *in vitro* stimulation, thus GARP expression was supposed to be useful for selectively discriminate activated human FOXP3+ Tregs. Expression of activation-induced molecules on Tregs may indicate that Tregs in cancer patients have activated phenotype or are differentiated from activated T cells. In support of this possibility, human CD4+FoxP3+CD45RA- T cells, and mouse Klrg1+ Tregs were reported to be terminally differentiated. Thus, Tregs or a fraction of Tregs in cancer patients may be terminally differentiating activated T cells. In some studies, Tregs at the tumor environment showed an effector phenotype and were appeared to have +er suppressive activity than circulating Tregs in patients with head and neck cancer, and acute myelogenous leukemia. In some mouse tumors, intra-tumoral Foxp3+ Tregs expressed + levels of the costimulatory molecule OX40 while CD4+Foxp3- T cells and CD8+ T cells expressed lower levels of this molecule. Expression of TNFR2 is also reported on a +ly suppressive subset of CD4+CD25+FoxP3+ Tregs in mouse tumor[42]

Tregs from the peripheral blood of renal cell carcinoma patients expressed Helios.[43] Expression of Helios was also detected in Tregs accumulated in human ovarian carcinomas. In a xenogeneic mouse model of malignant human brain tumor, the majority of tumor associated Tregs expressed Helios, but their frequency decreased when thymectomy was done before tumor implantation, thus it was concluded that thymus-derived, rather than tumor-induced, Tregs, are predominant in the brain tumors. In another study, however, expression of Helios was detected in peripherally induced Foxp3+ Tregs. In a murine colon adenocarcinoma model, most of tumor infiltrating Foxp3+ Tregs expressed low levels of Helios and neuropilin-1. Increased presence of Foxp3+ Tregs expressing low levels of neuropilin-1 was reported in the tumor tissue, while spleen Tregs were predominantly neuropiline-1+.

There are a few reports on immunophenotype of CD8+ Tregs in cancer patients. CD8+ Tregs in the peripheral blood, ascites, and tumor draining lymph nodes of ovarian cancer patients and also Tregs from the peripheral blood of healthy donors were CD45RO+CCR7+. In prostate cancer patients, CD8+CD25+FOXP3+ Tregs expressed CD122, and partly GITR. In colorectal cancer patients, CD8+CD25+FOXP3+ Tregs had increased expression of CTLA-4, GITR, and TGF-β in comparison with Tregs isolated from healthy individuals.

Roles of Tregs in Tumor/Cancer

T cells recognizing self-antigens are present in peripheral tissues and their activity can result in severe autoimmune disorders. But, various peripheral tolerance mechanisms are in work to suppress these potentially autoreactive T ells and also effector immune cells to avoid immune-mediated damages. Tregs are the most studied immune cells with immunosuppressive function. These cells have a crucial role in maintaining peripheral tolerance to both auto-antigens and foreign antigens [8]. As most tumor-associated antigens are self-antigens, it can be postulated that Tregs are participated in tolerance against tumor cells bearing self-antigens. Tregs are also able to suppress antitumor function of effector immune cells. However, it is not well known whether the presence of Tregs in the tumor sites per se leads to tumor growth. Identification of Treg activities, their tumor antigen specificity, as well as their frequency in tumor bearing animals and cancer patients may illuminate effects of Tregs in cancer development/progression. Assessing the correlation between Treg levels and disease outcome in cancer patients can also point out the roles of Tregs in cancer.

Mechanisms of Immunosuppression Mediated by Cancer Associated Tregs

Induction of immunosuppressive CD4+ T cells (Tr1 cells) and suppression of antitumor immunity was associated with IL-10 expressed at early tumor tissue. Suppressive activity of CD4+CD25+CTLA4+ Tregs from ovarian and non-small-cell lung cancer patients was partly mediated by TGF-β. CD4+CD25+ Tregs from patients with pancreatic cancer or breast cancer, expressed CTLA-4, IL-10, and TGF-β. *In vitro* inhibition of cytolytic T lymphocyte proliferation by autologous CD4+CD25+ Tregs derived from a colorectal carcinoma patient was reported to be mediated by TGF-β. IL-10-secreting Tr1 and CD4+CD25+ Tregs were found in Hodgkin lymphoma infiltrating lymphocytes and peripheral blood mononuclear cells and their suppressive function mediated by IL-10 secretion, cell-to-cell contact, and CTLA-4. Tregs from tumor draining lymph nodes of Hodgkin's lymphoma suppressed T cells via CTLA-4 and IL-10. CD4+CD25+ Tregs in the circulation of chronic lymphocytic leukemia suppressed T cells through CTLA-4. The suppressive activity of CD4+CD25+Foxp3+CD62L+ Tregs from tumor draining lymph nodes of fibrosarcoma bearing mice was mediated via CTLA-4 and CD86. Increased frequencies of IL-10 producing CD4+CD25+ Tregs were strongly associated with disease stage in gastro-esophageal cancers. Circulating IL-10 producing Tregs with specificity to tumor associated antigen-specific Tregs have also been identified in metastatic melanoma patients.[44] Tregs isolated from the spleen of tumor bearing rats inhibited *in vitro* T cell immune responses against immunogenic tumor cells through a cell-to-cell contact mechanism which was dependent on TGF-β but not IL-10, and delayed *in vivo* rejection of immunogenic tumors. In a mouse melanoma model, CD4+CD25+ Treg-mediated suppression of antitumor immunity was not mediated through IL-10. CD4+CD25+ Tregs from tumor bearing mice were able to suppress NK cell activity via a TGF-β-dependent manner. CD4+CD25+ Treg-mediated suppression of the cytotoxicity of tumor specific CD8+ T cells in a murine transgenic colon carcinoma model was also appeared to be through TGF-β signaling. Intra-tumoral CD4+CD25+ Tregs isolated from B cell non-Hodgkin's lymphoma patients suppressed *in vitro* proliferation, and cytokine (IFN-γ and IL-4) production of infiltrating CD4+CD25- T cells. PD-L1 was induced on *in vitro* activated intra-tumoral CD4+CD25+ Tregs in B cell non-Hodgkin's lymphoma patients. Besides, PD-1 was constitutively expressed on intra-tumoral CD4+CD25- T cells and blocking the interaction between PD-1/PD-L1 partly attenuated the suppressive effect of Tregs on CD4+CD25- T cells. CD4+CD25+FOXP3+ Tregs isolated from the peripheral blood of cancer patients, upregulated the expression of FasL and inhibited the proliferation of CD8+ responder T cells through a Fas/FasL-mediated apoptosis but the suppression of CD4+ responder T cell proliferation was independent of Fas/FasL mechanism. iTregs from colorectal cancer patients was reported to secrete PGE2, which can suppress T cell responses. In prostate cancer patients, CD8+ Tregs mediated immunosuppression via both cell-to-cell contact and soluble factors other than TGF-β and IL-10. The suppressive function of CD8+ Tregs could be reversed by human TLR8 signaling. Interactions of CTLA-4 on Tregs with the costimulatory molecules CD80/CD86 on dendritc cells can maintain dendritic cells in an immature phenotype, and induce expression of IDO in dendritic cells. IDO-expressing leukemic dendritic cells induced Tregs, which impaired leukemia-specific immune responses. In colon cancer, Tregs that recruited to tumors by tumor derived CCL5 killed CD8+ T cells via TGF-β. Tregs from head and neck squamous cell carcinoma showed increased ectonuleotidase expression and activity. CD39 expressed on Tregs inhibited NK cell activity and promoted hepatic metastatic tumor growth in mice. Increased expression of CD39 on CD4+ T cells was associated with poor prognosis in chronic lymphocytic leukemia. CD39 was also involved in suppressive activity of tumor infiltrating CD8+ Tregs. Perforin and granzyme B were reported to be involved in the Treg-mediated suppression of antitumor immunity. Foxp3+ Tregs induced perforin-dependent death of dendritic cells in the tumor draining lymph nodes. Deficiency of neuropilin-1 on CD4+Foxp3+ Tregs resulted in impaired tumor growth in mouse melanoma. It is appeared that several other molecules are also participated in the immunosuppressive function of Tregs.

Other Activities of Tregs in Tumors

A potential role for T cells in tumor angiogenesis was proposed by observation that human peripheral blood T cells and cancer infiltrating lymphocytes can express VEGF.[45] Subsequently, tumor infiltrating Tregs were associated with VEGF overexpression and intratumoral angiogenesis in breast, and endometrial cancers. Tregs suppressed IFN-γ-dependent antiangiogenic effects of Th1 effector cells. Furthermore, Tregs that recruited into tumor tissue under the effect of tumor hypoxia-induced CCL28 promoted tumor angiogenesis and tumor growth in ovarian cancer. Mouse CD4+CD25+ Tregs suppressed osteoclast differentiation induced by activated T cells or recombinant RANKL and M-CSF in vitro. In mice inoculated with human prostate cancer into bone marrow, adoptive transfer of Tregs led to increased bone mineral intensity, while depletion of Tregs reduced bone density in xenograft mouse models. Because increased levels of CD4+CD25+ Tregs were found in the bone marrow microenvironment of prostate cancer patients with bone metastasis, these results may indicate that Tregs can suppress osteoclast differentiation or function in prostate cancer patients with bone metastases. In a breast cancer model, tumor infiltrating Tregs stimulated metastasis of cancerous cells to the lung. Metastasis was mediated through RANKL-RANK signaling as blockade of RANKL diminished metastasis. Furthermore, loss of Foxp3 expression and acquisition of effector functions has been observed in Tregs. Recent evidences on diversity of Treg functions and homeostatic properties in different contexts indicate that Tregs can become specialized for different environmental conditions.[46] Thus, Tregs may have many unidentified roles in the +ly complicated tumor environment.

Tumor Antigen Specificity in Tregs

So far, in numerous studies + frequencies of Tregs have been reported in cancer patients or tumor bearing mice, however, antigen specificity of these Tregs has not been illustrated. Thus, there is a paucity of data addressing the tumor specificity of Tregs in tumor bearing animals and cancer patients. An evidence for existence of tumor antigen-specific Tregs was firstly provided by observation of Treg clones specific for the tumor antigen LAGE-1, a cancer/testis antigen, which were generated from tumor infiltrating lymphocytes from melanoma patients.[47] Tumor-derived ARTC1 peptide-specific CD4+ Treg clones were also generated from tumor infiltrating T cells. Furthermore, tumor antigen-specific CD4+ Tregs were reported from patients with melanoma, human papillomavirus related-cervical cancer, acute myeloid leukemia, and colorectal cancer. Increased frequencies of circulating CD8+ Tregs specific for heme oxygenase-1 were identified in patients with melanoma, renal or breast cancers and these Tregs exhibited a stronger suppressive function than CD4+CD25+ nTregs. Recently, tumor-specific CD4+ Tregs and CD8+ Tregs were detected in patients with Merkel cell cancer, which is a virally induced, rare but +ly malignant skin cancer. In addition, tumor antigen-specific Tregs were induced or expanded in response to cancer vaccines in murine models as well as in patients with human papillomavirus-related cervical cancer, and melanoma.

Peptide-specific activation of CD8^{+} Tregs was found in tumors. Antigen priming was appeared to be important for accumulation of iTregs at the tumor sites. Accumulation of iTregs possessing TCRs restricted to a defined antigen expressed by tumor cells within the tumor microenvironment was reported, which was associated with suppression of CD4^{+} T cell responses to tumor vaccine. Analysis of TCR repertoires in Tregs and CD4^{+}CD25- conventional T cells infiltrated to mouse carcinogen-induced fibrosarcoma tumors, as well as melanoma tumors showed that the TCR repertoires are distinct between the two cell populations. These finding show that Tregs can express tumor antigen-specific TCRs. Nevertheless, tumor associated/ specific antigens have not been identified in most types of cancers; thus, evaluation of tumor specificity of Tregs in patients with these cancers is difficult.

Correlation between Increased Frequency of Tregs and Disease Outcome in Cancer Patients

In multiple studies, + frequencies of Tregs were correlated with poor prognosis and decreased survival rates in patients with esophageal, gastric, or intestinal cancers.[48,49] Increased frequencies of Tregs or Foxp3^{+} cells were also correlated with tumor burden or tumor growth in esophageal and gastric cancer patients. But, infiltrating Foxp3^{+} cell numbers were appeared to not be a predictive factor for patient's survival in esophageal squamous cell carcinoma in another study.[50] In ovarian cancer patients, CD4^{+}CD25^{+}FOXP3^{+} Tregs were found in the peripheral blood, malignant ascites, tumor tissue, and tumor draining lymph nodes and increased frequencies of Tregs were associated with reduced survival.[30] In ovarian tumor tissue, a + CD8^{+} cell/Treg ratio was associated with favorable prognosis and increased levels of FoxP3 expression, assessed by RT-PCR, were associated with poor prognosis. In other studies, increased levels of Tregs were correlated with worse disease outcome or poor survival in patients with pancreatic ductal adenocarcinoma,[51] breast cancer, hepatocellular carcinoma, renal cell carcinoma, cervical cancer, and non-small cell lung cancer. Elevated levels of Tregs in the peripheral blood of patients with head and neck squamous cell carcinoma were associated with a worse prognosis. Low ratios of CD8^{+} T cell/CCR4^{+} Treg in the tumor tissue of oral squamous cell carcinoma patients were associated with worse survival. Glioblastoma patients with +er density of Foxp3^{+} cells in the tumor tissue showed relatively shorter progression-free survival and overall survival.[52] Foxp3^{+}CD8^{+} Tregs were contributed to disease progression in patients with prostate and colorectal cancers. Increased liver-infiltrating FoxP3^{+}CD8^{+} Tregs were associated with tumor stage in patients with hepatocellular carcinoma. In patients with primary melanoma, low levels of FOXP3 expression in melanoma tissues were associated with better disease-free survival and overall survival. In contrast, in some studies increased levels of tumor infiltrating Tregs were associated with favorable prognosis in cutaneous malignant melanoma, head and neck cancer, gastric cancer, colorectal cancer, ovarian cancer, and breast cancer. Stage IV melanoma patients with low rate survival had low percentages of CD4^{+}CD25^{+} Tregs in the peripheral blood.[53]

Increased frequencies of CD4^{+}CD25^{+} Tregs were correlated with worse disease outcome in some hematologic malignancies, such as myelodysplasic syndrome, acute myeloid leukemia, and multiple myeloma. Lower levels of Tregs in the peripheral blood were also associated with long-term survival in multiple myeloma patients. In contrast, increased levels of Tregs were associated with improved survival in patients with Hodgkin's lymphoma, follicullar lymphoma, diffuse large B-cell lymphoma, and cutaneus T cell lymphoma.[54]

Upon curative surgery, the increased frequencies of CD4^{+}CD25+ Tregs were significantly reduced in gastric and esophageal cancer patients. But, frequencies of Tregs were again increased in patients having a relapse after tumor resection showing a correlation between increased Tregs and cancer relapse in these types of cancers. Increased numbers of tumor infiltrating Tregs were associated with adverse prognosis in resectable gastric cancer. High CD8^{+} cell/FOXP3^{+} cell ratio in the tumor tissue was correlated with disease-free survival and overall survival in colorectal cancer patients after curative resection. Increased levels of Tregs in the peripheral blood of patients with non-small cell lung cancer were correlated with advanced stage disease. Percentage of CD4^{+}CD25^{+}FOXP3^{+} and CD8^{+}CD28- Tregs in the peripheral blood of non-small cell lung cancer patients increased with tumor stage and markedly reduced after surgery. Increased tumor infiltrating Foxp3^{+} Tregs were associated with a worse survival rate and increased chance of relapse in non-small cell lung cancer patients after curative resection. Intra-tumoral balance of Tregs and cytotoxic T cells has been associated with prognosis of hepatocellular carcinoma after resection. The presence of low Foxp3^{+} cell density in combination with +-activated CD8^{+} cell density in the tumor tissue was associated with improved disease-free survival and overall survival, while + density of Foxp3^{+} cells and low density of CD8^{+} cells was associated with disease progression and worse patient's survival after curative hepatic resection. The disease-free survival rate was lower in patients with + numbers of Foxp3^{+} cells in the tumor tissue than in patients with low Foxp3^{+} cells in hepatocellular carcinoma patients after curative resection. Decrease in Tregs was observed after resection of tumors in kidney transplant recipients. Tregs in lymphoid infiltrates surrounding primary breast tumors were correlated with an adverse clinical outcome. Infiltration of CXCR4-expressing FoxP3^{+} Tregs was correlated with human papillomavirus infection and progression of cervical cancer. The levels of circulating Tregs in advanced melanoma are +er than minimal residual disease. But, in advanced melanoma patients received + dose IL-2 in combination with gp-100 peptide vaccine, patients who showed better response to the treatment had +er frequencies of Tregs. In a clinical trial, low frequency of FOXP3^{+} Tregs prior to vaccination with tumor associated peptides for renal cell cancer, was correlated with better T cell responses and disease control in vaccinated patients.[55]

It has been shown that Tregs are associated with tumor growth in several murine tumor models. In a chemically induced tumor model, progressively growing tumors contained +er percentages of Tregs than rejecting tumors [56]. In a murine model of pancreatic cancer, disruption of homing Tregs inhibited tumor growth. In a murine transgenic model of prostate dysplasia, increased levels of peripheral blood Tregs were correlated with tumor progression. Tumor associated macrophages-mediated recruitment of Tregs into colorectal tumor tissue was also related to promotion of colorectal cancer development in mice. Furthermore, Tregs infiltrated to skin tumors, which was appeared to be mediated by tumor infiltrating monocytic MDSCs, favored tumor growth in mice.

Controversial Aspects of Tregs in Cancer Development/ Progress

As disserted in the previous section, Tregs have been found at + levels in patients with various types of malignancies and in most cases they were associated with poor prognosis, but in some reports increased levels of Tregs were associated with improved survival. In breast cancer patients, increased levels of Tregs were associated with worse clinical outcome in many studies. However, in a study it has been

shown that in the hormone-negative group of breast cancer patients, FOXP3 mRNA expression levels were not correlated with patient's survival. In another study, the percentages of CD4+CD25+ cells in the total CD3+ or CD4+ cells in the peripheral blood of breast cancer patients were not +er than that in healthy individuals. But, +er percentages of CD4+CD25+ cells in the total CD3+ or CD4+ cells were detected in the peripheral blood of patients with recurrent non-small cell lung cancer than that in healthy individuals. Likewise, there was no significant correlation between the densities of Foxp3+ cells in the tumor tissues and disease-free/overall survival in triple-negative breast cancer patients.[57] In contrast, + numbers of FOXP3+ T cells were associated with favorable prognosis in estrogen receptor-negative breast cancer, however, tumor infiltrating FOXP3+ T cells were strongly associated with tumor infiltrating CD8+ cells. As tumor infiltrating CD8+ cells have been correlated with good prognosis in breast cancer, it is possible that CD8+ T cells have been implicated in the favorable prognosis in estrogen receptor-negative breast cancer patients. Presence of + numbers of FOXP3+ cells in the tumor tissue was also associated with improved patient's survival in triple-negative breast cancer patients.

In patients with head and neck squamous cell carcinoma, increased levels of tumor infiltrating Tregs were associated with favorable prognosis. Interestingly, in one report, increased frequency of tumor infiltrating CD8+ T cells and +er CD8+ cell/Treg ratio was contributed to the better clinical outcome in tonsillar squamous cell carcinoma patients. High levels of Tregs and low ratio of CD8+ T cell/Treg in the peripheral blood of patients with human papillomavirus-related head and neck squamous cell carcinoma were also associated with a better survival. In contrast, in another study, elevated levels of Tregs in the peripheral blood of patients with head and neck squamous cell carcinoma were associated with a worse prognosis. In oral squamous cell carcinoma patients, + levels of CCR4+Tregs and low levels of CD8+ cells in the tumor tissue were associated with worse patient' survival. Glioblastoma patients with +er density of Foxp3+ cells in the tumor tissue showed relatively shorter progression-free survival and overall survival. Patients with +er density of CD8+ cells showed no significant differences in survival.[52] In other study, FoxP3+ cells were found in tumor tissues of glioblastoma patients but not in low-grade astrocytoma or oligodendroglial tumors. There was no significant association between FoxP3+ cells and patient prognosis, but + level of CD4+ cells combined with low level of CD8+ cells was associated with poor prognosis in glioblastoma patients.

In colorectal cancer patients, Tregs were associated with favorable prognosis in most studies, but not in others. Increased numbers of Tregs compared to surrounding healthy mucosa have been detected in colorectal cancers. In one of these studies, Treg infiltration was +er in colorectal cancer tissue than in healthy colon. Treg infiltration in the tumor tissue was significantly +er in limited colorectal cancer than metastatic colorectal cancer. However, Treg infiltration was not correlated with survival. There was also no association between Treg/CD8+ T cell ratio and survival.[58] Another report suggests, in colorectal cancer patients, a FOXP3 mRNA expression level is not correlated with survival.[59] In patients with stage II and III colon carcinomas treated with adjuvant chemotherapy, the density of FoxP3+ cell infiltration was similar in the tumor stroma and epithelia and the impact of FoxP3+ cells on survival is dependent on CD8+ density. High density of CD8+ cells was correlated with favorable survival regardless of the level of FoxP3+ infiltration. Tumor infiltrating FoxP3+ cells was not prognostic when + density of CD8+ T cells is detected within tumors, while patients harboring tumors infiltrated with + density of FoxP3+ cells and low density of CD8+ cells had

significantly improved overall survival rate. The +est survival rate was observed when tumors had + density of either FoxP3+ cells or CD8+ cells, and the survival rate was lowest when the density of both cell populations was low. In contrast, in other studies Tregs accumulated in the tumor tissues were reported to be correlated with tumor progression in colorectal cancer patients. The frequency of CD4+CD25+Foxp3+ Tregs was also significantly +er in tumor draining lymph nodes than that in peripheral blood but lower than that in tumor tissues of colorectal cancer patients. Reports show that number of FOXP3+ cells in the colorectal tumor tissue is positively correlated with lymph node metastasis and + ratio of CD8+ cell/FOXP3+ cell in the tumor tissue was correlated with disease-free survival and overall survival in patients after curative resection.[60]

In hematological malignancies, the presence of Tregs is also controversial. In patients with myelodysplasic syndrome, progression to more aggressive disease is in accordance with increased frequencies of CD4+CD25+FOXP3+ Tregs [61]. Increased levels of Tregs were correlated with advanced disease stage in patients with multiple myeloma, and B cell chronic lymphoid leukemia. An increased frequency of CD4+CD25+ Tregs is associated with disease relapse after allogeneic stem cell transplantation for chronic myeloid leukemia. CD4+CD25+ Tregs are associated with poor prognosis in patients with acute myeloid leukemia. Increased frequency of CD4+CD25+FOXP3+ Tregs in the peripheral blood of multiple myelomas is associated with low survival rate. Study shows that in multiple myeloma patients, lower levels of Tregs and +er levels of Th17 cells in the peripheral blood were associated with long-term survival. In contrast, low frequencies of FOXP3+ cells and + frequencies of cytotoxic T lymphocytes in the lymph nodes of Hodgkin's lymphoma patients are correlated with poor overall survival.[62] In other report, increased levels of intra-tumoral FOXP3+ cells were associated with increased survival in follicular lymphoma, germinal center-like diffuse large B cell lymphoma, and Hodgkine's lymphoma. It should be noted that, these FOXP3+ cells were not confirmed to be Tregs by assessing CD25 expression or by functional assays. In several other studies, + numbers of tumor infiltrating FOXP3+ Tregs were associated with improved survival in different lymphoma patients. Reduced numbers of Tregs were reported in patients with Sézary syndrome, a variant of cutaneous T cell lymphoma, which was appeared to be accountable for the more aggressive nature of Sézary syndrome campared to other cutaneous T cell lymphomas. Moreover, observation of dysfunctional Tregs in multiple myeloma, and lack of suppressive CD4+CD25+FOXP3+ T cells in advanced stages of primary cutaneous T cell lymphoma indicates a beneficial role of Tregs in these types of malignancies.

Tregs are capable to have different functions depending on signals that they receive from the microenvironment. Thus, Tregs may have diverse activities depend on their localization regions in cancer patient. In breast cancer, a + density of Tregs within lymphoid infiltrates surrounding primary tumors was associated with an adverse clinical outcome, while a + density of Tregs distributed elsewhere in the tumor exhibited no association with disease outcome. In colorectal cancer, a + density of Tregs within tumor tissue was correlated with improved outcome, while elevated Treg density within normal mucosa was associated with poor prognosis. Thus, evaluation of Tregs in different areas of a cancer tissue including the tumor bed, stroma, aggregates of lymphoid cells, and the normal tissue adjacent to malignant region is recommended for determining their prognostic significance in various types of cancer.

The relative proportion of Tregs to effector T cells or total T cells and its correlation with disease outcome has been studied in various

cancers. A + CD8^{+} cell/Treg ratio was associated with favorable prognosis in ovarian cancer patients.[63] The presence of Foxp3^{+} cells in the tumor tissue was not associated with disease prognosis when assessed independently of total tumor infiltrating CD3^{+} cells in early stage-non-small cell lung cancer patients; but a +er Foxp3^{+} cell/CD3^{+} cell ratio was associated with cancer metastasis and worse patient's survival. Lack or low level of CD3^{+} cells in the tumor tissue was associated with a + risk of disease recurrence after curative resection. In one study on colorectal cancer, Treg infiltration was not correlated with survival. Accordingly, there was no association between Treg/CD8^{+} T cell ratio and patient'survival,[58] however, in colorectal cancer patients after curative resection, + ratio of CD8^{+} cell/FOXP3^{+} cell in the tumor tissue was correlated with disease-free survival and overall survival [60]. Increased levels of tumor infiltrating Tregs and + Foxp3^{+}/CD8^{+} ratio were associated with adverse prognosis in resectable gastric cancer. Increased numbers of intraepithelial-tumor infiltrating Tregs and a low CD8^{+} T cell/Treg ratio were associated with worse survival in cervical cancer patients. Low ratios of CD8^{+}/Treg in the tumor tissue, but not total Tregs, were associated with worse survival of oral squamous cell carcinoma patients. Patients with advanced stage breast cancer had +er levels of CD4^{+}CD25^{+} Tregs and a lower ratio of CD4^{+} T cell/Treg in the peripheral blood in comparison with stage I, II, or III breast cancer patients. Increased density of Foxp3^{+} cells and + ratio of Foxp3^{+} cells to CD4^{+} or CD8^{+} cells were also associated with unfavorable clininicoopathological parameters. Interestingly, increased density of Foxp3^{+} cells and + ratio of Foxp3^{+} cells to CD4^{+} or CD8^{+} cells in the tumor periphery were correlated with decreased patients' 5-year disease-free survival rate, while no association was found between Foxp3^{+} cell density or Foxp3^{+} cell/CD4^{+} or CD8^{+} cell ratios in the tumor bed and disease free survival. Increased frequency of tumor infiltrating CD8^{+} T cells and +er CD8^{+}/Treg ratio was contributed to the better clinical outcome in tonsillar squamous cell carcinoma patients. High levels of Tregs and low ratio of CD8^{+} T cell/Treg in the peripheral blood of patients with human papillomavirus-related head and neck squamous cell carcinoma were associated with a better survival. CD4^{+}CD25^{+}FoxP3^{+} Treg/CD4^{+}CD25- T cell ratio was related to immunosuppression in patients with B cell acute lymphoblastic leukemia. Therefore, assessing the relative proportion of Tregs to effector T cells in the tumor infiltrate, lymph nodes, and peripheral blood and their correlation with disease outcome is a valuable prognostic factor for different cancer patients.

It is important to note that some epithelial cells also express Foxp3 that is used as a specific marker for the detection of Tregs in tumor tissues. In a recent study, + density of Tregs in gastric cancer predicts a poor prognosis, but expression of FoxP3 protein in gastric cancer cells predicts better survival.[64] Thus, assessing the expression of Foxp3 may not be solely indicative of Tregs in tumor tissues.

Beneficial Effects of Tregs during Inflammation-Induced Carcinogenesis

Tregs may have diverse effects on carcinogenesis and cancer progress. Results of numerous studies in cancer patients and tumor models show that Tregs are involved in cancer progression. However, recent findings also indicate that Tregs can have a protective role during carcinogenesis. In some conditions, chronic inflammation and immune responses promote cancer development.[65] Chronic inflammation is linked to cancer development and progression in a mouse model of colon cancer, and prostate cancer. Induction of genetic instability and alterations by cancer-related inflammatory mediators is recently proposed. In the tumor microenvironment, inflammation

may contribute to proliferation and survival of malignant cells, angiogenesis, and metastasis.[66]

Diverse innate immune cells such as NK cells, dendritic cells, macrophages, mast cells, neutrophils, and eosinophils are found in tumor microenvironment.[67] These cells have critical roles during inflammation, but their function may also lead to tumor immune evasion. Dendritic cells, the most potent antigen presenting cells, in the tumor microenvironment fail to stimulate T cells. As previously discussed, Tregs can modulate dendritic cells. B cells have been associated with both stimulation and inhibition of tumor growth. It has been proposed that carcinogenesis promoted by chronic inflammation is B cell dependent as they have a major role in recruiting innate inflammatory cells to tumors. CD4^{+}CD25^{+} Tregs directly suppressed B cell immunoglobulin responses. CD4^{+}CD25^{+} Tregs also killed antigen presenting B cells in coculture experiments. In an *in vitro* experiment, it was found that human CD4^{+}CD25^{+} Tregs induced monocyte differentiation toward alternatively activated macrophages with potent antiinflammatory potential that may promote tumor growth. Human CD4^{+}CD25^{+} Tregs were reported to be capable to inhibit lipopolysaccharide-induced monocyte survival; but Tregs were also suggested to be capable to suppress immune cells such as macrophages that are involved in the tumor progression.

It has been recently suggested that control of inflammation may prevent tumor development or progression in colon.[68] Therefore, it is assumed that microbial flora-induced Tregs can modulate inflammation and subsequent carcinogenesis in colorectal tract, as Tregs has been proposed to suppress proinflammatory and tumor promoting Th17 responses against gut microbiota in cancers of colorectal, and even oral cavity. There are some evidences supporting protective roles of Tregs during carcinogenesis. Increased levels of Tregs are found in tissues with inflammation, as increased levels of Tregs have been reported in inflammatory bowel disease. iTregs were essential for tolerance induction in experimental colitis. Adoptive transfer of CD4^{+}CD25^{+} Tregs inhibited development of microbially induced colon cancer in Rag2-deficient mice. CD4^{+}CD25^{+} Tregs also induced regression of intestinal tumors in ApcMin/[+] mice. In CEA-transgenic mice, adoptive transfer of Tregs possessing CEA-specific chimeric antigen receptors suppressed the severity of induced colitis and hindered colitis-associated colorectal cancer development.[69] Thus, Tregs can be beneficial in the control of inflammation-induced carcinogenesis by suppression of immune responses and inflammation. On the other hand, tumor volume is correlated with expansion of CD4^{+}CD25^{+} Tregs in lymphoid tissues in a rat tolerogenic tumor model of colon carcinoma. Tregs isolated from the spleen of tumor bearing rats inhibited *in vitro* T cell immune responses against immunogenic tumor cells and delayed *in vivo* rejection of immunogenic tumors. In addition, recruitment of Tregs into colorectal tumor tissue is mediated by tumor-associated macrophages, which led to promotion of colorectal cancer development in mice. These findings suggest that Tregs can promote cancer growth.

Plasticity of Tregs in Cancer Setting

TCR stimulation, various cytokines, metabolic factors and other signaling agents can affect the phenotype and function of Tregs. Indeed, Tregs have the potential to adapt to their environmental conditions based on signals that they receive from other cells, cytokines or other factors, and consequently acquire specialized phenotype and function.[70] On the other hand, Tregs can lose Foxp3 expression and differentiate into effector T cells. Recent findings indicate that Treg plasticity is attributed to a population of Foxp3-expressing conventional T cells.[11] These Foxp3^{+} T cells are presumably are differentiated iTregs that do

not undergo the Treg-specific epigenetic modulations such as DNA hypomethylation in the Foxp3 locus.

Continued expression of Foxp3, as well as epigenetic modifications, is necessary for maintenance of transcriptional and functional program in mature Tregs.[71] Loss of Foxp3 expression and acquisition of effector functions has been observed in Tregs *in vivo*, although another study indicated stability of Treg linage *in vivo*. These diverse observations may be due to the fact that the stability of Foxp3 expression is different between committed Tregs and uncommitted Tregs. Most naturally occurring, thymic, Tregs are committed Tregs as they have relatively stable Foxp3 expression, while uncommitted Tregs, including recently-peripherally induced Tregs, can show plasticity upon exposure to certain stimuli.

Some extrinsic signals are important in controlling Foxp3 expression and there are evidences emphasizing that Tregs can differentiate into effector T cells such as Th1 cells, Th17 cells, and follicular B helper T cells. Differentiation of TGF-β induced-iTregs into Th1-like cells was reported.[72] TCR restimulation in iTregs, from apoptosis-defective mouse strains, resulted in loss of Foxp3 expression, defective suppressive function, and production of Th1-fating effector cytokines.[72] TGF-β inhibited FasL expression and subsequent AICD in conventional T cells as well as restimulated iTregs. In addition, TGF-β limited iTreg conversion into effector T cells after TCR restimulation by triggering apoptosis by induction of Bim/Bcl2L11-dependent apoptosis in iTregs.[73] Human CD25+FOXP3+ Tregs were shown to differentiate into IL-17 producing cells. nTregs differentiated into Th17-like cells in the presence of IL-6. IL-17+Foxp3+ T cells are appeared to be an intermediate differentiation stage between Th17 cells and Tregs as Tregs were reported to differentiate into IL-17+Foxp3+ T cells that phenotypically and functionally resemble Th17 cells. Generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches was reported. Plasticity in Tregs is also observed in tumor context. A shift of Tregs from a protective antiinflammatory phenotype to a cancer-promoting proinflammatory phenotype was reported in polyposis.[74] A substantial fraction of FOXP3+IL-17+ T cells were isolated from colorectal cancer samples. Mast cells contribute to the reversion of the antiinflammatory function of Tregs toward promotion of inflammation in colorectal cancer.[75] Loss of Foxp3 expression in a minor population of tumor associated Tregs has been reported in mice bearing melanoma tumor, although the majority of Tregs in the tumor retained Foxp3 expression. A gradual shift from a Th17 response toward a predominant infiltration of Tregs has also been observed during ovarian cancer growth. These findings indicate that Tregs, or a fraction of Tregs, can show plasticity by differentiation into other subset and vice versa, as also mentioned previously, under cancer-derived conditions. Plasticity in Tregs can be accountable for the difficulty of the determination of Tregs and their association with cancer development/growth and explains some controversial observations on the correlation between increased levels of Tregs and disease outcome in cancer patients. For example, accumulated evidences show that in tumor tissue, Tregs are capable to differentiate into Th17 cells, or vice versa. It should be pointed out that the role of Th17 cells in the cancer context is controversial. It has been suggested that Foxp3+IL-17+ cells promote development of cancer-initiating cells. Elevated levels of IL-17 produced by Th17 cells promoted myeloma cell growth. IL-17 was also reported to promote tumor growth. Colorectal cancer-derived Foxp3+IL-17+ T cells were able to suppress tumor specific CD8+ T cells. High frequency of Th17 cells was predictive of poor prognosis in colorectal cancer.[76] In contrast, other studies suggest that IL-17 inhibit tumor growth and metastasis.[77] Tumor-specific Th17-polarized cells were reported to eradicate large established tumors. Thus, evaluation of other T cell subsets along with Tregs are essential for identifying the accurate correlation between increased levels of Tregs and disease outcome in various cancer patients.

In vivo Depletion or Blocking Tregs Augmentation Antitumor Immune Responses

In the past decade, manipulation of Tregs has been shown to enhance the efficacy of cancer immunotherapy approaches. Various drugs or agents such as denileukin diftitox (ONTAK, an IL-2-diphtheria toxin fusion protein),[78] LMB-2 (a conjugate of an anti-CD25 single chain antibody and Pseudomonase exo-toxin), daclizumab, Paclitaxel, basiliximab, and cyclophosphamide have been reported to deplete Tregs or block the suppressive activity of Tregs. In a clinical trial, depletion of CD4+CD25+ T cells by ONTAK enhanced immune responses to tumor vaccine, but, in another study, ONTAK was found to be ineffective in depleting CD4+CD25+ T cells.[79] Reversion of the suppressive function of human CD4+CD25+ Tregs as well as CD8+CD25+ Tregs by TLR8 ligands was also reported.[80] Depletion of intra-tumoral T cells particularly Tregs was reported after administration of anti-CTLA-4 antibodies in mice.[81] however, analysis of cellular mechanisms of antitumor responses induced by CTLA-4 blockade in patients with renal cell cancer or metastatic melanoma revealed that anti-CTLA-4 antibody did not reduce Treg numbers in the peripheral blood and the immunosuppressive activity of Tregs was not inhibited *in vivo* or *in vitro*.[82] Combinational administration of anti-CTLA-4 and anti-CD25 monoclonal antibodies synergistically enhanced antitumor immune responses; indicating that suppressive activity of Tregs and inhibitory effects of CTLA-4 signaling pathway in effector T cells act as distinct mechanisms in suppression of antitumor immune responses. Stimulation of Tregs through agonistic anti-GITR antibody abrogated Treg-mediated suppression. A soluble form of GITRL blocked suppressive activity of mouse Tregs in vitro. In several murine tumor models including melanoma, fibrosarcoma, colon carcinoma, and pancreatic cancer, a reduction in the numbers of tumor infiltrating Tregs or attenuation of Treg suppressive function was reported in mice treated with agonistic anti-GITR antibody. Combinational administration of blocking anti-CTLA-4 and anti-PD-1 antibodies led to reduction in Tregs and myeloid cells within mouse melanoma tumors.[83] Depletion of CD4+CD25+ Tregs by anti-folate receptor-4 monoclonal antibody led to induction of antitumor immunity to mouse fibrosarcoma and colorectal tumors. Administration of anti-OX40 antibody reduced intra-tumoral CD4+Foxp3+ Tregs as well as CD4+Foxp3- T cells in mouse B cell lymphoma, melanoma, and colon tumor models, while in other studies there was no major changes in intra-tumoral Treg numbers after administration of anti-OX40 antibody. In most of other studies, administration of anti-CD25 monoclonal antibody has been used for targeting Tregs.

Depletion of Tregs reduced the efficacy of some cancer immunotherapeutic modalities,[84] which may be due to the depletion of both Tregs and effector T cells by the depleting agent. Nonetheless, in clinical trials, percentages and numbers of CD4+FoxP3+ Tregs were +er in the peripheral blood of cancer patients that did not respond to the adoptive immunotherapy in comparison with patients that did show response[85] indicating that reduction of Treg before adoptive immunotherapy may beneficial. Immunotherapy with chemokine fusion protein in combination with CD25+ T cell depletion led to regression of established tumors in mice.[86] Depletion of tumor associated Tregs augmented the efficacy of cytotoxic T cell adoptive immunotherapy in murine acute myeloid leukemia.[87] Enhanced efficacy of dendritic cell vaccine after administration of anti-CD25

antibody was observed in a preclinical acute myeloid leukemia murine model.[88] In many other studies, depletion or blocking of Tregs has been resulted in enhanced efficacy of several immunotherapeutic approaches including tumor vaccines, cytokine therapy, gene therapy, or adoptive immunotherapy in murine tumor models and cancer patients. These findings clearly prove negative effects of Tregs on antitumor immunity.

In our study, administration of anti-CD25 monoclonal antibody before establishment of tumor led to inhibition of tumor growth, but there was no prominent inhibition in the tumor growth after administration of anti-CD25 monoclonal antibody in establishes tumors (unpublished data). This finding is in agreement with the previous reports.[89,90] suggesting that CD4$^+$CD25$^+$ Tregs have a crucial role in tumor growth in early stages of tumor development. Depletion of CD4$^+$CD25$^+$ Tregs enhanced immune responses to tumor antigens and generated cross-reactive antitumor immunity in mice.[91] Furthermore, depletion of CD25$^+$ Tregs led to inhibition of melanoma tumor growth in mice.[92] Intra-tumoral depletion of CD4$^+$ cells also led to the rejection of late-stage tumors with + tumor infiltrating Tregs.[93] CD4$^+$CD25$^+$ Tregs suppressed concomitant antitumor immunity against rechallenge with the same tumor in a poorly immunogenic mouse melanoma model. Depletion of CD4$^+$ T cells induced concomitant antitumor immunity in mice bearing progressive melanoma. Depletion of CD25$^+$Tregs prior to tumor inoculation reduced, but not prevented, tumor growth in a transgenic mouse model of prostate tumor.[94] In a mouse model of brain tumor, increased numbers of tumor infiltrating Tregs were observed and mice treated with anti-CD25 monoclonal antibody survived more than tumor bearing control mice.[95] In a chemically induced tumor model, tumors were rejected in mice pretreated with anti-CD25 monoclonal antibody.[56] In a neu-transgenic mouse model of breast cancer, Treg depletion with denileukin diftitox immediately after tumor inoculation led to immune-mediated tumor rejection.[96] In a recent study, oral administration of homogenized fibrosarcoma tumor tissue led to systemically increased CD4$^+$CD25$^+$ Tregs which award a tumor growth advantage in mice. Depletion of Tregs post-tumor tolerization and post-tumor induction led to complete tumor regression in tumor bearing mice.[97] In most other studies, depletion of Tregs or blocking of their suppressive function led to tumor growth inhibition. These findings show that Tregs are implicated in tumor/cancer growth in most types of cancers, and manipulation of Tregs can augment cancer immunity. However, more investigations are needed for determining effects of Treg manipulation on cancer patients.

From these findings, it can be concluded that Tregs have a beneficial role in cancer prevention when inflammation and immune responses promote carcinogenesis and/or cancer progression; however, Tregs become detrimental where abrogation of antitumor immune responses leads to promotion of cancer development and growth. It is also possible that, at least in some stages of cancer or some types of cancers, Tregs are not involved as a major component in cancer progression/suppression; rather, they are only present in the tumor sites as a bystander cell, thus presence of Tregs not indicates their positive or negative role on cancer progression in these situations. Treg activity may also be diverse at different anatomical sites. Similar to other T cell subsets, Tregs can differentiate into other T cell subsets even with opposite phenotype, and vice versa, which makes the definition of their roles in cancer conflicting. Nonetheless, depletion or blocking of Tregs in most studies led to improved antitumor responses. Therefore, in cancer patients that an association between increased levels of Tregs and poor prognosis exists, targeting Tregs may improve the survival rate of patients and also may augment the efficacy of certain anti-cancer therapies.

Conclusion

Tumor cells and tumor stromal cells in the tumor microenvironment can attract, differentiate, and/or expand Tregs. In accordance, + levels of Tregs have been reported in cancer patients. In some types of cancer, Tregs are involved as a major component in cancer progression as they have a crucial role in cancer promotion via suppression of anti-cancer immune responses and even nonimmune-mediated mechanisms. But, Tregs are also beneficial in certain types of cancer or in distinct stages of cancer development. This opposite role of Tregs may be due to the fact that immune responses during inflammation can provoke carcinogenesis or cancer progress in certain types of tumor or in distinct developmental stages; consequently, Tregs have favorable impact on these cancers by suppressing immune responses and inflammation. Tregs also show plasticity in some conditions. Thus, evaluation of the effects of immune responses on cancer development as well as plasticity of T cells is necessary to illustrate Treg roles in various malignancies. Tregs also exhibit nonimmune activities in the tumor environment such as promotion of angiogenesis. Indeed, Tregs are capable to have different functions depending on their microenvironment and signals that they receive from the environment. Tregs may have a diverse prognostic significance at different types of cancer and even in a single cancer depend on their localization regions within the cancer tissue. Application of appropriate markers for accurate identification of Tregs and study of frequency and various functions of Tregs during carcinogenesis, different cancer growth stages, and different anatomical sites are essential for precise determination of negative and positive roles of Tregs in various cancers. Increased knowledge in the biology of these cells and their roles in cancers would be helpful in better manipulation of these cells in various cancer patients. Yet, modulation of Treg-inducing factors in the tumor microenvironment and depletion or blocking of Tregs may be valuable approaches for induction of anti-cancer immunity and improving the efficacy of immunotherapies in which increased levels of Tregs are associated with poor disease outcome.

Conflicts of Interest

There is no conflict of interest.

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