Abstract. The immune system prevents establishment and progression of cancer through innate and adaptive surveillance. However, some cancerous cells successfully evade the immune system. Regulatory T-cells (Tregs) facilitate such evasion. Tregs may be the factor responsible for the limited success of human tumor immunotherapy to date. To improve immunotherapy, it is thought that the number of Tregs and their functions should be inhibited in patients with advanced cancer. In this review, we focus on recent immunotherapy efforts targeting Tregs.

Certain T-cells react to self-antigen. Consequently, the immune system consists of various regulatory systems that suppress induction of autoreactive cytotoxic T-lymphocytes (CTLs). One such regulatory system is composed of regulatory T-cells (Tregs). Tregs play an essential role in maintaining immunological unresponsiveness to self-antigens. Since many cancer antigens are self-antigens, Tregs are thought to suppress antitumor immunity (e.g. CTLs) and promote tumor progression. In this review we provide a brief summary of the importance of Tregs in cancer and focus on therapy targeting Tregs.

Fundamental Role for Tregs

Tregs represent a unique CD4+ T-cell subpopulation that suppresses the activation and proliferation of autoreactive lymphocytes and induces self-tolerance. There are two kinds of self-tolerance, central tolerance and peripheral tolerance.

The former contributes to lymphocyte differentiation through the elimination and inactivation of autoreactive lymphocytes in primary lymph organs. The latter contributes to immune tolerance to self-antigen in mature lymphocytes circulating in peripheral blood. Tregs are believed to contribute to the induction and maintenance of peripheral tolerance. Generally, Tregs are recognized as naturally occurring Tregs (nTregs), having differentiated from CD4+CD8+ T-cells in the thymus. nTregs, like all T-cells, arise from progenitor cells in the bone marrow and undergo lineage commitment and maturation in the thymus (1). nTregs comprise a small population, just 5-10% of peripheral CD4+ T-cells (2), but play a critical role. nTregs migrate from the thymus into the periphery when just 3 days old, and thymectomy of mice at day 3 results in lethal autoimmunity due to the lack of peripheral Tregs (3). Recently, inducible Tregs (iTregs) have attracted attention regarding tumor immunity. iTregs are derived from naïve CD4+ T-cells (Tn cells) that have migrated from the thymus to the periphery, where they differentiate and induce cytokines such as transforming growth factor-β (TGF-β). The function of nTregs and iTregs are quite similar and it is difficult to distinguish nTregs from iTregs. CD4+CD25+ forkhead box P3 (FOXP3) + Tregs include nTregs and iTregs and are thought to be the major Tregs population. nTregs differentiate in the thymus and decrease in number with age since the thymus undergoes rapid atrophy after adolescence. On the other hand, the decrease of iTregs that is induced in the periphery with age is slow compared to the one of nTregs. Therefore, conversion of memory T-cells to iTregs is thought to be required for Tregs maintenance (4-5). FOXP3+ T-cells with suppressive activity have been described based upon their cytokine induction profile. Type 1 regulatory T cells (Tr1) are induced by interleukin-10 (IL-10) (6), and T-helper 3 (Th3) cells, are induced by TGF-β (7). Natural killer (NK) T-cells have also been studied for their regulatory properties (8). Although the majority of NK T-cells express CD4, most of the remaining cells express neither CD4 nor CD8, although in humans there is a small subset of CD8+ NK T-cells (9-10).
There are other Tregs that are not of the CD4+ lineage. An in vitro study identified a subset of human CD8+ T-cells (CD8+/CD28−) that was able to confer tolerance by preventing up-regulation of the co-stimulatory markers CD80 and CD86 on antigen presenting cells (APCs), by CD4+ T-cells (11). Unlike CD4+ iTregs, CD8+ Tregs are dependent on interferon-γ (IFN-γ) to secrete TGF-β (12). How CD8+ Tregs are generated is still unknown, however, and multiple subsets of CD8+ Tregs, both thymus-derived and peripherally induced, have been described in human and mouse (13). γδ-T-cells which can be developed extrathyrmically also seem to be important Treg elements of the immune system (14-15). It has been shown that γδ-T-cells are capable of enhancing inflammatory responses in autoimmune disease, such as systemic lupus erythematosus, rheumatoid arthritis (15, 16), graft-vs-host disease (17), and delayed-type hypersensitivity (18). Figure 1 summarizes the differentiation pathways of Tregs described in this review (19).

Tregs in Cancer

Tregs comprise 5-10% of CD4+ T-cells in peripheral blood and are normally found in lymph organs (20-23). The reason why Tregs are enriched in lymph nodes may be due to the fact that they express homing receptors, including CC chemokine receptor1 (CCR1), CCR2, CCR4, CCR5, CCR6, CCR8, CCR9, CXC chemokine receptor3 (CXCR3), CXCR4, CXCR5,CXCR6, α4β1 integrin, αEβ7 integrin, α4β7 integrin, and the P- and E- selectin ligands (24). The study also showed that dendritic cell (DC) preferentially attract Tregs by secreting chemokines CCL17 and CCL22, which are ligands for the CCR4 receptor (24). Tregs numbers in peripheral blood mononuclear cell (PBMC) from patients with non-small cell lung cancer or ovarian cancer are higher than the ones observed in healthy volunteers (25). Similar results have been noted in breast cancer, colon cancer, esophageal cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma, malignant melanoma and pancreatic cancer (26-28). Moreover, Tregs may increase in malignant ascites (25, 29). There are three possible reasons why Tregs accumulate in cancer. Firstly, iTregs are induced or proliferate locally due to cytokines such as TGF-β, IL-10, and vascular endothelial growth factor (VEGF), which are reportedly important factors for induction and promotion of iTregs differentiation. Secondly, immature DCs exposed to TGF-β, IL-10, and VEGF induce Tregs. Much current vaccine-based immunotherapy for cancer is dependent on DC function, hence the need for modulating Tregs to maximize the effect of such vaccines (30). Thirdly, Tregs proliferate through signaling of T-cell receptor (TCR), CD28, and IL-2. Thus, Tregs may promote self-tolerance to impede immune surveillance against cancer in healthy individuals and suppress potential responsiveness to autologous tumors in cancer patients (31).

Many investigators define T-cells that express FOXP3+ as Tregs, and suggest a negative correlation between prognosis and the number of tumor-infiltrated Tregs (32-36). On the other hand, several recent studies present conflicting prognostic data for some hematological malignancies, especially B-cell lymphoma in which an elevated number of FOXP3+ cells were shown to correlate with improved survival (37). The assertion that there is a correlation between Tregs and prognosis is still controversial. Importantly, a reduced ratio of CD8+ T-cells to CD4+CD25+FOXP3+ in Tregs, as well as Tregs numbers in tumors, correlates with poor prognosis in patients with breast (35), gastric (38), ovarian (32, 35, 38-39), and colon cancer (40).

Immunotherapy Targeting Tregs

This review focuses on therapy directed at Tregs aiming to reduce and eliminate them. Figure 2 summarizes potential approaches for the elimination and inhibition of Tregs, as described below.

Anti-CD25 therapy. Basiliximab which is an anti-CD25 monoclonal antibody is the arsenal of current immunotherapies being used in kidney transplant patients (41). Bluestone et al showed that basiliximab caused a transient loss of FOXP3+ and FOXP3+CD25+ T-cells in the circulation (41). Denileukin dinitox (Ontak) is a recombinant fusion protein product of diphtheria toxin and IL-2 that selectively binds to the IL-2 receptor of cells and, following internalization, inhibits protein synthesis (42). Rasku et al showed that Ontak caused a transient depletion of Tregs (43). Telang et al showed the promising result of Ontak in patients with unresectable stage IV melanoma in phase II trial (44).

Immunotoxin LMB-2 is another agent used for relative selective destruction of Tregs. LMB-2 consists of a single-chain Fv fragment of anti-CD25 monoclonal antibody fused to a truncated form of the bacterial Pseudomonas exotoxin A, from which two amino acids have been deleted (45). Major trials testing this immunotoxin in patients with CD25+ hematological malignancies (46) and refractory hairy cell leukemia (47) have shown promising results.

However, one of the problems is that CD25 can be expressed in activated effector T-cells, thus anti-CD25 therapy may also affect activated T-cells.

Monoclonal antibodies against Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4). CTLA-4 is expressed on Tregs and is thought to play a pivotal role in their suppressive function. Ipilimumab, a monoclonal antibody against CTLA-4, was evaluated extensively as a possible therapeutic agent for the treatment of several kinds of melanoma (48). A phase III study of ipilimumab was published, and significant improvement in overall survival and drug tolerance among patients with
metastatic melanoma was observed (49). The US Food and Drug Administration approved ipilimumab injection for unresectable or metastatic melanoma on March 25, 2011. Another monoclonal antibody against CTLA-4, tremelimumab, a fully human IgG2 antibody developed by Pfizer Pharmaceuticals, is undergoing clinical investigation (48).

**Antibody against glucocorticoid-induced tumor necrosis factor (TNF)-receptor.** Glucocorticoid-induced TNF-receptor (GITR) contributes to the immunosuppressive function of Tregs. Interestingly, anti-GITR antibody may ameliorate the suppressive function of Tregs in vivo and in vitro (50). Preclinical evidence has demonstrated that signaling through GITR such as anti-GITR antibody, soluble GITR ligand could modulate the activity of Tregs with loss of FOXP3 expression (51).

**FOXP3 vaccination.** FOXP3 is a Tregs-specific marker and may offer the most rational approach to target Tregs. The concept of Tregs depletion via vaccination is to enhance the efficiency of previous antitumor vaccinations that have led to patented products (48). Patent WO 2008/081581 describes the invention of a vaccine using nonapeptides and decapetides derived from FOXP3 that bind HLA molecules (48).

**Toll-like receptor 8 (TLR8).** The TLR8-myeloid differentiation factor 88 (MYD88)-Interleukin-1 receptor associated kinase 4 (IRAK4) signaling pathway can reverse the suppressive function of different Tregs populations (52). It is not entirely clear why only TLR8 ligands can reverse the suppressive function of Tregs. One reason may be that Tregs express a relatively high level of TLR8 (52). Poly-G oligonucleotides or similar ligands might be useful in clinical settings to enhance the efficacy of immunotherapy directed toward cancer.

**Drug-induced Tregs inhibition.** Recent effects of drug-mediated Tregs inhibition have been reported. Cyclophosphamide increases antitumor effects by reducing Treg numbers and function (53). Cyclosporine A and tacrolimus also reduce the Tregs numbers as a function of

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**Figure 1.** A schema illustrating how regulatory T-cells (Tregs) differentiate in both the thymus and periphery. Natural killer T-cells: NK T-cells,forkhead box P3; FOXP3.
IL-2 secretion and inhibition of IL-2 signaling (54, 55). Tregs are sensitive to paclitaxel (56) and imatinib suppresses FOXP3, a master gene of Tregs (57). Dasatinib suppresses inhibition of Tregs proliferation and reduces FOXP3 expression by inducing G0/G1 arrest (58).

Other possible molecular mechanism that may target Tregs.

**p38 mitogen-activated protein kinase (MAPK) pathway:** The p38MAPK pathway in Tregs is activated more so than in typical CD4+ T- cells. Recent study revealed that depletion of CD25+ Tregs in combination with treatment with a p38 chemical inhibitor is necessary to completely block the immunosuppressive function of IL-10-producing anergic CD25+ iTregs (59).

**Hypoxia inducible factor-1α (HIF-1α):** Ben-Shoshan showed that in vivo expression of HIF-1α induced FOXP3 expression and an increase in the number of functionally active FOXP3+CD4+CD25+ Tregs (60).

**Notch signaling pathway:** Notch is a morphogen and contributes to cancer initiation and progression by regulating FOXP3 expression via interaction through the FOXP3 promoter (61). Notch signaling, therefore, may be a target for regulating both cancer progression and antitumor immunity.

**OX40.** OX40 (CD134) is a co-stimulatory TNF receptor family molecule that is constitutively expressed on Tregs (62). OX40 activation inhibits FOXP3 gene expression and limits Tregs suppression of effector T-cells (63). Furthermore, intratumoral injection of anti-OX40 monoclonal antibody strongly suppressed tumor growth (64). This result suggests the OX40 receptor may be a target for antitumor immunotherapy.

**Exosome.** Exosomes are endosome-derived organelles of 50-100 nm that are actively secreted through an exocytosis pathway by many cell types (65). They are very rigid and resistant to enzymatic degradation in blood, ascites, and effusions. (66). These biophysical properties allow exosomes...
to play an important role in cell to cell communication, in particular communication between immune cells (67). In fact, exosomes express many cell–cell communication-related molecules, including MHC class I and II, CD86, tetraspanins, and heat-shock proteins (65, 66). Recently, tumor-derived exosomes were shown to contribute to maintaining Tregs numbers and suppressive function in malignant effusions. It appears that surface-bound TGF-β1 on tumor-derived exosomes mediates FOXP3 expression (68). Thus, elimination of malignant effusion derived exosome, or control of such exosomes expressing TGF-β1, may be new immunotherapy therapeutic strategies for advanced cancer with malignant effusions.

**Vascular endothelial growth factor receptor 2 (VEGFR2) expression on Tregs.** We identified VEGFR2 as a potential marker expressed on the surface of Tregs (69). VEGFR2 is expressed selectively on CD4+FOXP3<sup>-high</sup> cells and has strong immunosuppressive functions on allogeneic T-cells. We reported that anti-VEGF antibody (bevacizumab) inhibited Tregs expansion, suggesting that VEGF contributed to Treg induction (70). Taken together, VEGFR2 may be a useful target since it is expressed on the cell surface of only the FOXP3<sup>high</sup> population. Moreover, peptide vaccination using VEGFR2 is undergoing clinical investigation in a phase I study (71).

**Conflict of Interest Statement**

The Authors declare no conflict of interest.

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