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REVIEW

Regulatory T Cell as a Target for Cancer Therapy

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Abstract Advances in our understanding of $CD4^+$ $CD25^+Foxp3^+$ regulatory T cells (T_{Regs}) enabled the characterization of their activities in maintaining peripheral tolerance, preventing autoimmune diseases, and limiting chronic inflammatory diseases. Ironically, an effective action of these cells during tumor development can limit beneficial responses by suppressing immunity and limiting antitumor resistance, whereas one of the main functions of the immune system is to eliminate malignant cells. During the last years, the immunological role, mechanism of action, and clinical importance of these cells were profoundly characterized and the relationship between this subset of lymphocytes and cancerous cells arises as a key factor that influences tumor development. Recent insights obtained from clinical studies and experimental mouse models expand our perception of the potential role of T_{Regs} in cancer treatment. In this review we describe the basic mechanisms of T_{Reg} origin and differentiation, their potential role in cancer, as well as the future perspectives concerning the modulation of these cells as a potential approach for anticancer strategies.

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Introduction

Cancer is an important cause of morbidity and mortality in children and adults that originates from the uncontrolled proliferation and spread of clones of transformed cells. Although the tumor arises from changes in self cells, the immune system reacts against the tumor by developing an adaptive response, especially as a result of the expression of tumor-associated antigens (TAAs) recognized by the immune system of the tumor bearer (De Visser et al. 2006; Hamerman et al. 2005). Nonetheless, tumors can escape immune surveillance. The process of evasion may result from various mechanisms, demonstrated by some experimental evidence in murine models indicating that the immune responses to tumor cells provide selective pressures that result in their survival and spread. Among the mechanisms known, regulatory T cells (T_{Regs}) have an important role in the suppression of the T-cell response to tumors. Evidence from mice models and cancer patients indicates that the population of T_{Regs} increases in parallel with tumor growth. Some experiments showed that depletion of T_{Regs} in mice affected by tumor increases antitumor immunity and reduces tumor growth (Bennett et al. 2001; Brunkow et al. 2001; Wildin et al. 2001).

The role of T_{Regs} is concerned with the development of autoimmunity, allergy, and rejection as well as the suppression of immune responses to cancer (Sakaguchi et al. 2008; Yamaguchi and Sakaguchi 2006). T_{Regs} are a specialized subset of $CD4^+$ T cells implicated in the suppression of immune response. This population is differentiated from other $CD4^+$ cells by the high expression of

CD25 and by the expression of the transcription factor Foxp3 (forkhead/winged helix transcription factor). These cells have essential roles in the maintenance of immune homeostasis, working as regulating effector T cells which respond through a variety of processes, such as the production of inhibitory cytokines, such as interleukin (IL)-10, IL-35, and transforming growth factor (TGF)-β, cytolysis induction by the production of granzymes, disruption of effectors cell metabolism, and dendritic cell (DC)-mediated suppression (Feuerer et al. 2009; Fontenot et al. 2003; Yamaguchi and Sakaguchi 2006).

Significant progress has been made over the past few years in delineating the molecules and mechanisms that T_{Regs} use to mediate immune suppression (Gavin et al. 2006; Hori et al. 2003; Rubtsov et al. 2008; Vignali et al. 2008). In this review we outline our current understanding of the mechanisms used by T_{Regs} to mediate suppression and the challenges that lie ahead in defining their mode of action. We also discuss whether T_{Regs} are likely to depend on one, a few, or many of these mechanisms. Unless otherwise stated, we primarily focus on the mechanisms of the $CD4^+CD25^+Foxp3^+$ T_{Reg} subsets.

Ontogeny of T_{Regs}

T cells are responsible for cellular immunity, developing their activity via recognizing antigen through cell-surface receptors (TCRs). Most T cells also express glycoproteins, CD4 or CD8, which are important for TCR signaling (Egerton et al. 1990; Huesmann et al. 1991). The expression of CD4 or CD8 defines two T-cell lineages which differ in their MHC specificity and function. $CD4^+$ T cells originate from blood marrow as whole lymphocytes at the end of fetal life during hematopoiesis (Alam et al. 1996; Kisielow et al. 1988; Teh et al. 1988). The process of differentiation is complex and involves several steps. As $CD4^+$ and $CD8^+$ T cells are derived from a common precursor pool of double-positive (DP) thymocytes, there is a series of events that culminates in a lineage choice during the differentiation of DP thymocytes into $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive (SP) thymocytes, a process known as positive selection. This is a process in which thymocytes that express TCRs for self peptide–MHC complex are rescued from cell death, while thymocytes whose receptors do not recognize self peptide-MHC complex are killed due to the lack of recognition. This positive selection ensures that mature T lymphocytes are restricted by MHC molecule, the $CD8^+$ T cells to MHC I and $CD4^+$ T cells to MHC II. By contrast, negative selection occurs when thymocytes that express an autoreactive TCR are actively eliminated (Borgulya et al. 1991; Bosselut 2004; Von Boehmer 1994, 1996). The correct conformity of these steps guarantees the lineage choice between $CD4^+$ and $CD8^+$ T cells.

When DP thymocytes turn into SP thymocytes and then the $CD4^+$ T cells are finally differentiated, the question arises of how the subsets of $CD4^+$ T cells are shaped. Most subsets of $CD4^+$ T cells are thought to differentiate from a common precursor, naïve $CD4^+$ T cells, depending on the cytokines present during the primary antigenic stimulation of the naïve T cells. After interaction with related antigen presented by antigen-presenting cells, $CD4^+$ T cells can differentiate into a variety of effectors subsets, including Th1, Th2, and, more recently, Th17 cells, follicular helper T cells, and T_{Regs} (Fig. 1) (Mahic et al. 2008; Zhou et al. 2009b).

Some categories of T_{Regs} have been characterized and two subsets are best known. One category, naturally occurring in the thymus is identified as nT_{Regs} and the other, which differentiates in the periphery, is inducible by some cytokines and is identified as adaptive T_{Regs} (T_{Regs}^{Adapt}). Both subsets are characterized by the expression of the forkhead transcription factor Foxp3 (Pacholczyk et al. 2006; Zhou et al. 2009b).

nT_{Regs} are generated during the early stages of fetal and neonatal T-cell development (Sakaguchi et al. 2001). These cells are generated in the thymus and then exported to peripheral tissues, where it is proposed that they normally function. The thymus-induced regulatory cells are $CD4^+$ and they typically express high levels of CD25 as well as the co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4), the tumor-necrosis factor (TNF) superfamily member GITR (glucocorticoid-induced TNF receptor family related protein, TNFRSF18), and the x chromosome-encoded forkhead transcription factor forkhead boxp3 (Foxp3) (Fontenot et al. 2005; Pacholczyk

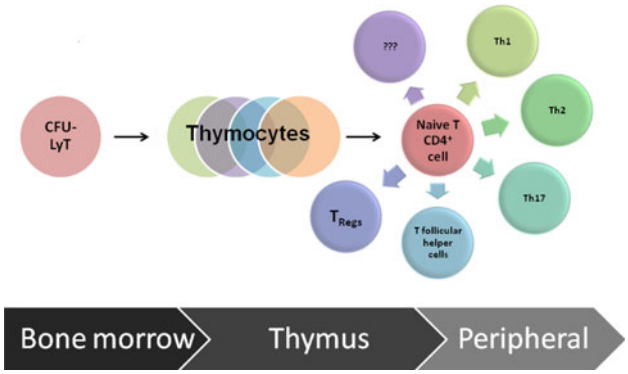

Fig. 1 Summary of $CD4^+$ T-cell subset differentiation. Initially, CFU-LyT, which is formed in the bone morrow, differentiates into thymocytes. During thymocyte development, immature thymocytes that express both CD4 and CD8 genes must choose to be either a helper $CD4^+$ or cytotoxic $CD8^+$ T cells (not shown). After activation, $CD4^+$ T cells can differentiate into different lineages of T-helper cells with distinct biological functions

et al. 2006; Ziegler 2006). Foxp3 has been demonstrated to be an essential factor for the suppressive phenotype of nT_{Regs}. This was corroborated when mutations in the Foxp3 gene led to fatal autoimmune disease in rodents and humans. On the other hand, Foxp3 is transiently induced upon activation of $CD4^+CD25^-$ T cells and is also present in T_{Regs}^{Adapt} (Hsieh et al. 2006; Lin et al. 2007) (Fig. 1).

nT_{Regs} are known as a "natural" population because they are always present in normal individuals and carry out their regulatory function during the normal surveillance of self antigens (Bluestone and Abbas 2003). Despite the certainty of these cells, the signals that are responsible for the generation of T_{Regs} in the thymus are undefined. However, there are some investigations that indicate that CD28 controls nT_{Regs} in the thymus (Bluestone and Abbas 2003). Furthermore, T_{Regs}^{Adapt} are generated from mature $CD4^+$ T-cell populations under certain conditions of antigenic stimulation and they can be induced ex vivo by culturing mature $CD4^+$ T cells with antigen or polyclonal activators in the presence of immunosuppressive cytokines (Feuerer et al. 2009). Despite what is already known, there is a remaining question of how immature thymocytes are selected to be T_{Regs}.

T_{Regs} in Cancer

Although mechanisms of natural and acquired immunity destroy tumor cells in vitro, the major challenge for immunologists is to determine which of these mechanisms may contribute to protective responses in vivo against the tumor. Cancer cells are known to express several foreign-looking antigens, the TAAs, recognized by the host immune system. These TAAs can stimulate either an innate or an adaptive immune response (Stevanovic 2002).

Initially, the mechanisms that trigger the innate immune response in cancer are unclear. Despite this, it is known that there is a major involvement of DCs, natural killer (NK) cells, macrophages, and mast cells (MCs). NK cells destroy many types of tumor cells, especially those that have reduced expression of MHC I, but express ligands for activating receptors of NK cells, such as MICA, MICB, and ULB, which are ligands for the receptor activator NKG2D (Raulet 2004). Several studies have demonstrated the importance of NK cells in tumor response. Smyth showed that NK cells are critical for an effective resistance to sarcoma formation (Smyth et al. 2001) and Kim demonstrated that transgenic mouse with defective natural killing cells failed in clearing transplanted MHC I-deficient tumor cell lines (Kim et al. 2000). Concerning macrophages' actions in cancer, it is well characterized that they have the potential to destroy tumor cells in culture, although these cells have a paradoxical role in the immune system during cancer development. First, the macrophage, as any innate immune cell, acts when tissue homeostasis is perturbed, releasing soluble mediators such as cytokines, chemokines, matrix remodeling proteases, and reactive oxygen species, and initiating phagocytosis, among others. Secondly, on the other hand, they contribute to the maintenance of inflammation, leading to neovascularization, which is fundamental for tumor growth (Błach-Olszewska 2005; Mantovani et al. 2002; Sica et al. 2008). Another innate cell, the DC, plays a critical role in eliciting innate and adaptive cellular immune responses, taking up TAAs and migrating to lymphoid organs, where it presents its antigens to lymphocytes involved in adaptive immunity. Finally, MCs were already identified in a diverse array of tumors in humans and animal models. Interestingly, accumulation of these cells in tumors is associated with poor prognosis, indicating that they have important biological roles in cancer development. MCs are known to regulate the early functions of DCs, consequently controlling the rise of tumor-specific T cells (Banchereau and Palucka 2005; Imada et al. 2000).

Innate immunity sets the stage for the activation of the more sophisticated adaptive immune system. T-cell immunity is the key to the protective immune responses against tumors. Traditionally, this function has been referred to $CD8^+$ T cells (CTLs) with cytotoxic activities that are restricted by MHC class I molecules. However, relative importance has recently been given to $CD4^+$ T cells due to their capability in protective anti-tumor response. Moreover, hosts which development tumor may induce antibody production against TAAs, which indicates an important defense mechanism against cancer (Coulie and Connerotte 2005; Harris 2004) (Fig. 2).

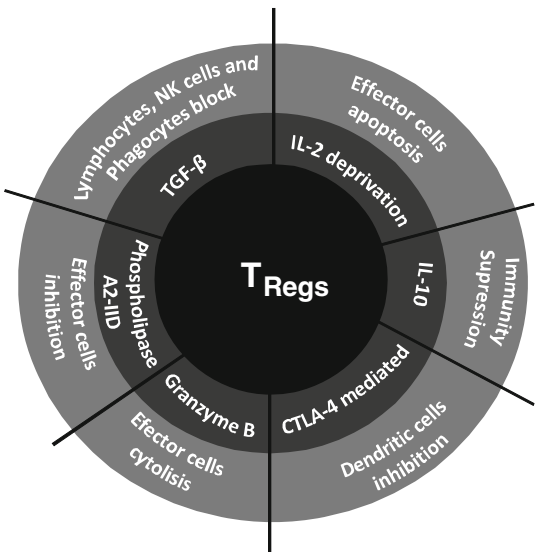

Fig. 2 Schematic representation summarizing T_{Reg} actions and their postulated mechanisms

However, some questions still intrigue immunologists. If the host immune system recognizes these TAAs and respond to them, why does cancer develop? Even though a host immune response against specific malignant antigens exists, it is well established that malignant cells commonly induce immune tolerance to escape the host response to these TAAs. Cancer is characterized by the lack of cell-cycle controls, which leads to excessive cell proliferation. This characteristic by itself is sufficient to break the immune system, as the cell production is greater than the host could eliminate via immunological mechanisms. Another fact is that most of the antigens expressed by cancer cells are not immunogenic and do not elicit an immune response. Nevertheless, several other observed characteristics of malignant cells are thought to be involved in the escape of these cells from host immunity.

Different types of defects in the expression of classical MHC class I antigens, resulting in total or partial loss of their expression, are related to tumor evasion from immune response. Because the correct expression of this protein is necessary for the proper action of $CD8^+$ CTLs, these changes may provide malignant cells with mechanisms to escape T-cell recognition and destruction (Seliger et al. 2002). The downregulation of other co-stimulatory molecules necessary for the immune response is also observed in malignant cells and is probably another mechanism by which these cells escape host antitumor immunity (Airoldi et al. 2003).

Other mechanisms involved in the process of immunotolerance observed in malignant cells are well described. The release of inhibitory cytokines has been found in a variety of cancer types. For example, secretion of TGF-β, which downregulates many of the processes necessary for CTL activation, is known to be a crucial step for tumor evasion (Weller and Fontana 1995).

Nowadays, a mechanism which is much discussed by which cancer cells escape the host immune response is the activity of T_{Regs}. As widely described earlier, this lymphocyte subset is known to inhibit the global immune response, which brings us to the following question: How influential are they in malignant development? For more than three decades, several efforts have been made to describe how these cells influence cancer progression, and this is the subject of the next section of the present review.

The Role of T_{Regs} in Cancer

In the 1980s, the involvement of regulatory lymphocytes in the suppression of the immune response against cancers started to be elucidated. Trying to explain the paradoxical observation that immunogenic tumors develop in immunocompetent hosts, Robert North and coworkers published a series of in vivo results that suggested the involvement of T lymphocytes in the suppression of the antitumor immune response (Berendt and North 1980; North 1982; North and Bursuker 1984). They observed that intravenous infusion of sensitized T cells from immunocompetent donors caused complete regression of large established tumors only in thymectomized T cell-deficient recipients and also that T cell-mediated regression of established tumors in T cell-deficient recipients can be inhibited by an infusion of splenic T cells from tumor-bearing donors. These data strongly suggested an involvement of subpopulations of T cells in the suppression of immunity against cancers.

More recently, after the identification of the $CD4^+$ T cells expressing CD25 as a regulatory cell, the function of this subset of T_{Regs} in cancer immunity has been further studied. Using an anti-CD25 monoclonal antibody, Onizuka and colleagues caused the depletion of T_{Regs} in animal models and observed the regression of tumors that grew progressively in syngeneic mice (Onizuka et al. 1999). This study suggested that $CD4^+CD25^+$ immunoregulatory cells were involved in tumor growth. In the same year, this idea was supported by the observation in vivo and in vitro that removal of $CD4^+CD25^+$ T cells, also using an anti-CD25 antibody, can break immunological unresponsiveness to syngeneic tumor cells (Shimizu et al. 1999). These results originally indicated that the T_{Regs} involved in the regulation of the antitumor response can, at least in part, be $CD4^+CD25^+$ T cells.

T_{Regs} exert their immunosuppressive effects by widely described mechanisms, which are probably related to their inhibition of host antitumor immunity. In the cited article by Shimizu (Shimizu et al. 1999), removing $CD4^+CD25^+$ T cells leads to the generation of two distinct types of effectors cells, $CD8^+$ CTLs and $CD4^-CD8^-$ NK-like cells, which can make one suppose that T_{Regs} negatively influence effector cells. Several lines of evidence support the notion that T_{Regs} can exert their regulatory activity by a cytolytic action over effector cells. Gene expression arrays showed an overexpression of granzyme B, a cytolytic protein, in mouse T_{Regs} (McHugh et al. 2002). Later, Noelle and coworkers (Gondek et al. 2005) reported that T_{Regs} derived from granzyme-B-deficient mouse had reduced suppressive activity in vitro. Recently it was documented that T_{Regs} suppress anti-tumor activity of NK cells and CTLs and that this suppression is a result of the granzyme-B-dependent cytolytic action of T_{Regs} (Cao et al. 2007). The cytolytic effects of T_{Regs} are well described, but the exact mechanism by which this event occurs and how this can influence the immune response against cancer are not entirely understood.

Not only effector T cells, but also NK cell activity was demonstrated to be hampered by the influence of T_{Regs}, and as previously discussed, NK cells are crucial for effective

antitumor immunity. NK cells cultured with T_{Regs} have been demonstrated to be less efficient due to a decrease in their cytotoxicity and diminished interferon (IFN)-γ secretion, which explains the observation that T_{Regs} depletion ameliorates NK cell-mediated lysis of tumor cells. Also, Foxp3-knockout mice exhibited increased NK cell proliferation, and similar results were achieved by pharmacologically depleting T_{Regs} with anti-CD25 monoclonal antibody (mAb) or immunostimulatory doses of cyclophosphamide. Finally, results obtained in humans support these findings once a lower concentration of circulating T_{Regs} in response to NK cell stimulation was observed in patients bearing gastrointestinal tumors. All these data were published by Ghiringhelli and colleagues in a single paper which provided crucial information concerning the interaction between T_{Regs} and NK cells (Ghiringhelli et al. 2005).

As mentioned in the introduction, T_{Regs} constitutively express CTLA-4, and recently it was shown that CTLA-4 is an important effector molecule mediating T_{Reg} actions in immunity. The control of T_{Reg} activity mediated by CTLA-4 was studied by Wing and colleagues. They generated CTLA-4 conditional knockout (CKO) mice in which CTLA-4 was specifically deleted in T_{Regs}. The CKO mice showed severe disruption of immunological equilibrium, dying prematurely at 7 weeks of age and showing severe splenomegaly, lymphadenopathy, cardiomegaly, liver congestion, and lymphocyte infiltration in several tissues due to a T cell-mediated autoimmune condition. When testing tumor immunity in these knockout mice it was proved that CTLA-4 deficiency in T_{Regs} leads to enhanced antitumor immunity. It was also observed that splenic DCs, when cultured with CTLA-4-deficient T_{Regs}, do not downregulate CD80 and CD86 expression, as commonly observed when DCs are co-cultured with normal T_{Regs} (Wing et al. 2008). In summary, these data strongly support the idea that the inhibitory function of CTLA-4 in T_{Regs} is due to the downregulation of CD80 and CD86, which limits the activation of naive T cells via CD28, and also that this pathway is probably involved in antitumor immunity.

Another postulated mechanism by which T_{Regs} can downregulate an immune response is the induction of apoptosis in immunological cells. As cited above, CD25 acts as a receptor for IL-2, and this characteristic of T_{Regs} made some authors hypothesize if they could induce IL-2 deprivation. In this way, Pandiyan et al. (2007) documented that, in fact, T_{Reg} consumption of IL-2 reduces its concentration, which directly stimulates effector cell apoptosis. Although these observations provide a better understanding of T_{Reg} actions in the immune system, we can only suppose if these mechanisms are involved in the inhibition of cancer immunity.

Suppression of $CD4^+CD25^-$ T-helper cells by T_{Reg} cells was already observed in vitro and is also a possible mechanism of immunosuppression induced by T_{Regs}. As cited above, $CD4^+$ T-helper cells are involved in the immune response against tumors and its suppression by T_{Regs} in the tumor's microenvironment possibly disrupts this antitumor activity. T_{Reg} suppressive action on $CD4^+$ cells is mediated by a contact-dependent mechanism and is dependent on the secretion of TGF-β (Nakamura et al. 2001). More recently it was postulated that T_{Regs} can suppress the proliferation of $CD4^+$ and $CD8^+$ cells by secreting phospholipase A2-IID, which inhibits the effector cells in vivo and in vitro (von Allmen et al. 2009).

Inhibitory cytokines such as IL-10 and TGF-β have been receiving considerable attention as mediators of suppression induced by T_{Regs} and were already proposed as key factors for the inhibitory actions of T_{Regs}. Both IL-10 and TGF-β are known to have several immunosuppressive effects that can promote the growth of several types of cancer (Hawrylowicz and O'Garra 2005; Strauss et al. 2007; Thornton and Shevach 1998). As an example, TGF-β appears to limit the antitumor activity of cytokine-induced killer cells (Hilchey et al. 2007), mediate the inhibition of NK cell proliferation and activity (Ghiringhelli et al. 2005), and its production by T_{Regs} has also been implicated in limiting antitumor immunity in clinical studies (Li et al. 2007).

As we can see, T_{Regs} are known to downregulate the antitumor immune response by several mechanisms which, in the end, can facilitate the development of cancer (Fig. 2). These observations bring us to the following question: What is the clinical significance of T_{Regs}?

Clinical Significance of T_{Regs}

For some years, several studies have been attempting to correlate the presence of infiltrating T lymphocytes in tumors with patients' outcome. It is easy to understand that different types of lymphocytes will influence cancer progression in different ways. For example, Zhang and colleagues (Zhang et al. 2003), measuring $CD3^+$ effector T cells in ovarian tumor specimens from patients with advanced disease, observed that patients with tissues containing $CD3^+$ cells had an overall survival of 38%, while tissues where infiltration of $CD3^+$ cells was absent predicted a survival of 4.5%. The obvious conclusion is that effector lymphocyte infiltration in ovarian cancer is associated with a better prognosis. One year later, accumulation of $CD4^+CD25^+$ cells in ovarian cancer was documented by Curiel et al. (2004). This study showed a high concentration of functional T_{Regs} in patient's ascites and tumors concomitantly to a low concentration in tumor-draining lymph

nodes, suggesting a preferential recruitment of these cells to the tumor mass or associated ascites. Analyzing tumor-associated survival, they reported that T_{Regs} were a significant predictor of death. In this same article it was documented that tumor T_{Regs} inhibit TAA-specific T-cell immunity also by blocking the production of IFN-γ and IL-2 by CTLs. We can observe that although infiltration of $CD3^+$ cells signifies a good prognosis for ovarian cancer patients, the infiltration of $CD3^+CD4^+CD25^+Foxp3^+$ cells predicts a worse progression, strongly suggesting that the lymphocyte-mediated immune response is an important factor for cancer development.

Several other studies regarding T_{Reg} infiltration in solid tumors have been published. A high concentration of T_{Regs} was already demonstrated in tumors collected from patients with hepatocellular carcinoma (HCC). Performing immunohistochemical staining of Foxp3 in paraffin-embedded tissues from HCC patients, Zhou et al. (2009a) observed an accumulation of these cells in the intratumoral region, while in the peritumoral and nontumoral regions they were rarely observed. A significant inverse correlation between intratumoral $Foxp3^+$ T_{Reg} density and overall survival or disease-free survival was also observed, and a higher intratumoral prevalence of these cells was correlated with the presence of cirrhosis or later cancer stages. These data indicate that the accumulation of these cells is associated with disease progression and also that T_{Regs} in the tumor's microenvironment results in impairment of tumor-specific cell-mediated immunity in HCC. In this study there was also observed an association between macrophage and T_{Reg} infiltration, probably via IL-10 production.

Peterson and coworkers (Petersen et al. 2006) observed in non-small-cell lung cancer an increased prevalence of T_{Reg} cells infiltrating the tumor and also that a higher ratio of T_{Regs} and total tumor-infiltrating T lymphocytes is associated with the development of metastasis and a reduction in cancer-specific survival. Interestingly, an abnormal count of T_{Regs} in cancer patients is not only observed in the tumor area, but also in peripheral blood. For example, a recent study also made with lung cancer patients showed an increase in frequency of T_{Regs} in the peripheral blood of non-small cell lung cancer patients. In fact, several other cancer sites, such as gastrointestinal (Tokuno et al. 2009), pancreas, and breast cancer (Liyanage et al. 2002) and melanoma (Javia and Rosenberg 2003), were also screened for the prevalence of these immunosuppressive cells with positive results.

Interestingly, the results observed in studies with some hematological cancers go in a different direction. The presence of T_{Regs} in high concentrations seems to improve patients' prognosis. Studying lymphoma tissue from 926 patients immunohistochemically, Tzankov et al. (2008) observed that higher numbers of T_{Regs} correlated with improved survival for follicular lymphomas and classical Hodgkin's lymphomas. In another study with patients with classic Hodgkin lymphoma, similar data were obtained and a low infiltration of $Foxp3^+$ cells was supposed to represent a biological marker predicting an unfavorable outcome. This benefit of high T_{Reg} concentration in B-cell lymphoma patients can be explained by the suppressive effects of T_{Regs} on B-cell function, which is overproduced in these malignancies.

Overall, almost all studies support the idea that infiltration of T_{Regs} is associated with disease progression in patients with solid cancers. Data collected over the last decades opened the possibly of using T_{Regs} as a target for the development of new therapeutic strategies. Targeting these cells is a promising approach and some clinical trials are already being made.

T_{Regs} and Anticancer Therapy

Recent studies concerning the relation between T_{Regs} and cancer progression strongly indicate a preponderant importance of this subset of lymphocytes in the suppression of anticancer immunity. This conclusion opens a very promising new perspective in the field of cancer therapy: modulation of T_{Reg} activity. Even before T_{Regs} started being discussed as a possible target for treatment, several drugs acting on these cells were routinely used. Observations made with chemotherapeutics commonly applied in the treatment of several cancers showed that some of these drugs directly modulate T_{Reg} activity.

Cyclophosphamide is a valuable alkylating agent with cytotoxic action used in the treatment of a large number of carcinomas and other cancers. This drug is known to have strong immunosuppressive action, inducing a pancytopenic state. Some studies, however, indicate that the use of low doses of cyclophosphamide selectively reduces T_{Reg} concentration, not influencing the total number of lymphocytes and NK cells (Ghiringhelli et al. 2007). The same research group, 3 years before publishing these data, originally observed in vivo the effect of cyclophosphamide on T_{Reg} count (Ghiringhelli et al. 2004). Audia and coworkers also made interesting observations concerning the possible immunostimulatory effects of low doses of cyclophosphamide. Histological analysis of tumors from some patients included in the study showed that administration of cyclophosphamide reduced the infiltration of T_{Regs} while it induced $CD8^+$ lymphocytes (Audia et al. 2007), reflecting the positive results of the drug in tumor immunity. These studies were realized with different therapeutic regimens, but their results tend to demonstrate an immunomodulatory action of low doses of cyclophosphamide dependent of T_{Reg} inhibition.

The fact that cyclophosphamide can selectively suppress T_{Regs}, besides having its known cytotoxic effect, seems to reinforce the clinical application of this drug in oncologic therapy. A recent study used an animal mesothelioma model to assess the activity of cyclophosphamide and gemcitabine on cancer and suggested that a successful chemotherapeutic approach may depend on the status of T_{Regs} (van der Most et al. 2009). Cyclophosphamide cured tumor-bearing immunocompetent mice, while gemcitabine did not, probably due to the inhibitory activity towards T_{Regs} observed only in subjects treated with cyclophosphamide. Overall, this observation induces us to believe that different clinical outcomes in patients treated with similar drugs can be explained by immunological effects on T_{Regs} and, consequently, that the screening of drugs with immunological effects could make cancer therapy more rational.

Interesting results were obtained when studying the antineoplastic fludarabine. This drug is efficiently used in the therapy of chronic lymphocytic leukemia and macroglobulinemia, although sometimes it results in decreasing $CD4^+$ lymphocytes. Beyer et al. (2005) observed in a clinical study that fludarabine suppression of $CD4^+$ cells is greater in cells which are also $CD25^+$, i.e. T_{Regs}. This partially selective decreasing of T_{Regs} by fludarabine is probably advantageous for the treatment of neoplastic conditions.

Nowadays, even drugs recently approved by the Food and Drug Administration (FDA) are being screened for possible actions on T_{Regs}. This fact exemplifies the growing attention given by researchers to these cells' actions. Recently, lenalidomide and pomalidomide, two new drugs with known ability to enhance immune function, were submitted to several in vitro assays in order to assess if inhibition of T_{Regs} could explain, at least in part, their actions on the immune system. A decrease in the expansion of T_{Regs} in response to treatment with the two drugs and inhibition of their function were observed (Galustian et al. 2009). It is interesting to relate that both drugs had shown activity in cancer clinical trials and that lenalidomide is currently approved by the FDA for the treatment of patients with myelodysplastic syndromes and multiple myeloma, and these observations could clarify the obscure mechanism of action responsible for their therapeutic effects.

Perspectives

Due to the enormous amount of observations suggesting that the inhibition of T_{Regs} could have therapeutic usefulness, major projects are being realized to develop novel agents that directly target T_{Regs}. Several possible strategies could be used to concretize this idea. Reduction of T_{Reg} function, blockade of T_{Reg} effector functions, blockade of T_{Reg} differentiation, redirection T_{Reg} differentiation, and antigen-specific T_{Reg} elimination are possible effective approaches.

Chimeric Proteins

Immunotoxin LMB-2 is a very promising perspective drug under study which could provide relative selective destruction of T_{Regs}. This agent was originally described in 1993 and consists of a single-chain Fv fragment of the anti-CD25 mAb fused to a truncated form of the bacterial *Pseudomonas exotoxin* A, in which two amino acids are deleted (Kreitman et al. 1993). This toxin acts by inactivating elongation factor 2 and consequently inhibiting protein synthesis. The Fv fragment of the the anti-CD25 mAb implies that the toxin binds only in $CD25^+$ cells, including T_{Regs}; consequently the toxin preferentially enter cells expressing this antigen.

Some clinical trials with LMB-2 are already concluded, while others are still ongoing. Initially, major trials tested the use of this immunotoxin in patients with $CD25^+$ hematological malignancies (Kreitman et al. 2000) and refractory hairy cell leukemia (Kreitman et al. 1999), in which cancerous cells are known to express CD25. Both trials showed promising results, with relevant reductions in malignant circulating cells and improvement of global health status. Although these studies showed promising results, they cannot be related to a possible toxic action towards T_{Regs} once the patients enrolled in these studies presented cancers in which the cancerous cells express CD25. More recently, in 2007, an interesting clinical trial was published showing that treatment with LMB-2 of patients with metastatic melanoma reduced circulating and tumor-infiltrating T_{Regs}. Indeed, this trial was realized to assess if LMB-2 induced T_{Reg} depletion and elicited a better immune response to a peptide vaccination, but the results did not support this hypothesis (Powell et al. 2007). Moreover, the usefulness of this immunotoxin in association with vaccines is not discarded, and new clinical trials with dose optimization or even different peptide vaccines in the associated treatment can possibly bring promising results. In fact, there are still some active clinical trials sponsored by the United States National Cancer Institute that could obtain better results.

Denileukin diftitox (DAB389IL-2, Ontak) is another agent constructed by molecular engineering and composed of portions of diphtheria toxin linked to IL-2 chains. This chimeric protein was designed to act toxically only on eukaryotic cell lines that bear high-affinity surface receptors for IL-2; in this way, the diphtheria toxin receptor-binding domain was replaced by IL-2 sequences, so the

toxin is internalized only by these cells (LeMaistre et al. 1998). Although one may think the selectivity of denileukin diftitox is similar to that of LMB-2, that is not true. This engineered protein is partly made up of IL-2 chains, so it is addressed to cell lines expressing any IL-2 receptor (CD25, CD122, or CD132), while LMB-2, as an immunotoxin acts, only in cells expressing CD25. The main similarity between these agents resides in the fact that diphtheria toxin, although present in a different organism, also acts by inactivating eukaryotic elongation factor 2, resulting in similar effects observed with *P. exotoxin* A.

This agent was created by collaboration between researchers from Boston University and Seragen Inc. in 1988 (Bacha et al. 1988). Ten years later, the results of a phase I trial with DAB389IL-2 realized in patients between 16 and 81 years old with histologically confirmed relapsed cutaneous T-cell lymphoma or Hodgkin's disease shown to express IL-2 receptor were published (Frankel et al. 2003). This trial evaluated the safety and pharmacokinetics of the agent and also its anticancer activity, with remissions documented in patients with cutaneous T-cell lymphoma, but not in patients with Hodgkin's disease. Phase II and III clinical trials were then realized and in 1999 the FDA approved denileukin diftitox for clinical use in cutaneous T-cell lymphoma.

Since approval of denileukin diftitox by the FDA, several preclinical and clinical studies started in order to analyze its possible activity in other diseases. Fludarabine-refractory chronic lymphocytic leukemia patients had already been used in a more recent clinical trial (Attia et al. 2005). Denileukin diftitox infusions in these patients resulted in a rapid decrease in leukemic cells in peripheral blood and bone marrow, a prolonged progression-free interval, and better quality of life. These leukemic cells are known to express IL-2 receptor, explaining the good activity of the tested drug. In 2005, an article was originally published using denileukin diftitox in nonhematological malignancies based on the possible effects of this drug on T_{Regs} (Gerena-Lewis et al. 2009). Unfortunately, a study realized with metastatic melanoma-bearing patients showed no association between the use of denileukin diftitox and a decrease in T_{Reg} inhibition and cell immunity or any other positive clinical response. Another study with previously treated non-small-cell lung cancer (Dannull et al. 2005) also showed limited activity of the drug for these patients. However, utilization of the chimeric toxin as a modulatory agent over T_{Regs} was not excluded, and in the same year a promising result was related. Denileukin diftitox was used to eliminate T_{Regs} prior to vaccination, and the results showed that this new approach could result in an interesting application of the drug. In patients with metastatic renal carcinoma, a significant reduction of T_{Regs} after the use of the toxin was observed and this reduction significantly improved the vaccine-mediated stimulation of the T-cell response (Litzinger et al. 2007). Similar results were already obtained with a murine model. A reduction of T_{Regs} in several compartments after denileukin diftitox treatment was documented and also the advantage of using this agent prior to cancer vaccines in order to enhance the T-cell immune response.

mAbs Against CTLA-4

The antigen CTLA-4 is a cell-surface protein found in T cells, including T_{Regs}. It is known that this protein has inhibitory effects on the immune system once it induces T-cell anergy through a mechanism involving CD80 and CD86, as previously described (Chambers et al. 2001). The fundamental importance of this antigen, not only for the correct functionality of T_{Regs}, but to total immune regulation, has brought it to the status of a possible therapeutic target in oncologic research. The rationale behind this idea is that CTLA-4 blockade could influence the immune balance in favor of immune stimulation, tolerance breakdown, and tumor eradication (Leach et al. 1996). In this context, monoclonal antibodies targeting CTLA-4 are an interesting perspective in the field of immunotherapy, and at least two monoclonal antibodies are under intensive study: ipilimumab (also known as MDX-010) and tremelimumab (also known as CP-675,206).

Ipilimumab was developed in the mid 1990s by the biopharmaceutical company Medarex, now a subsidiary of Bristol-Myers Squibb. This monoclonal antibody was constructed using the UltiMAb® technology for human antibody development and since then had been extensively studied in clinical trials (Leach et al. 1996). Ipilimumab was most extensively evaluated as a possible therapeutic agent for the treatment of several kinds of melanomas. A phase I/II study of ipilimumab published in the Journal of Clinical Oncology in 2008 evaluated the safety and effectiveness of this agent in a group of 88 patients with unresectable stage III or IV melanoma and good response and drug tolerance among the patients were observed (Weber et al. 2008). The association of this immunotherapeutic with other agents, such as dacarbazine and IL-2, is also in clinical trials (Weber 2008).

Other cancer types are also being investigated for treatment with ipilimumab. A trial realized by the National Cancer Institute showed in a cohort of patients with metastatic clear cell renal cancer that CTLA-4 blockade with ipilimumab induces cancer regression (Yang et al. 2007). In another trial the effects of this immunotherapeutic on metastatic hormone-refractory prostate cancer were tested and preliminary results showed a decline in PSA values. Pancreatic cancer, urothelial carcinoma, lung cancer, and

hematological cancer are the focus of several other completed or active trials using ipilimumab, with no results published yet.

Another monoclonal antibody against CTLA-4, tremelimumab, a fully human IgG2 antibody developed by Pfizer Pharmaceuticals, is also under clinical investigation. Although the ideal therapeutic regimens are still unknown, recently published data concerning tremelimumab's effectiveness in patients with metastatic melanoma showed a promising and durable antitumor activity (Camacho et al. 2009). The utilization of this agent was already associated with the infiltration of effector T cells, above all cytotoxic $CD8^+$ T lymphocytes (Ribas et al. 2009), and it seems to restore effector and memory T_{Reg}-resistant $CD4^+$ and $CD8^+$ T-cell proliferation (Ménard et al. 2008), both studies made in patients with melanoma. Despite the growing amount of data supporting the efficacy of tremelimumab in patients with melanomas, some groups are focusing on the application of this antibody associated with other anticancer drugs to the treatment of other malignancies, such as colorectal neoplasm, HCC, prostate cancer, bladder cancer, lung cancer, and renal carcinoma.

Foxp3 Vaccination

The agents discussed above were not developed to target T_{Regs} directly and specifically, as IL-2 receptor and CTLA-4 are found not only in this subset of lymphocytes. This lack of specificity obviously reduces their use for the clinical modulation of T_{Regs}, and the most rational approach to attack T_{Regs} specifically is targeting Foxp3. Once Foxp3 is not expressed on the cell surface, antibodies or ligand-based reagent cannot be used to eliminate Foxp3-expressing cells in vivo. A possible approach is to create a vaccine using Foxp3 chains that would stimulate a specific cytotoxic response via $CD8^+$ cells, leading to the elimination of Foxp3-expressing cells. To test this hypothesis, Nair and coworkers used DCs transfected with Foxp3 mRNA to immunize mice against Foxp3 (Nair et al. 2007). Despite the fact that Foxp3 is a self antigen, the immunization was successful and a cytotoxic T cell-mediated effect on Foxp3-expressing cells was observed. Further, splenocytes from the immunized mice killed $CD25^+Foxp3^+$ but not the $CD25^-Foxp3^-$ cells, showing that T_{Regs} are really susceptible to cytotoxic T lymphocytes stimulated by the vaccine. It was hypothesized that the developed vaccine could enhance vaccine-induced antitumor immunity, and to test this, mice implanted with B16/F10.9 tumor cells were vaccinated for Foxp3, TRP-2 (tyrosinase-related protein-2, the main antigen of the tested tumor), or both antigens. The results showed that a single vaccination with Foxp3 or TRP-2 slightly inhibited tumor growth, but only a combined vaccination was capable of totally inhibiting tumor growth in a way that a proportion of the subjects remained tumor-free. These observations strongly supported the notion that Foxp3 vaccination may be associated with antitumor vaccines to optimize the immunologic response vaccination induces.

The concept that depletion of T_{Regs} via vaccination could be useful to enhance the efficiency of studied antitumor vaccination has already led to patented products. As an example, patent WO 2008/081581 describes the invention of a vaccine using nonapeptides and decapeptides derived from Foxp3 protein that can bind HLA molecules. This vaccine could suppress the functions of T_{Regs} and be used in cancer immunotherapy.

Concluding Remarks

Approaches that provide insight into the immunological mechanisms of T_{Regs} are crucial for understanding its contribution to cancer immunotherapies. The mechanisms discussed here have been evaluated to some therapies with great expectations of clinical application; moreover, the development of a greater number of clinical studies to further assess the beneficial effects of these drugs when associated with cancer vaccines is fundamental to expand its clinical uses. Novel agents targeting other T_{Reg}-specific antigens, such as GIRT, are also important to prospect the immunotherapeutic applications of T_{Regs}.

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