Therapy with CD4+CD25+ T regulatory cells – should we be afraid of cancer?

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This review focuses on the role of regulatory T cells (Tregs) in the process of carcinogenesis. The controversy of this issue arose due to the increasing therapeutic use of Tregs in humans (inter alia, in the treatment of autoimmune diseases). It is mainly due to potential dangers related to immunosuppressive activity of these cells, especially regarding cancer. The natural function of regulatory T cells (which is the suppression of excessive activity of the immune system) is purportedly linked to an increased risk of cancer initiation. This work brings together and summarizes the most important reports of researchers dealing with this problem and attempts to explain doubts and fears related to Tregs and their uncertain connection with cancer initiation and progression. It is clearly shown that regulatory T cells are associated with acceleration of existing tumors (they are attracted by microenvironments created by cancer cells) but cannot initiate them on their own.

Key words: T regulatory cells, immunotherapy, cancer, immunosuppression, tumor induction.

Introduction

Regulatory T cells (CD4+CD25 [high]FoxP3+ Treg cells) are lymphocytes with predominantly suppressive activity in the immune system. There are two major subpopulations, natural T regulatory cells generated during thymocyte development in the thymus (nTreg or tTreg) and those induced from naïve T cells in the periphery during the immune response (iTreg).

Treg cells express many molecules which are good marker candidates. The most characteristic include CD25 (IL-2 receptor of α chain), CTLA-4 (CD152), GITR molecule (glucocorticoid-induced TNFR family related gene), CD45RO, CD122 (IL-2 receptor of β-chain), HLA DR, CD62L, PD1, Notch, OX40 (CD134), CD103 and low to no expression of IL-7 receptor [1, 2]. CD4+CD25+ Treg cells can also produce IL-10, TGF-β and IL-4, but not IL-2 [3]. Yet, the most characteristic marker important for the activity of Treg cells is the transcriptional factor FoxP3 [4]. It is well known that mutations of the gene encoding the Treg-specific *foxP3* cause immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome [5].

In the body, Treg cells are mainly responsible for maintaining immune tolerance, which means unresponsiveness of the immune system to auto-antigens together with an efficient robust response to non-self-antigens and also the response to exogenous antigens in order to reduce the reaction and chronic immune activation [5]. This way an antigenic challenge is able to ignite the response during infections or after vaccination and there is no response to self-tissues, a fetus or transplanted organs [6]. This is one reason why Treg cells are currently being intensively tested in clinical trials as a novel cellular drug in autoimmune diseases and transplantation [7]. On the other hand, Treg cells are linked to tumorigenesis as the properties of these cells can also impose tolerance of malignant cells and therefore facilitate progression of tumors. A large number of Treg cells can be found in and around tumor tissues including local lymphoid tissue draining the tumor [8–10]. Moreover, the abundance of these cells in malignancy is often associated with worse prognosis for the patient [11].

There are many mechanisms that cause the development of cancer. The fundamental abnormality is the uncontrolled continuous proliferation of malignant cells. Cancer cells infiltrate and attack normal tissues by growing and dividing in an uncontrolled manner and then spread as metastases through the whole body, impairing the function of particular systems, which can be fatal. In general, loss of growth control is the result of genomic instability of malignant cells, but the immune system constantly surveys the tissues and usually eliminates such cells at a very early stage of anaplasia [12]. For some reasons, in some rare cases such surveillance is inefficient and cancerous cells are able to sneak through the tight control of immune cells, which results in a clinically evident tumor.

Some researchers believe that the induction of tumors is possible due to enhanced activity of Treg cells suppressing the immune response, while others conclude that the volume of the tumor must be big enough to attract Treg cells and the suppressive activity of these cells is only responsible for the late stages of tumor growth and metastases. So, is there tumor or Treg overactivity first? This "egg or hen" dilemma is important as tolerogenic therapies with Treg cells or agents inducing these cells *in vivo* are currently on the way to the clinic [7] and eventual tumorigenesis would be an extremely serious adverse effect hampering the routine use of such treatment.

Correlation between immunosuppression, regulatory T cells and cancer

There is a clear correlation between the level of immunosuppression and the possibility of cancer. For example, patients with solid organ transplants have a higher risk of developing cancer due to the immunosuppression administered to maintain the function of the allograft. Extensive research in kidney transplantation covering 42 countries and 200,000 patients demonstrated up to a 12-fold greater risk of development of lymphoma in recipients of the graft as compared to the general population [13, 14]. Importantly, the immunosuppression in this example was administered as unspecific pharmacological agents which, apart from the localized effects around the allograft, affect the whole body.

While Tregs have a suppressive role, it can be suggested that they can induce tumors similarly to pharmacological agents. Moreover, epidemiological data are in favor of such a hypothesis. It has been shown that the risk of cancer development increases with the patient's age [15]. Interestingly, the level of regulatory T cells also increases with age. Importantly, the more severe frailty in ageing is, including oncologic diseases, the higher is the level of Treg cells [16–18]. Of course, the growing number of Tregs is one of many elements related to the aging of the immune system (immunosenescence) and reduced immunological surveillance which may affect the increased risk of cancer. Hence, apparently the evidence from nature suggests that the increased level of Treg cells in the elderly may be associated with higher incidence of tumors at this age.

Regulatory T cells do not initiate cancer but promote its progression

Considering the fact that the level of Treg cells is associated with the incidence of cancer, the main question is 1) whether they can induce cancer or 2) they are a silent witness to the entire cancer process or 3) they are active players in the advanced stages of the disease only.

Treg cells are able to inhibit the anti-tumor effector function of immune cells. For example, natural killer (NK) cells are inhibited in tumor-bearing hosts by TGF-β bound to the Treg cell membrane. *In vivo* studies showed that in mice bearing tumor and treated with Treg cells more lung metastases were observed than in untreated tumor-bearing controls, which was associated with the activity of NK cells. In addition, the depletion of Treg cells improved both the course of the disease and the activity of NK cells [19]. This confirms that increased levels of Treg cells definitely promote the progression of already existing tumors. However, it should be emphasized that in the cited study and many later reports, this happened only when the cancer was already present and Treg cells could drive its progression.

Nevertheless, there is no good proof that Treg cells can induce cancer in a healthy tumor-free body. It is rarely reported in animal models that healthy animals treated with adoptive transfer of Treg cells develop cancers. Also, no link between the induction of tumors and Treg cells has been confirmed in humans. There are a number of ongoing or already completed clinical trials with adoptive transfer of Treg cells and only one of them reported diagnosis of tumors after administration of Treg cells [7, 20]. For example, in our trial with children with newly diagnosed type 1 diabetes, these patients were administered intravenously one or two doses containing from 1×10^7 to 3×10^7 autologous Treg cells per kilogram of body weight. As one of the important inclusion criteria was no history of neoplasm, we are sure the children were otherwise healthy. No severe side effects of therapy, notably tumors, were observed during the two-year follow-up period and also post-trial screening has been negative until today, which is more than 5 years after infusion [21, 22]. The only human trial that reported tumors after administration of Treg cells is inconclusive. It was performed in the treatment of graftversushost disease after bone marrow transplantation due to hematological malignancies [23]. This indicates that these patients were heavily immunosuppressed with many pharmacological immunosuppressants and chemotherapy long before the therapy with Treg cells was commenced [23]. It is therefore unlikely that Treg cells were the only reason for tumor induction but it is possible that the infusion of these cells accelerated the development of already existing but not diagnosed tumors. This should alert all physicians attempting to use tolerogenic cells in the treatment that the patients must be very carefully screened for eventual tumors at recruitment to the therapy.

Paradoxically, some researchers observed protective effect of Treg cells against some types of malignancy. It has been found that the increased level of Treg cells, especially Treg cells infiltrates in the affected lymphoid tissue, can be a positive predictor of patient's survival in follicular lymphoma, and a marked reduction in Treg cells was observed on transformation to aggressive diffuse large B-cell lymphoma (DLBCL) [24]. An increased number of Treg cells may also be an advantage in colorectal cancer (CRC). It has been reported that a large number of Treg cells is associated with lower metastatic scores in this tumor [25]. This effect probably depended on the inhibition of Th17 response by Treg cells. *Ex vivo* studies showed that Treg cells have the ability to inhibit the secretion of pro-inflammatory cytokines (IL-17 and IL-22) by Th17 cells in this environment [26]. To be fair, this may be a unique feature of this particular kind of tumor, in which the inflammation promotes metastases. Apart from the increased number, also the proportion of particular subsets of Treg cells infiltrating the tumor in the CRC may have an impact on the final prognosis [27, 28]. Moreover, there have been ob-

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served beneficial effects of IL-10 therapy, which is one of the major suppressive cytokines secreted by Treg cells, in patients with CRC. In the mouse model, treatment based on PEGylated IL-10 has shown an increase in CD8$^+$ T cell activation and IFN-γ production [29], whereas a clinical trial in humans indicated an increased amount of IFN-γ T cells while lowering IL-17 production [30].

The reason that Treg cells do not induce tumors and only promote the existing ones seems to be implicated by the biology of these cells. Although Treg cells suppress the activity of the main subsets involved in immune surveillance against tumors, this regulation is very much localized and precise. A local mode of the suppression exerted by Treg cells is of special importance. The studies on graft rejection in animals showed that these cells mainly accumulate and activate in the graft and in the local lymphoid tissues [31]. In addition, Treg cells regulate cytotoxic subsets, such as CD8$^+$ T cells and NK cells, mainly through direct cell-to-cell contacts [32]. Both CD8$^+$ T cells and NK cells show reduced cytotoxicity and lower production of IFN-γ and perforin when co-cultured with Treg cells [32]. Studies show also that when Treg cells do not have direct contact with other cells, they do not show inhibitory features. Their suppressive effect depends on the direct cell-to-cell contact and very easily disappears after separation from CD8 or NK cells by transwell. Hence, the suppression is selective and not generalized, as confirmed also in other studies [33]. This selectivity has also been confirmed in our trial with diabetic patients. While the infused cells were able to delay the ongoing autoimmune process in the pancreas, the immune memory (after immunization), induced before the treatment with Treg cells, remained intact [21]. Importantly, Treg cells themselves are not active and do not perform their inhibitory function until they receive an activation signal (e.g. from ongoing inflammation). Treg cells exert suppressive activity only when activated, which is suggested by their need for IL-2 [34]. This cytokine is secreted and provided by other cells mainly during an ongoing immune response and inflammation [22]. Hence, there must be inflammation or some antigenic challenge, which induces production of cytokines including IL-2, and only after that are Treg cells attracted to the site of inflammation and exert suppression. For these reasons, it seems unlikely that Treg cells induce tumors, as it seems that without a prior stimulus they do not suppress and remain "silent" in the body. There must be a tumor first that ignites some level of inflammation, which subsequently activates Treg cells. From this angle, Treg cells should strongly accelerate existing tumors with a high proinflammatory profile such as melanoma. Indeed, a high level of Treg cells in this tumor is noted in late stages of the disease [35] and the blockade of CTLA-4, a major receptor of Treg cells, is one of the most effective treatments when combined with other forms of immunotherapy [36, 37].

The plasticity of Treg cells can also explain why these cells alone are unlikely to induce formation of tumors. Under conditions of prolonged stimulation, as in the tumor environment or inflammation, these cells lose their Treg phenotype and, consequently, also their function [38]. It may be a mechanism facilitating clearance of the infection and reducing the time of the inflammation to a minimum. The Treg cells' lineage stability is complicated and still not fully understood and can be influenced by many factors [39]. For example, even slight changes in the environment, such as temperature, strongly affect the changes in the phenotype and function of Treg cells [40]. Moreover, Treg cells can turn into a Th17-like pro-inflammatory phenotype and produce pro-inflammatory cytokines [41]. A swap from suppressive to inflammatory phenotype is a serious and confirmed problem in autoimmune diseases and cancers [42].

Treg cells in tumors are heterogeneous and many of them are non-classical Treg cells. Researchers have noted that the Treg population present in the tumor is in part different from the thymic natural regulatory T cell population. For example, they can also be Tr1 cells characterized by high immunosuppressive abilities. In studies in a CRC, it was shown that up to 30% of tumor-infiltrating lymphocytes were Tr1. They are able to produce IL-10 and TGF-β. Some reports suggest that the suppressive abilities of these cells could be 50 times higher compared to autologous FoxP3$^+$ Treg cells [43]. In addition, Treg cells expressing latency-associated protein (LAP) are found in CRC and have some association with poor prognosis in patients [43–45]. In many types of cancers regulatory cells were characterized by low expression and even lack of FoxP3 expression [27]. On the other hand, studies showed that also CD8$^+$ T cells can express FoxP3 and have inhibitory action towards CD4$^+$ T cells [46]. Such cells are also identified in tumor tissues. *In vitro*, CD8$^+$ T effector cells co-cultured with ovarian tumor cells have been shown to convert to CD8$^+$FoxP3$^+$ T cells [47]. This demonstrates not only that the tumor microenvironment plays an important role in modulating the immune response but also that this microenvironment is the initiating factor for these modulations. Thus, it supports the idea that Treg cells alone cannot induce cancer and can only be an intensifying factor in the development of already existing tumors.

There is no evidence that Treg cells can induce cancer. It is more accurate to say that they accelerate the progression of existing cancers.

Does the tumor induce regulatory T cells?

The ability of Treg cells to inhibit immune responses sustained by cytotoxic lymphocytes is a mechanism commonly used by tumor cells. Regulatory T cells present in the tumor microenvironment may cause further progression of the cancer [48], and as a consequence, an increased risk of metastasis and poor patient survival [49]. Scientists have shown that partial or complete removal of these cells reduces tumor progression [19, 36, 37]. It is therefore important to gain deep knowledge on how tumor cells use Treg cells to defend against anti-tumor responses.

Tumors attract Treg cells

It has been proven that chemokines effectively recruit Treg cells into a tumor. The first axis was CCR4 and its ligands CCL22 and CCL17. Firstly, it has been shown that the expression of the CCR4 receptor is higher on Treg cells than on any other immunoreactive cells. In addition, tumors

produce large amounts of the ligands for CCR4, which are CCL22 and CCL17 [49]. Curiel *et al.* have shown that the production of CCL22 induces Treg recruitment to the cancer site. The blockade of this interaction with monoclonal antibodies against CCL22 in ovarian cancer resulted in a decrease of Treg cells recruitment and an increased antitumor response [49].

The specificity of the tumor architecture and its microenvironment should be taken into account when specific chemokines are considered. For example, hypoxia and angiogenesis increase the levels of CCL28, the ligand for CCR10 expressed by Treg cells. Hence, advanced tumors with hypoxic regions and/or building their own microcirculation can attract Treg cell migration using this axis [50].

However, not all chemokines present in the tumor clearly attract Treg cells. For example, the CXCR4-CXCL12 axis can explain specific recruitment of Treg cells. The studies have shown that the population of FoxP3+ T cells expressed a high level of CXCR4 receptor in several carcinomas such as advanced cervical cancers. However, Curiel *et al.* used a monoclonal antibody blocking CXCL12, the ligand for this receptor, in ovarian cancer without any significant effect on Treg cell migration [49]. Regarding this issue, a research group also examined the role of CCL21/CCR7 signaling but the results were inconclusive [51].

This can be even more complicated if cytotoxic cells use chemokine signals similar to Treg cells to navigate towards tumors. For example, CCR5 receptor and its ligands CCL3, CCL4 and CCL5 are involved in Treg activity. A selective blockade of either ligands or receptor decreased the percentage of Treg cells infiltrating the tumor but also impaired the activity of anti-tumor cells [52]. According to these results, additional activity of CXCR3-CXCL9/CXCL10/CXCL11 axes is needful for effector cells to enhance the anti-tumor response. Therefore, therapies based on blocking these axes might be risky, even if they are sufficient to clear Treg cells from the tumor environment [53].

The data indicate that receptors such as CCR4, CXCR4 and CCR10 are mainly responsible for the accumulation of intra-tumor Treg cells. The detailed knowledge about pathways mentioned above can be used in therapies based on depletion and blocking the activity of Treg cells, but we must be sure that the same mechanisms are not necessary to ignite anti-tumor cytotoxic responses of effector cells.

Tumors induce proliferation of Treg cells

Some reports show that Treg cells not only migrate to the tumor from the periphery but it is also possible that they can proliferate in the tumor. It has been shown that this is a mechanism dependent on indoleamine 2,3-dioxygenase (IDO). IDO production by mature monocyte-derived dendritic cells (moDCs) enhances proliferation of CD4+FoxP3+ T cells [54]. Immature dendritic cells (DCs) are not as efficient as mature DCs. IDO is an important factor in immune balance and down-regulating effector T cells. This action is based on tryptophan catabolism and kynurenine formation. Chung *et al.* demonstrated the importance of an IDO-dependent mechanism, which can be used for DC-based immunotherapy. They have shown that

Treg cells' activity depends on direct contact between DCs and suppressive T cells. The IDO-dependent mechanism of Treg cell proliferation requires at least two signals: the direct interaction of CTLA-4 on Treg cells with CD80 and/or CD86 receptors on DCs and endogenous IL-2. In the presence of the transwell inserts separating Tregs from DC, T effector cell proliferation has been observed. On the other hand, the absence of the transwell and direct contact promoted suppression. Similarly, it has been clearly demonstrated that using a monoclonal antibody against IL-2 blocked Treg cell activity, which highlighted the need for IL-2 [54]. A recent player in this action is a novel suppressive population of suppressors called myeloid-derived suppressive cells (MDSCs). The interaction of intratumoral IDO and Treg cells has been found to be important in the recruitment and activation of MDSCs [55]. MDSCs can then further promote Treg cell proliferation, which creates a positive feedback loop for an enhanced immunosuppressive environment in the tumor [56].

Another mechanism surprisingly involved in intra-tumor Treg cell proliferation is associated with angiogenesis and the VEGFA-VEGFR pathway. To emphasize the fact that Treg cell proliferation depends on the VEGFA-VEGFR pathway, it is worth mentioning that expression of VEGFR-1,-2 is increased in tumor-bearing mice in comparison to healthy controls. However, only VEGFR-2 is involved in VEGFA induced Treg cell expansion [57]. The data from a murine model of colorectal carcinoma also suggested that blockade of the vascular endothelial growth factor (VEGF) pathway significantly decreased the amount of regulatory T cells. Some therapies against different tyrosine kinases have been used to show a possible impact of VEGFA-VEGFR on the Treg cell population. According to that, anti-VEGF antibody and sunitinib (which targets VEGFR-1,-2,-3, PDGFR, c-kit and FLT3) were used in the study. The therapy caused a significant decrease in the Treg cell level and total amount in the spleen and tumor, whereas masitinib, which targets a number of tyrosine kinases other than VEGFR, did not reduce the Treg cell ratio. This very important study suggests that novel therapies based on tyrosine kinase blockade could reduce Treg cell numbers in cancer patients [58]. Finally, in patients treated using chemotherapy with bevacizumab (a monoclonal antibody against VEGF) a decreased level of Treg cells in peripheral blood is observed. It is crucial that the treatment has no influence on Treg cell function and it does not limit the cells involved in the anti-tumor response.

Altogether, it is clear that Treg cells are used by the tumor to progress. Microenvironments created by malignant cells attract this suppressive subset mainly via produced chemokines. Recruited intra-tumor Treg cells additionally expand due to several mechanisms including mainly IDO and VEGF pathways.

There is strong evidence that Treg cells accelerate the progression of existing cancers.

Conclusions

Novel therapies with tolerogenic Treg cells require strong proof of safety to develop further. Having in mind

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that immunosuppressive therapies are often associated with increased risk of cancer, it is advisable to continuously check the safety. Currently, it seems that Treg cells are an ideal therapeutic tool as they exert precise suppression in a localized way, which excludes the possibility of generalized immunosuppression with its adverse effects, notably tumorigenesis. The evidence from performed studies suggests that Treg cells cannot induce tumors. Nevertheless, they are very efficient in the progression of existing tumors. Hence, Treg cells can be safely used as therapeutics in tumor-free patients, but the therapy must be preceded by a careful check for any record of malignancy. If there are any doubts that such a history exists, as in graft-versus-host disease after bone marrow transplant, the risk-to-benefit ratio should always be thoroughly assessed before the final decision.

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