# REGULATORY T CELLS: POTENTIAL TARGET IN ANTICANCER IMMUNOTHERAPY

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## SUMMARY

The concept of regulatory T cells was first described in the early 1970s, and regulatory T cells were called suppressive T cells at that time. Studies that followed have demonstrated that these suppressive T cells negatively regulated tumor immunity and contributed to tumor growth in mice. Despite the importance of these studies, there was extensive skepticism about the existence of these cells, and the concept of suppressive T cells left the center stage of immunologic research for decades. Interleukin-2 receptor α-chain, CD25, was first demonstrated in 1995 to serve as a phenotypic marker for $CD4^+$ regulatory cells. Henceforth, research of regulatory T cells boomed. Regulatory T cells are involved in the pathogenesis of cancer, autoimmune disease, transplantation immunology, and immune tolerance in pregnancy. Recent evidence has demonstrated that regulatory T cell-mediated immunosuppression is one of the crucial tumor immune evasion mechanisms and the main obstacle of successful cancer immunotherapy. The mechanism and the potential clinical application of regulatory T cells in cancer immunotherapy are discussed. [*Taiwan J Obstet Gynecol* 2007;46(3):215–221]

**Key Words:** CD25, Foxp3, immunotherapy, regulatory T cells

## Introduction

There is accumulating evidence that many tumor antigens recognized by autologous cytotoxic lymphocytes are antigenically normal self-constituents [1,2]. Furthermore, immunotherapy of cancer by vaccination with tumor antigens or transfusion of *ex vivo* propagated cytotoxic lymphocytes often leads to the appearance of autoimmunity because of antigenic cross-reactions between tumor antigens and normal tissue antigens [3,4].

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T cells were initially subdivided into two broad classes, i.e. helper T cells and cytolytic/suppressor T cells, depending on the expression pattern of certain cell surface molecules. T cells bearing CD4 molecules (Lyt $1^+2^-3^-$ in mice in early days) were classified as T helper cells, whereas $CD8^+$ T cells (Lyt $2^+$ in mice in early days) were classified as cytolytic/suppressor T cells. Thus, suppressor T cells were initially thought to be mostly $CD8^+$. North's group first showed that T cells bearing the helper phenotype (Lyt $1^+2^-3^-$) can function as suppressor T cells in a mouse tumor model [5,6]. That human $CD4^+$ T cells can also function as "suppressor" or "regulatory" T cells were shortly demonstrated in the human tumor system [7–9].

Regulatory T cells (previously known as T suppressor cells) re-emerged as a dominant form of immunologic tolerance in the mid-1990s, when it was identified that the transfer of $CD4^+$ T cells depleted of the $CD25^+$

subset into *nu/nu* recipient mice induced the development of organ-specific autoimmune diseases, which could be reversed by the subsequent transfer of $CD4^+$ $CD25^+$ T cells [10]. Thymectomy of adult rats and mice followed by fractionated X-irradiation could also produce autoimmune disease [11]. Collectively, these data were strongly suggestive of the existence of a thymically produced suppressive T cell population, which was responsible for the establishment and maintenance of peripheral self-tolerance. These regulatory cells appeared to migrate out from the thymus at a relatively late stage, when compared with conventional/autoreactive T cells (more than 3 days after birth in mice) [12]. $CD4^+$ $CD25^+$ T cells have been associated with cancer [13], autoimmunity [14], transplantation tolerance [15], and, most recently, with pregnancy [16]. $CD4^+$ $CD25^+$ T cells actively contribute to the maintenance of natural immunologic self-tolerance. Self-tolerance maintained by natural $CD4^+$ $CD25^+$ T cells may, however, impede development of tumor immunity by hampering the generation and activation of tumor-effector T cells recognizing autologous tumor cells. If this is the case, reduction of $CD4^+$ $CD25^+$ T cells or attenuation of their suppressive activity may enhance immune responses to autologous tumor cells.

Generally speaking, $CD4^+$ $CD25^+$ T cells ($T_R$ cells) possess the ability to suppress immune responses and can be divided into two types: naturally occurring and adaptive regulatory cells [17]. Naturally occurring regulatory cells are generated in the thymus under poorly understood conditions. Adaptive regulatory cells (also referred to variously as "Th3" or "Tr1") are generated outside of the thymus under specialized activation conditions [12].

## Surface Phenotype and Subsets of Regulatory T Cells

Initially, CD5 was proposed as a marker for $T_R$ cells by demonstrating that the otherwise normal lymphocytes depleted of $CD5^{high}$ $CD4^+$ cells elicited autoimmunity when transferred to athymic nude mice [18]. Unfractionated $CD4^+$ cells (which contain CD5 high expressers) prevented the induction of autoimmunity when co-transferred along with the $CD5^{low}$ cells, implying that $T_R$ cells were contained specifically within the $CD5^{high}$ compartment. Similarly, the CD45RB molecule appears to divide T cells into two distinct functional subsets: $CD45RB^{high}$ and $CD45RB^{low}$ cells [19]. The $CD45RB^{high}$ population triggers inflammatory bowel disease when transferred to lymphopenic mice, by eliciting an immunopathologic reaction against normal gut flora, whereas the $CD45RB^{low}$ counterpart prevents such disease induction. To date, the most functionally useful surface marker for $T_R$ cells has proven to be their constitutive expression of the interleukin (IL)-2 receptor $\alpha$-chain (IL-2R), CD25. Approximately, 5–10% of $CD4^+$ peripheral T cells constitutively express CD25 in normal naïve mice and healthy humans [10,20].

Interestingly, CD25 does not appear to be merely a marker for $T_R$ cells, but rather reflects an absolute dependence on IL-2 for their peripheral maintenance and function. This is dramatically demonstrated by the loss of $T_R$ cells and consequent autoimmunity, which occurs if IL-2 signaling is perturbed, e.g. in the case of knock-out mice [21] or antibody blockade [22].

More recently, a number of other cell surface molecules have been shown to be associated with $T_R$ cells, which includes CTLA-4 (CD152), $\alpha_E\beta_7$-integrin (CD103), glucocorticoid-induced tumor necrosis factor family receptor (GITR), and neuropilin-1 (a receptor involved in axon guidance) [23–26]. It should be noted, however, that no single uniquely expressed cell surface molecule has, thus far, been identified for $T_R$ cells, and in many cases, relatively specific molecules such as GITR or CD25 are also upregulated to high levels on activated non-regulatory T cells as well. This problem is made especially acute in humans who naturally show large numbers of activated $CD25^+$ T cells, and, therefore, a definitive identification is usually only possible by sorting the highest $CD25^+$ expressers [27].

There are significant differences in the expression of CD25 on humans as compared with mouse T cells, and these differences influence the techniques in isolating $T_R$ cells from human peripheral blood. Fluorescence-activated cell sorting profiles of T cells from mouse spleen and human peripheral blood stained with antibodies against CD25 and CD4 are not equivalent. In the mouse, $CD4^+$ $CD25^+$ T cells are seen as a distinct population of cells that is easily distinguished from the $CD4^+$ $CD25^-$ cells and comprise approximately 10% of splenic $CD4^+$ T cells. Thus, the isolation of mouse $T_R$ cells is rather straightforward. In contrast, human $CD4^+$ T cells exhibit a continuous and primarily low expression of CD25 in which 2–4% express high levels of CD25, while up to 30% express low levels of CD25. This staining continuum makes it more difficult to determine whether all or only a subset of the $CD25^+$ cells should be included in the $CD25\,T_R$ population. The analysis of other cell surface proteins expressed on the surface of $CD4^+$ $CD25^{high}$ or $CD4^+$ $CD25^{low}$ subsets isolated from peripheral blood indicates that the $CD25^{high}$ subset is homogenous, as over 95% of the cells express CD45RO, CD62L, and CD122, and includes the majority of cells that express HLA-DR and the transferring

receptor (CD71) [20,28]. In contrast, the $CD25^{low}$ subset contains a more heterogeneous mixture of cells, as demonstrated by expression of CD45RO (80%), CD62L (80%) and CD122 (28%).

Moreover, it has long been known that there are major differences in the proteins expressed by humans when compared with mouse T cells, most strikingly in regard to HLA-DR and CD45RA/CD45RO. In contrast to the studies in the mouse demonstrating high levels of expression of CD62L and CD38 by $CD4^+$ $CD25^+$ T cells, these same surface markers have not been useful for isolation of human regulatory cells [29,30].

Two populations of Foxp3-expressing regulatory T cells have been characterized. One lineage, designated as natural suppressor cells, arises in the thymus in a pathway that requires T cell receptor stimulation by peptide-loaded major histocompatibility class II proteins, IL-2, and CD28, and might be mediated through dendritic cells associated with Hassall's corpuscles [31]. The maintenance of these cells after thymic export involves additional IL-2, CD40, and probably transforming growth factor-β (TGF-β). A second group of regulatory T cells might be induced in the periphery from Foxp3-negative $CD4^+$ T cells; prolonged, non-inflammatory antigen exposure and high local concentrations of TGF-β promote this conversion [32].

## Mechanisms of Suppression by Regulatory T Cells

The mechanism of suppression by $CD4^+$ $CD25^+$ T cells is still poorly understood. The most basic feature indicated by *in vitro* models is that suppression requires direct cell contact between the $CD4^+$ $CD25^+$ $T_R$ cell and the target T responders. Furthermore, it appears that human $CD4^+$ $CD25^+$ $T_R$ cells must be activated through their T cell receptors in order to be operationally suppressive [27]. Another *in vitro* analysis has concluded that $CD25^+$ suppressor T cells are anergic, i.e. they do not proliferate in culture when stimulated with antibodies to CD3 or antigens unless supplemented with high doses of IL-2. In the absence of exogenous IL-2, stimulated $CD25^+$ T cells suppress the proliferation of $CD4^+$ as well as $CD8^+$ T cells by a reaction that is independent of IL-10 and TGF-β secretion, as has been shown with suppressor T cells from IL-10- and TGF-β-deficient mice, which seem to suppress effectively [33].

However, others have postulated an essential function for cell-bound TGF-β on the basis of inhibition of suppression by antibodies to TGF-β [34]. The suppression of proliferation requires direct cell contact between suppressor and suppressed cells, as suppression does not occur when cells are separated by a permeable membrane. The presence of antigen-presenting cells (APCs) is not required, as suppression occurs in APC-free cultures. In all cases, the suppression requires activation of suppressor T cells by T cell receptor ligands or antibodies to CD3. No role has been shown for the surface molecules GITR, CTLA4, or PD-L1 using currently available blocking antibodies, as the addition of increasing numbers of $CD4^+$ $CD25^+$ $T_R$ cell causes suppression even in the presence of these reagents [35,36].

Cytolytic activity has been invoked as a possible mechanism of suppression. Human $CD4^+$ $CD25^+$ Foxp3-expressing T cells can be activated by a combination of antibodies to CD3 and CD46 to express granzyme A and kill activated $CD4^+$ and $CD8^+$ T cells by a perforin-dependent mechanism in a reaction that does not involve Fas–Fas ligand binding. Antibodies to CD18 interfere with the killing, suggesting that CD18 is involved in the interaction of suppressor T cells with their targets. Antibodies to CD3 and CD46 are superior to antibodies to CD3 and CD28 in inducing granzyme- and perforin-mediated killing. It remains to be determined whether activation by antigen can have similar consequences [37].

## Regulatory T Cell and Cancer Immunity

It is becoming increasingly clear that while T effector cell "boosting" strategies are capable of raising high levels of antitumor T cells, tumors often grow despite such lymphocyte induction. The inability of most cancer patients to mount an effective immune response can largely be attributed to tumor evasion strategy. Several tumor immune evasion mechanisms have been defined and include: (1) downregulation of major histocompatibility complex class I, (2) loss of tumor antigen expression, (3) downregulation of adhesion/accessory molecules by tumor and/or APC, (4) induction of anergy or clonal deletion of responding T cells, (5) changes in T cell signal transduction molecules, (6) secretion of immunosuppressive cytokines, and (7) the recruitment or activation of $T_R$ cell [38–40].

It is now clear that many tumor-associated antigens recognized by autologous T cells are normal self-constituents, and thus, presumably within the remit of control by self-reactive $T_R$ cells. $T_R$ cells may even be actively recruited by tumors as a means of immune evasion. Because tumor-associated regulatory T cells express Foxp3 mRNA and protein, it is possible that these cells traffic to tumors from the thymus, bone marrow, lymph nodes, and peripheral blood [41]. The

desirable elimination of tumors by cytotoxic effector T cells may thus be impeded by the action of $T_R$ cells, and evidence is accumulating that this, in fact, appears to be the case [42,43]. The early successful attempts to deplete $CD4^+$ $CD25^+$ $T_R$ cells and, hence, induce tumor regression involved the systemic administration of anti-CD25 monoclonal antibody (mAb) prior to tumor challenge [44–46].

But what is the evidence, other than the simple presence of $T_R$ cells in the peripheral blood of cancer patients, that they have an impact on immunity against human cancer? Three observations collectively provide strong evidence that $T_R$ cells do, in fact, ameliorate immunity against a wide variety of human tumors. First, the frequency of $T_R$ population is increased in the peripheral blood of cancer patients and/or enriched in frequency among tumor-infiltrating lymphocytes or within tumor-draining lymph nodes. Second, an accumulation of $T_R$ cells in tumor-associated tissue predicts poor prognosis or survival. Third, $T_R$ cells with specificity for antigens expressed by human tumors have recently been identified. Numerous studies in recent years have found increased frequencies of $CD4^+$ $CD25^+$ T cells with some or all of the features of $T_R$ in the peripheral blood of patients with a wide array of cancers, including head and neck cancer [47], lung cancer [48], gastrointestinal cancers [49], pancreatic and breast cancer [50], and skin cancer [51].

Moreover, experiments suggest that the simple elimination of $T_R$ cells from splenocyte preparations by treatment with anti-CD25 mAb results in productive responses to syngeneic tumors when transferred together to lymphopenic recipients [45,52,53].

One crucial question regarding human tumors and the increased frequency of $T_R$ cells within tumor microenvironments is the extent to which they are generated within, versus recruited to, the tumor microenvironment. One study of patients with ovarian carcinoma found that tumor cells and infiltrating macrophages secreted the chemokine CCL22, which was shown to be chemotactic for $T_R$ cells, all of which expressed the relevant CCR4 receptor [54]. Thus, there is a precedent for recruitment of $T_R$ cells to a tumor microenvironment. In addition to enhanced migration, it is also possible that the increased number of $T_R$ cells within the tumor microenvironment might represent antigen-induced expansion of natural $T_R$ cells or conversion of non-regulatory T cells into $Foxp3^+$-induced $T_R$ cells, because many tumors produce high levels of TGF-β (necessary to induce $T_R$ cells *in vitro*) [55].

Intra tumor infiltration/expansion of $CD4^+$ $CD25^+$ $Foxp3^+$ $T_R$ cells also potentially constitutes an immune evasion mechanism in the murine mesothelioma model. A strong correlation between tumor size and percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ $T_R$ cells was noted, implicating that increase in $T_R$ cells within the small murine mesotheliomas allows the evasion of the host antitumor effector immune response, thus leading to tumor growth [56].

## Therapeutic Scheme Involving the Use of Regulatory T Cells

The immune responses to cancer mount two major systems. The innate response, composed of granulocytes, macrophages, dendritic cells, natural killer cells, and complements, is rapidly triggered into action, detecting tissue disturbance through a set of germline-encoded pattern recognition receptors. The adaptive reaction, consisting of antibody-producing B cells and T lymphocytes, is slower to develop but manifests exquisite specificity and memory [57]. Emerging evidence implicates a critical role for immune regulatory circuits in attenuating antitumor immunity. These circuits primarily function to maintain tolerance to normal tissues, but since cancer arises from self, antitumor immune responses are similarly subject to regulation.

As detailed earlier, a large body of data from murine studies have demonstrated the potent ability of both natural and adaptive regulatory cells to control immune response under a wide range of clinically important conditions (Table).

Currently, it is tempting to speculate that part of the antitumor efficacy of current cytotoxic treatments might involve antagonizing regulatory T-cell function; previous work indicated that cyclophosphamide disrupts immune regulatory circuits [58], and thus, patients with weak regulatory T-cell but strong $CD8^+$ T-cell reactions might respond to this manipulation. Nonetheless, robust regulatory T-cell infiltrates will likely require more potent and specific strategies, and a number of critical immunoregulatory molecules expressed on the surface of regulatory T cells may well be suited to this purpose. Indeed, initial clinical testing of a fully human monoclonal antibody that blocks CTLA-4 has already established the ability of this scheme to effectuate substantial tumor destruction, although at the expense of some autoimmunity and potential severe side effects (e.g. enterocolitis) [59].

Another strategy is using anti-CD25 mAb. Anti-CD4 mAb has been administered systemically or intra tumorally to treat different tumor cell lines [44–46,56]. Surprisingly, intra tumoral delivery of anti-CD25 mAb is potentially feasible. This treatment regime is in line with the current thinking that $T_R$ cells are active at the site of immune regulation, and attempts to modulate

**Table.** Potential clinical applications of $CD4^+$ $CD25^+$ $T_R$ cells

| | Target | Potential therapeutic strategies |
|---|---|---|
| Increase of $T_R$ cell potency | Transplantation | *Ex vivo* gene transduction of Foxp3 (expression marker of $T_R$ cell) to conventional T cells |
| | Autoimmune disease | *Ex vivo* generation of regulatory cells from conventional T cells using cytokines, pharmacologic agents or modified dendritic cells |
| | Allergy | Expansion of host $T_R$ cells *ex vivo* using transplantation antigens or known autoantigens plus growth factors such as interleukin-2, granulocyte-macrophage colony-stimulating factor |
| Reduction of $T_R$ cell potency | Cancer | Decrease of $T_R$ cells or transient elimination of $T_R$ cells and/or perturbation of suppressive function *in vivo* (anti-CD25 mAb, anti-CTLA4 mAb) |
| | Infectious disease | Rendering effectors cells resistant to suppression (signaling through GITR with anti-GITR mAb or GITRL) |

*mAb = monoclonal antibody; GITR = glucocorticoid-induced tumor necrosis factor family receptor.*

the effects of these $T_R$ cells should be targeted to these sites. Moreover, intratumoral treatment may avoid potential side effects incurred by systemic depletion of $T_R$ cells using systemic delivery of anti-CD25 mAb.

Current tumor antigen vaccination strategies have begun to incorporate strategies that combine depletion of $T_R$ cells followed by tumor vaccination, to foster better expansion and/or effector function of vaccine-induced tumor immunity. In this regard, IL-2 diphtheria toxin fusion protein (ONTAK) has shown efficacy in the treatment of non-Hodgkin's lymphoma and cutaneous T cell lymphoma [60]. Moreover, signaling directed through Toll-like receptor 8 via a CpG motif can reverse $T_R$ cells function *in vitro* and *in vivo* [61], further demonstrating that it might be feasible to manipulate $T_R$ cells activity to enhance the efficacy of cancer vaccines. This evidence also has implications for the potential additional effectiveness of DNA-based vaccines, because they are likely to contain CpG motifs [62,63].

## Conclusion

It is now clear that $T_R$ cells actively hinder tumor immunity in cancer patients. Emerging data indicate that $T_R$ cells might be generated to the same tumor-associated (self) antigens that comprise many candidate cancer vaccines. Removal of $T_R$ cells lead to the activation of not only tumor-specific $CD8^+$ cytotoxic T lymphocytes, but also, presumably, tumor-specific $CD4^+$ helper T cells. Using this strategy can help devise a novel immunotherapy for cancer in humans or make the current immunotherapies more effective, e.g. by cytokine gene transduction in tumor cells, DNA vaccination, vaccination with tumor antigens/peptides, or tumor antigen-pulsed dendritic cells.

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