Regulatory T Cells in Cancer Biology: A Possible New Target for Biochemical Therapies

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Abstract: $CD4^+CD25^+$ T cells are essential for maintenance of self-tolerance and therefore have been referred to as regulatory T cells (Treg). Experimental tumor models revealed that Treg are potent inhibitors of an anti-tumor immune response. Treg are expanded in human cancer. Currently, a variety of strategies for the induction of a specific anti-tumor immune response are tested in preclinical and clinical settings. Biochemical strategies modifying and/or depleting Treg in cancer patients for an enhancement of vaccine-based therapeutic concepts will be discussed in detail in this review.

Keywords: Biochemical modification, regulatory T cell, immune evasion, cancer.

IMMUNEPARALYSIS IN CANCER PATIENTS

Most cancer patients harbor significant numbers of $CD8^+$ and $CD4^+$ T cells recognizing specific tumor antigens (Ags), such as WT-1 in acute myeloid leukemia (AML) [1,2], bcr-abl in chronic myeloid leukemia (CML) [3,4] or Her2/neu in breast cancer [5,6]. Unfortunately, so far, in most cases, tumor-reactive T cells (mechanisms of CTL-mediated tumor cell killing are depicted in Fig. **1**) fail to eradicate the tumor *in vivo*. Those tumor-reactive T cells seem to be actively maintained in an unresponsive state. It is well documented that tumors use a wide variety of strategies for evading the host's anti-tumor immune-response. The secretion of immuno-suppressive factors, such as TGF-β, for example supports the generation of anergic T cells [7]. Growth factors affecting dendritic cell (DC) differentiation and function [8], such as the vascular endothelial cell growth factor (VEGF), are also highly expressed by a wide variety of malignant tumors [9]. Moreover, tumors can also induce apoptosis in T cells infiltrating malignant tissue, for example, by direct activation of death receptors *via* FasL [10] and/or by high expression of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase [11], which degrades the essential amino-acid tryptophan, thereby increasing T cell cytotoxic metabolites (i. e. kynurenine) [12].

BIOLOGY OF REGULATORY T CELLS

Recently, regulatory T cells (Treg), which are characterized by the constitutive expression of CD25 (the interleukin-2 (IL-2) receptor α-chain) have also been attributed to contribute to cancer-related immuno-suppression [13-16]. Treg comprise 5–10% of the total population of $CD4^+$ T cells in mice and men and were primarily thought to be critically involved in the repression of autoimmune disorders, transplant rejection and inflammatory bowel disease [17]. In line with this idea, patients suffering from multiple sclerosis (MS) [18] and autoimmune-mediated mixed cryoglobulinemia in hepatitis C-positive individuals [19] display a numerical and functional Treg deficit. These examples demonstrate that a decrease of Treg number and/or function might promote the development of autoimmune diseases mediated by a decreased immune tolerance to auto-antigens.

TREG IN CANCER BIOLOGY – PRECLINICAL FINDINGS

In contrast to the findings in autoimmune diseases, Treg expansion might depict an important immune-evasion mechanism in cancer patients. This idea was first considered after the observation that Treg-depleted mice are characterized by an enhanced anti-tumor immunity [20,21]. Of note, at least in pre-clinical experimental tumor models, a tumor-antigen specific Treg expansion could be demonstrated [22]. Treg are capable to inhibit both $CD4^+$ and $CD8^+$ T cells in an antigen non-specific manner [23] and have also been described to modify NK [24] and B cells [25,26]. Immuno-suppression is thought to be primarily mediated by a combination of cell–cell contact and paracrine effects, with a predominant role for IL-10 and TGF-β [27,28]. Very recent reports support the idea that Treg are able to substantially interfere with the tumor-specific $CD8^+$ T cell immune response *in vivo*. Chen *et al*. elegantly demonstrated that Treg abrogate CD8 T cell-mediated tumor rejection by specifically suppressing their cytotoxic activity [29]. The molecular mechanism underlying this observation seems to be mainly mediated by TGF-β, as overexpression of a dominant-negative TGF-β receptor on tumor-specific $CD8^+$ T cells renders them resistant towards Treg-mediated inhibition. This in turn resulted in restoration of their cytotoxic activity with concomitant tumor rejection.

So far, an exact characterization of Treg has been hampered by the lack of specific cell surface markers. The observation that autoimmune diseases occur in both humans and mice lacking functional FoxP3 indicates that this transcription factor plays a crucial role in the regulation of T cell differentiation and function. Indeed, Fontenot *et al*. have generated $FoxP3^{-/-}$ mice, which succumb to an aggressive lymphoproliferative auto-immune syndrome almost identical to that of scurfy mice [30]. It was found that $FoxP3^{-/-}$ mice

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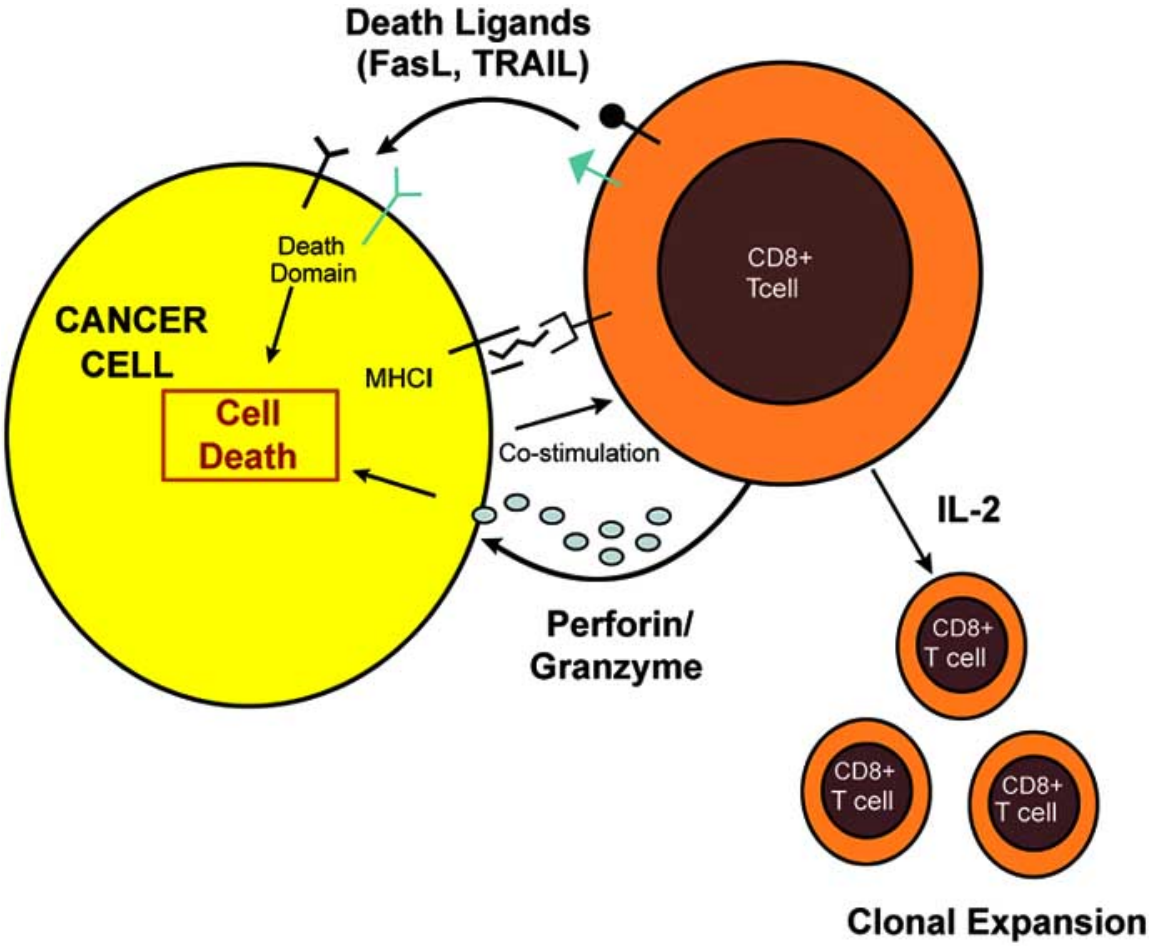

Fig. (1). Mechanisms of antitumor activity of cytotoxic lymphocytes (CTL).

lack a discrete $CD4^+CD25^+$ regulatory T cell population, which in turn leads to hyper-activation of $CD4^+$ T cells. Several recent reports have further shown that expression of FoxP3 is sufficient to confer suppressive activity on naive T cells [31,32]. Hence, FoxP3 fulfills the criteria of a Treg-specific marker, which is, at least in differentiated Treg, not known to be substantially regulated and represents a suitable surrogate marker for the indirect quantitation of Treg tissue-content.

TREG IN HUMAN CANCER

In the last years, it has become evident that patients suffering from cancer, who would require a functional T cell-mediated anti-tumor immune response, have a significant enlargement of the Treg pool. Woo and co-workers first demonstrated that significant numbers of regulatory T cells invade into malignant tissue of lung and ovarian cancers [33]. Evidence was recently provided that Treg numbers are increased in the peripheral blood of patients suffering from epithelial cancer [24]. These findings were corroborated for several cancer entities including pancreas and breast adenocarcinoma [34], head and neck cancer [35] hepatocellular carcinoma [36], gastrointestinal and esophageal cancer [37,38] as well as metastatic melanoma [39] and Hodgkin lymphoma [40]. Of note, it could be further demonstrated that the observed increase of Treg in peripheral blood is not simply due to redistribution of these cells between blood and tissue compartments, but is indeed caused by active cell division and proliferation (own data, manuscript submitted). Recently, Curiel and co-workers nicely demonstrated that human tumor-derived Treg suppress tumor-specific T cell immunity and may therefore contribute to growth of human tumors *in vivo* [41]. They further show that Treg preferentially move to and accumulate in tumor tissues and ascites, an effect which is probably mediated by the secretion of tumor cell and macrophage-derived CCL22. Therefore, local chemokine gradients may result in specific recruitment of Treg, representing a mechanism by which tumors may foster immune privilege. Notably, the functional relevance of these observations is demonstrated by a significant association of increased Treg numbers with a high death hazard and reduced survival in ovarian cancer [41].

The fact that tumor-specific Treg cells are present at the tumor site thereby controlling the induction of antigen-specific $CD4^+$ T cell responses [14,41] may at least in part explain why tumor-specific immune responses elicited by peptide or peptide-pulsed DC vaccines in cancer patients are only weak and transient. Although MHC class II-restricted tumor peptides are capable of stimulating a $CD4^+$ T cell response, it is completely unknown whether such peptides elicit either Ag-specific $CD4^+$ effector T cell or Treg responses or both. Until now, only little is known about the

physiological target antigens recognized by Treg in human cancer. Recently, LAGE1 and ARTC1 were identified as ligands for tumor-specific $CD4^+$ Treg cell clones generated from tumor-infiltrating lymphocytes (TILs) of cancer patients [22,42]. Further knowledge of Treg target antigens may open opportunities for the manipulation of antigen-specific immune responses directed to cancer as well as auto-immune and infectious diseases.

In summary, the observation the depletion of Treg enhances anti-tumor immunity in preclinical models as well as the content of Treg is increased in a variety of malignancies suggests that modulation and/or depletion of Treg might represent a suitable strategy for enhancement of the anti-tumor immune response in humans.

BIOCHEMICAL STRATEGIES MODULATING TREG

Denileukin [DAB(486)IL-2]

The human IL-2 receptor exists in three forms, low (CD25), intermediate (CD122/CD132) and high (CD25/ CD122/CD132) affinity. The high affinity form of this receptor is usually found on regulatory T cells, activated T and B lymphocytes and activated macrophages. DAB389-interleukin-2 (IL-2) is a fusion protein which targets the diphtheria toxin to the high affinity IL-2 receptor [43]. DAB(486)IL-2 fuses the fragments A and B (Met_1 -Thr_{387})-His of the diphtheria toxin to the sequence of IL-2 (Ala_1 - Thr_{133}) and is produced in an *E. coli* expression system. DAB(486)IL-2 has a molecular weight of 58 kD and has shown clinical activity in a variety of diseases, including B-cell non-Hodgkin's lymphoma, cutaneous T cell lymphoma (CTCL), Hodgkin's disease, psoriasis, rheumatoid arthritis, and HIV infection. DAB(486)IL-2 is known to be bound to cells expressing the high affinity receptor for IL-2 with subsequent internalization of the toxin into the cytosol of the target cell [44]. After internalization, the diphtheria toxin inhibits cellular protein synthesis, resulting in cell death. DAB(486)IL-2 is metabolized by proteolytic degradation. There are currently no data available on the *in vitro* or the *in vivo* effects of DAB(486)IL-2 on Treg. However, based on the expression of the high-affinity IL-2 receptor on these cells, it is conceivable that application of DAB(486)IL-2 might also deplete Treg. This might be of therapeutic value for immuno-modulation before immuno-stimulatory therapies, such as vaccination using DC or protein-based vaccines are applicated.

Cyclophosphamide

Following conversion to active metabolites in the liver, cyclophosphamide (Cy) functions as an alkylating agent, interfering with DNA replication and the transcription of RNA, ultimately resulting in the disruption of nucleic acid function. The drug exhibits phosphorylating properties which also enhance its cytotoxicity. Cy also possesses potent immuno-suppressive activity. It was in the late 90's when Awwad and co-workers demonstrated that Cy causes immunologically mediated regression of the immunogenic, Cy-resistant L5178Y lymphoma in syngeneic and semi-syngeneic mice [45]. Because the therapeutic effect of Cy could be inhibited by passive transfer of $CD4^+$ T cells from normal donor animals, it is apparent that the therapeutic effect of Cy is based on its ability to preferentially destroy $CD4^+$ suppressor T cells. These intruiging data from 25 years ago were recently corroborated by several research groups showing that low-dose Cy not only decreases cell number of Treg, but also leads to a decreased function of Treg. Cy treatment enhances apoptosis and decreases homeostatic proliferation of these cells. Expression of GITR and FoxP3, which are centrally involved in their suppressive activity, is downregulated after Cy administration [46]. These data have proven to be of relevance, as application of Cy enhances the effect of vaccination strategies *in vivo* by selecting low-frequency tumor-specific T cells, which are in the presence of normal numbers of Treg under the control of immuno-suppressive Treg, but can be expanded *in vivo* by vaccination strategies after Cy-mediated depletion of Treg [47,48]. Recently published data from a phase I/II study using allogeneic tumor-lysate-pulsed monocyte-derived DC demonstrated that application of Cy before vaccination of renal carcinoma patients improved survival-time by 3 months [49]. This observation might at least in part be explained by a Cy-induced depletion and/or modulation of immuno-suppressive Treg.

Fludarabine

Fludarabine is a cytotoxic analog of deoxyadenosine monophosphate and has high efficacy in the treatment of chronic lymphocytic leukemia [50]. It is phosphorylated intracellularly by deoxycytidine kinase to the active triphosphate 2-fluoro-ara-ATP.

It is assumed that all these mechanisms contribute to the inhibition of cell growth, with the inhibition of DNA synthesis being the dominant mechanism. A very recent report provided evidence that in CLL patients receiving fludarabine-containing therapy regimens, the inhibitory function of Treg was decreased or even abrogated [51]. In addition, Fludarabine markedly reduced frequencies of Treg after therapy with fludarabine, which has also been demonstrated to induce apoptosis of Treg *in vitro*. In light of the above described findings of Cy, combination therapies containing both, fludarabine and Cy might be further tested for the reduction of immuno-suppression prior to cancer immunotherapy.

Anti-CD25 Monoclonal Antibodies (mAb)

Two monoclonal antibody preparations against the α-chain of the IL-2 receptor (IL-2Rα) are available for use. Basiliximab and daclizumab, a chimeric and a humanized monoclonal antibody, respectively, are both glycoproteins produced by recombinant technology [52]. They specifically bind to and block the α-chain of the IL-2 receptor, which is expressed on the surface of activated T lymphocytes as well as on naturally occurring Treg. Both antibodies result in the depletion of $CD25^+$ T lymphocytes, which might be of value for the selective depletion of immunosuppressive Treg. However, the caveat that it might also deplete tumor-reactive $CD25^+$ T cells has to be carefully considered. There are currently no data available on the effects of these mAbs on the frequency and the function of Treg in humans.

siRNA Targeting FoxP3

RNA interference (RNAi) describes the sequence specific degradation of mRNA in animals and plants initiated by double-strand RNA molecules (dsRNA), which consist of

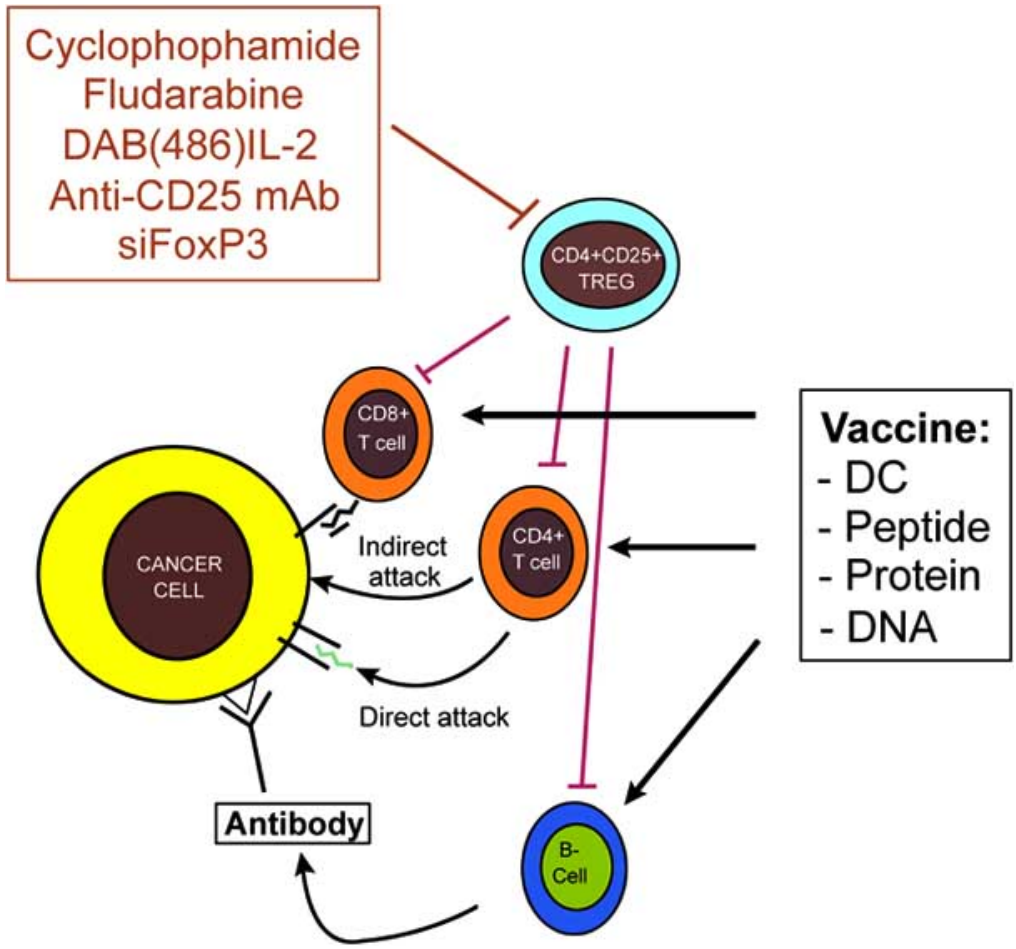

Fig. (2). Treg impair the antitumor immune-response by several mechanisms, i.e. by modulating CD4, CD8 T-cell as well as B-cell activity. Strategies modulating and/or depleting Treg are depicted and might improve antitumor immune responses induced by vaccination strategies.

two 21 to 22 nt long short-interfering RNA strands (siRNA) [53,54]. This mechanism was originally found in lower organisms and plants and represents a powerful tool for analyses of gene function in cell culture systems as well as in animal models. In this context, siRNA molecules can be derived from different sources: i) chemically synthesized dsRNA molecules are currently widely used but harbor the limitation that the silencing effect is only transient, and ii) vector-based systems encoding for siRNA under the control of polymerase III promoters by constructing short hairpin RNA (shRNA) forming a stem loop due to a non-complementary linker region have been used successfully in both transient [55] and stable settings [56,57]. However, the application of siRNAs is severely limited by their instability and relatively poor delivery of unmodified siRNA molecules into mammalian cells *in vivo*. To overcome this problem, recent reports demonstrated that noncovalent complexation of synthetic siRNAs with low molecular weight polyethylenimine (PEI) efficiently stabilizes siRNAs and enables their delivery into cells with full silencing activity *in vivo*. The systemic (intraperitoneal, i. p.) administration of PEI-complexed, but not of naked siRNAs targeting the c-erbB2/neu (HER-2) receptor results in a marked reduction of tumor growth through siRNA-mediated downregulation of HER-2 [58]. Alternatively, hydrodynamic application of non-modified siRNAs *via* i. v. -injection is also suitable for siRNA-mediated targeting of Fas or Caspase 8 in septic animals. The mRNA and protein was downregulated for up to 10 days with a decrease in apoptosis of hepatic and splenic cells. siRNA application resulted in a significantly improved survival of septic mice [59]. Thus, it was proposed that application of siRNA targeting the critical transcription factor FoxP3 might abrogate the immuno-suppressive activity of Treg *in vivo,* thereby facilitating the generation of an efficient anti-tumor immune response.

SUMMARY

Biochemical intervention strategies for the modification and/or inhibition of Treg function might substantially improve the efficacy of anti-tumor strategies aiming to target cancerous cells *via* help of the host's immune system (i. e. by dendritic cell vaccination or antibody therapies) (see Fig. **2**).

NOTE ADDED IN PROOF

After acceptance of the manuscript, two independent research groups reported on the value of Danileukin for the depletion of Treg in vivo. Attia and co-workers (J Immunother 2005 Nov-Dec; 28(6): 582-92) were not able to detect an *in vivo* depleting effect of the compound in melanoma patients. In contrast, Dunnull *et al.* (J Clin Invest 2005 Dec; 115 (12): 3623-33) described a Treg-depleting

effect of Danileukin, which in turn markedly enhanced the efficacy of a DC-vaccine in patients suffering from renal cell cancer. These contrasting results are most likely due to different application schedules used in the two different studies.

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