Targeting Regulatory T Cells for Anticancer Therapy

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Abstract: Regulatory T-cells (Tregs) comprise a group of either thymically derived or peripherally induced suppressor $CD4^+$ cells involved in the control of effector T-cells against both self- and foreign-antigens. They are found increased in tumor tissues and are thought to be involved in pathogenesis of cancer by providing tumors with a mechanism to evade immune detection and destruction. Despite the fact that mechanisms of Tregs regulation are still in progress, efforts are made aiming to develop approaches to deplete or inhibit tumor-associated Tregs function. This could lead to restore antitumor immunity and emerging strategies for therapeutic vaccination, and immunotherapeutic targeting of Tregs with specific drugs are underway.

Keywords: Targeted therapy, cancer, regulatory T cells, chronic lymphocytic leukemia, CD4, CD25, CD127.

DEFINING THE REGULATORY T CELLS

It is now well established that regulatory T cells (Tregs) are a distinct small subset of lymphocytes specialized in maintaining immune homeostasis [1]. In fact, Tregs appear to be able to suppress the function of self-reactive T-cells to protect the host from autoimmune disease [2]. These same cells may also prevent antitumor immune responses [3]. In humans, high levels of Tregs have been identified in lung, ovarian, breast, pancreatic tumor specimens, and in hematologic cancers [4].

Despite their existence as suppressive T-cells was proposed more than 30 years ago, a better identification of such cells lacked for several years because Tregs have a characteristic but not specific immunophenotype and not a single specific marker exists for its identification, at the present time. In the mid 1990s, Sakaguchi *et al* identified such a cell population as $CD4^+$ T-cells expressing surface interleukin-2 (IL)-2 receptor α chain (recognized by CD25) and termed them 'regulatory' T-cells [5]. However, CD25 is not exclusively restricted to Tregs because of its expression on the surface of T effector lymphocytes after activation [6]. Baecher-Allan *et al*, by means of flow cytometry and *in vitro* study of sorted cells, identified a very small subset of T cells with high expression of CD25 (Fig. **1**) that exhibited a strong regulatory function in humans [7-9]. $CD4^+$ $CD25^{+high}$ cells inhibited proliferation and cytokine secretion by activated $CD4^+$ $CD25^-$ responder T-cells in a contact-dependent manner. More recently, the intracellular transcription factor forkhead/winged helix box P3 (FoxP3) has been identified as the most accepted marker for Tregs [10-12]. Finally, the combination of CD127, that is part of the heterodimeric IL-7 receptor, CD4, CD25 and FoxP3, has been shown to better identify Treg cell population avoiding the contamination of such small subset of cells (about 1-4% of circulating CD4+ cells in humans) with activated T-cells [13,14]. Overall, Tregs may be defined as CD4+ T-cells expressing CD25 at high levels, cytoplasmic FoxP3, and very low to no CD127 on their surface. However, as reported in Table **1**, several other markers have been associated to Tregs, but none of them may be considered as a unique marker, as previously stated.

Two main subsets of Tregs have been described on the basis of their origin. Naturally occurring Tregs originate in the thymus as a consequence of the interaction with high-affinity antigens expressed in thymic stroma and constitutively expressing FoxP3 [15]. They suppress the response against self antigens. Tregs persist throughout life despite thymic involution after puberty in man. However, the subset of adaptive Tregs emerges also from the thymus but acquires its suppressive activity in the periphery regulating the response against self and non-self antigens [16].

By means of *in vitro* culture, Tregs have shown to suppress the proliferation of antigen-stimulated naïve T-cells [17-19]. Many possible mechanisms have been proposed for Treg-mediated suppression, but several views still remain

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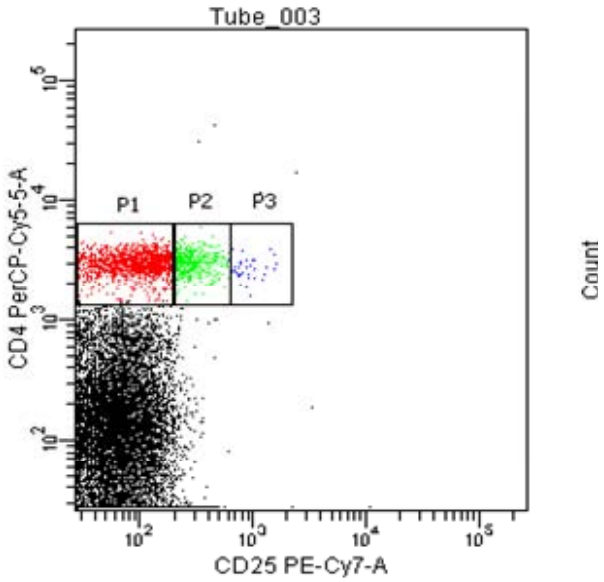

Fig. (1). Three main population of $CD4^+$ T-cells are gated according to the expression of CD25. Cells without expression of CD25 (P1), cells with low to medium expression of CD25 (P2) and cells with expression of CD25 at high density (P3). This latter has demonstrated to be Tregs by sorting and *in vitro* studies [7]. $CD4^+$ $CD25^{+high}$ cells inhibited proliferation and cytokine secretion by activated $CD4^+$ $CD25^-$ responder T-cells in a contact-dependent manner.

Table 1. Reported Immunophenotype of Human Tregs

Antigen	Expression
CD4	Positive
CD8	Negative
CD25	High
CD127	Low
FoxP3	Positive
GITR	High
IL-10	Positive
TGFβ	Positive
CD152 (CTLA-4)	High
CD154 (CD40L)	Negative
CD45RA	Negative
CD45RO	Positive

unclear [20]. First of all, Tregs need T-cell receptor (TCR) triggering to become suppressive [20,21], but there is no agreement on this topic [22]. Both natural and adaptive Tregs are antigen-specific, but once activated they exert their suppressive activity of activated $CD4^+$ or $CD8^+$ effector T cells in a non-specific way, which may be based on cell-to-cell contact or on the release of suppressive cytokines [23]. *In vitro* studies demonstrated that Tregs suppress proliferation and interferon (IFN)-γ production by effector T-cells through a direct cell-cell contact-dependent stimulation of effector cells. A crucial role is being played by glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR) and cytotoxic T-lymphocyte antigen-4 (CTL-4) surface antigens [24,25]. The ligation of CD80/CD86 complex on effector cells by CTL-4 on suppressive cells results in transmission of inhibitory signals of T cell function. Moreover, Tregs seem to modulate dendritic cells (DCs) function. The same ligation on DCs results in expression and activation of indoleamine 2,3-dioxygenase (IDO), a catabolic enzyme involved in tryptophan degradation [26]. On the other hand, other *in vitro* studies suggest a different mechanism of suppression by Tregs in which a critical role is played by production of soluble factors, such as IL-10 and TGF-β [27,28]. Activated Tregs may express granzyme A or perforin killing both activated $CD4^+$ and $CD8^+$ T-cells through perforin-dependent mechanism [29,30]. Finally, Tregs are able to perform a cytokine-induced block of maturation and activation of DCs. In fact, IL-10 and TGF-β are both capable to impair the antigen-presenting capacity downregulating major histocompatibility complex (MHC) class II and interfering in expression of costimulatory molecules [31,32].

TREGS AND CANCER

Tregs accumulating at tumor sites and in the circulation of patients with cancer are considered to be responsible for dampering antitumor defenses [33-37]. The mechanisms driving Tregs expansion in cancer are not fully understood [38]. It is hypothesized that the failure of the immune system to eradicate tumors is due to the immunosuppressive environment created by the growing tumor. Tregs accumulation in tumor site is probably due to chemoattractant cytokines, such as CCL22 and H-ferritin, secreted by tumor cells and tumor-infiltrating macrophages. Moreover, the interaction with prostaglandin E_2 (PGE_2)-induced tolerogenic DCs gives rise to the differentiation and peripheral expression of naïve Tregs into memory Tregs [39]. This latter together with tolerogenic DCs inhibit the generation of effector T-cells resulting in the induction of tolerance against the tumor. Curiel *et al*, found a large number of Tregs in malignant ascites and tumor masses of 104 patients with ovarian carcinoma [40]. In addition, infiltrating Tregs were associated with a high death hazard and reduced survival. On the contrary, improved survival was shown to correlate with Tregs higher number in B-cell lymphoma and in colorectal cancer [41-43]. The discrepancies between the prognostic impact of Tregs in different tumors remain to be better clarified. Moreover, tumor cells and microenvironmental macropahges were found to produce the chemokine CCL22, which mediates trafficking of Tregs to the tumor. Jarnicki *et al*, in a murine model of CT27 lung metastases found findings suggesting that tumor growth facilitates the induction or recruitment of CD4+ Tregs that secrete IL-10 and TGF-β and suppress effector CD8+ T-cell responses [44]. Moreover, Mailloux *et al* showed that Lewis lung carcinoma cells selectively

attracted a high number of Tregs through the secretion of CCL22 [45].

THE CASE OF THE CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in Western countries [46]. It is characterized by the accumulation of monoclonal B-lymphocytes in bone marrow, lymphoid organs and peripheral blood. Evidence is accumulating on the role of T-cells impairment in the pathogenesis and development of CLL [47]. Several authors reported that Tregs are increased in CLL patients [48-52]. We recently evaluated Tregs number in peripheral blood of 80 untreated patients with CLL [52]. A combination of CD4/CD25/CD127 monoclonal antibodies was used to evaluate Tregs by means of flow cytometry. CLL patients showed a higher absolute number of circulating Tregs compared to age and sex-matched controls (Fig. **2**). Moreover, Treg cell numbers were significantly correlated to more advanced Rai clinical stages, peripheral blood B-cell lymphocytosis, more elevated LDH levels, and absolute number of $CD38^+$ neoplastic B-cells. Taken together, this data showed that Tregs frequencies in the peripheral blood are increased in patients with advanced stages of disease, classically associated with a shorter overall survival. These are the clear features of active disease because of CD38 expression is now thought to be expressed specifically by the proliferative fraction of the disease [53].

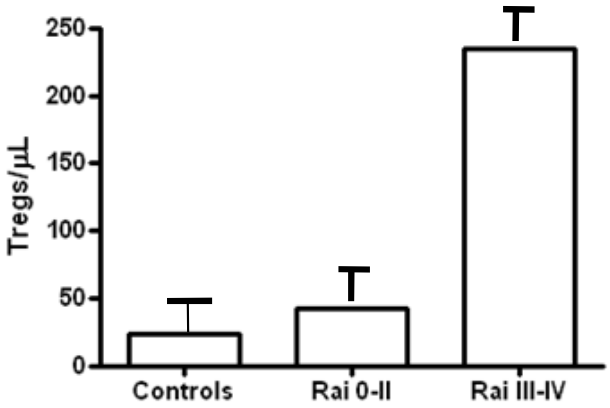

Fig. (2). Absolute mean number (/µL) of peripheral blood Tregs evaluated by means of flow cytometry in CLL patients, according to Rai clinical stage, and controls. Tregs were defined as $CD4^+$ lymphocytes expressing CD25 at high density and CD127 at low density or undetectable levels.

In light with the hypothesis that Tregs play a critical role in protecting CLL cells from getting killed by the immune system, is the evidence that they are reduced after therapy with fludarabine [48]. The same happens when patients with CLL were treated with thalidomide [54]. This drug and its analogues, such as lenalidomide, acts as immunomodulatory agents targeting the environment and both are shown to be effective in the treatment of CLL patients. A working hypothesis is that they may act modulating TNF [55].

Overall this data open new avenues in the field of the therapy of CLL patients combining conventional drugs, such as cyclophosphamide and fludarabine, with immuno-modulatory drugs aiming to reduce Tregs number and to modulate their function.

TARGETING TREGS TO CURE CANCER

Why the immune system fails to eradicate tumor cells in the body is still a matter of debate. Because increasing evidence shows that Tregs may play an important role in immune evasion mechanisms employed for tumor cell antigens, it is intuitive that may be therapeutic implications of intratumoral Tregs infiltrates. The modulation of Tregs function in cancer treatment has been studied using various approaches, with encouraging preclinical and clinical findings. Historically, cytotoxic chemotherapy and/or radiation therapy are used for cancer treatment. They act as killing cells and often are the causes of lymphopenia and neutropenia. It has long been thought to act on the immune system to cure cancer or to make the cancer treatment more powerful. More recently, attention is focused on the role of Tregs and their therapeutic implications [3, 56]. It is now noted that chemotherapy may have stimulatory effects in some cases. This is the case of cyclophosphamide that has shown to induce a depletion of Tregs [57]. Despite their exact mode of action is still not completely understood, other conventional chemotherapeutic drugs (i.e., methotrexate, gemcitabine, and mitoxanthrone) also show modulatory effects on Tregs. In addition, COX-2 inhibitors, such as indomethacin, showed ability to reverse Tregs-mediated anti-tumor suppression [58]. *In vitro* studies showed that tumor-derived PGE_2 increases FoxP3 expression and Tregs inhibitory activity.

The best approach to Tregs manipulation is under investigation [59]. Tregs inhibition or depletion: are these the two main choices. Depletion is usually made using monoclonal antibody targeting surface antigens on Tregs, such as CD25. Firstly PC61 was used in rodent models but its use was ineffective when tumor is well established and in progression [60,61]. Daclizumab is a monoclonal antibody targeting CD25 on T-cell surface and was used to treat cancer patients [62]. A single infusion of daclizumab in patients with metastatic breast cancer effectively depleted circulating Tregs. When a cancer antigen peptide vaccine was given during the daclizumab-induced Tregs nadir, a generation of cytotoxic T lymphocytes was observed, thus suggesting that this monoclonal antibody enhance immune responses to tumor antigen vaccination [63]. Another approach to administer chimeric proteins such as imunotoxins was proposed. Immunotoxins LMB-2 is constituted by a single-chain Fc fragment of the anti-CD25 monoclonal antibody fused to a truncated form of the bacterial *Pseudomonas exotoxin* A [64]. It acts by inactivating elongation factor 2 and consequently inhibiting protein synthesis, thus targeting only CD25+ T-cells. Hairy cell leukemia and other hematologic malignancies were treated in this approach [65,66].

Denileukin diftitox (DAB389IL-2, Ontak) is a chimeric protein composed by the fusion of portions of dipheria toxin and IL-2 chains. This drug was FDA-approved for clinical use in the treatment of cutaneous T-cell lymphoma in 1999. Fludarabine-refractory CLL patients were also successfully

treated. No beneficial effects, however, were observed in patients with metastatic melanoma and non-small-cell lung cancer [63,67,68].

Ipilimumab and tremelimumab are the two monoclonal antibodies targeting CTLA-4 [69]. CTLA-4 is a protein constitutively expressed on Tregs with inhibitory activity on the immune systems inducing T cell anergy through a mechanism involving CD80 and CD86 costimulatory molecules. Ipilimumab was successfully used to treat patients with melanoma, clear cell renal cancer, and metastatic hormone-refractory prostate cancer with good response and drug tolerance [70,71]. Another anti-CTLA-4 monoclonal antibody, tremelimumab, is under clinical investigation for metastatic melanoma [72]. This latter drug has shown to restore effector and memory $CD4^+$ resistant to Tregs inhibition and $CD8^+$ T-cell proliferation cells.

THE OTHER SIDE TO THE COIN

Since 1969, it is known that thymectomy give rise to the development of autoimmune disorders, such as thyroiditis, gastritis, orchitis, prostatitis, and sialoadenitis, in both neonatal and adult mice [73]. Inhibition of the development of these diseases may be obtained inoculating normal T cells from syngeneic animals in both experimental systems [74]. The onset of autoimmune disorders is due to the abrogation of $CD4^+$ T-cells development or, in adult, by destroying $CD4^+$ cells [75,76]. Moreover, the single gene FoxP3 mutation on the X chromosome in mice gives rise to a severe autoimmunity/inflammation disease. A similar disease, the so-called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome), characterized by autoimmune disease in multiple endocrine organs, is observed in humans when mutations of the human FoxP3 gene occurred [77]. This data clearly demonstrated the fundamental role of Tregs in maintaining the homeostasis of immunity and preventing the onset of autoimmune disorders. The possible onset of autoimmune disorders has been reported as consequence of Tregs depletion both in rodents and humans [78-80]. This issue assumes a critical relevance in the setting of new anticancer strategies designed to induce Tregs modulation, particularly in malignancies, such as CLL, that are prone to be complicated by either hemic (autoimmune hemolytic anemia, immune thrombocytopenia, pure red cell aplasia) and non-hemic autoimmune diseases. Moreover, fludarabine and alemtuzumab (campath), both drugs targeting T and B lymphocytes, the first inhibiting DNA repair, the second one acting through ligation of surface antigen CD52, have shown to be related to the risk of autoimmune disorders. In fact, since 1995 fludarabine has reported to increase the risk of autoimmune hemolytic anemia [81]. In addition, an increasing number of idiopathic trombocytopenic purpura (ITP) has been observed in patients treated with alemtuzumab for remitting/relapsing multiple sclerosis [82]. In the CAMMS223 trial, alemtuzumab therapy was suspended after ITP developed in 3 patients, one of which died of cerebral hemorrhage and a black-box warning on the Campath label appeared alerting on the risk of ITP. These data have to be taken into account in the hypothesis of combining agents modulating Tregs with fludarabine or campath, or both.

CONCLUSIONS

Tregs play a central role in maintaining peripheral tolerance to self-antigens and in regulating the immune response to non-self-antigens. It is now clear that Tregs are actively involved in the progression of cancer, and have an important role in suppressing tumor-specific immunity. Clinical strategies are developing to target Tregs with the aim to reduce or abrogate the antitumor suppression. One of the major challenges is to identify a unique marker of Tregs that can be used to more specifically target these cells with selective monoclonal antibodies. Combination therapies with conventional drugs and vaccination strategies are under investigation. However, there are still unanswered questions on the biology of Tregs and the risk of autoimmune disorders. For that reasons many efforts have to be made to better clarify them in order to drive anticancer therapy more efficiently.

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