Clinical Impact of Regulatory T cells (Treg) in Cancer and HIV

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Abstract The role of regulatory T cells, (Treg) in human cancer and HIV-1 infections has been under intense scrutiny. While the lack of a marker specific for human Treg has made it challenging to phenotype these cells, combinations of several markers and functional attributes of Treg have made it possible to assess their contributions to immune homeostasis in health and disease. Treg diversity and their plasticity create a challenge in deciding whether they are beneficial to the host by down-regulating excessive immune activation or are responsible for adverse effects such as suppression of anti-tumor immune responses resulting in promotion of tumor growth. Treg are emerging as active participants in several biochemical pathways involved in immune regulation. This review attempts to integrate current information about human Treg in respect to their activities in cancer and HIV-1. The goal is to evaluate the potential of Treg as targets for future immune or pharmacologic therapies for cancer or HIV-1 infections.

Keywords Treg · Immune Regulation · Cancer · HIV-1 · Immunotherapy

Introduction

Regulatory T cells (Treg) have dominated immunologic and clinical arenas in the last decade. Their participation in the regulation of autoimmune phenomena, cancer progression and infectious episodes has become clear, but the exact mechanisms underlying this regulation still remain to be defined. While initially Treg were considered a distinct subset of functionally-defined suppressor cells, today, a “division of labor” among Treg is a reality. Distinct Treg subsets have been defined accounting for specifically-targeted regulatory activities [1, 2]. As the understanding of the Treg role in health and disease expands, so does the awareness of their phenotypic and functional complexity. This system of immune check and balances exists to protect us from developing autoimmune diseases, prevent tissue damage resulting from intense inflammatory responses due to an infectious or non-infectious injury and to contain chronic inflammatory reactions that might promote tumor development [3, 4]. Treg are apparently capable of fulfilling all these requirements. They are characterized by a remarkable plasticity that allows them to readily adjust to demands of the local microenvironment [5]. As such, Treg represent a protective or rescue mechanism, although they can also be subverted to exercise suppressive functions that do not benefit the host, as is the case with Treg infiltrating tumor sites. Initially, the increased frequency of Treg among tumor-infiltrating lymphocytes (TIL) was considered to be a predictor of poor prognosis [6]. More recent data suggest that in colon or breast carcinomas, for example, Treg infiltrates into the tumor may be associated with improved outcomes due to Treg-mediated anti-inflammatory activity [7–9]. Thus, in cancer, Treg are emerging as both contributors

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to cancer progression, because of their ability to block anti-tumor immune responses, and also as inhibitors of cancer progression via their ability to suppress cancer-promoting inflammation [10, 11]. Which of these two roles Treg exercise in a given tumor microenvironment is unclear, and factors that govern Treg behavior in situ are still being investigated. In HIV infections, the role of Treg is highly controversial [12], and much remains to be learned about the factors which determine their functions in patients with chronic HIV-1 infections. The rationale for considering Treg in cancer and HIV in this presentation is to highlight certain similarities that exist between Treg in the tumor microenvironment and those operating in HIV infection. As chronic inflammation characterizes both conditions, Treg subsets are likely to exercise comparable effects.

One of the reasons for existing uncertainties about Treg in man is the fact that human, as opposed to murine Treg, are difficult to study. A lack of reliable markers able to identify human Treg and to separate them from other CD4⁺ T cells has created a situation, where only functional and not phenotypic studies can be trusted. Yet, these functional studies are not only difficult to perform, as Treg are a minor subset of CD4⁺ T cells, but are frequently non-specific in that the identity and phenotype of cells mediating suppression remain in limbo. To remedy this situation, we have focused on studies of a human Treg subset that is uniquely and selectively endowed with expression of CD39, an ectoenzyme present on the surface of Treg but not other CD4⁺ T cells. CD39 catalyzes hydrolysis of exogenous ATP to ADP and then to 5'-AMP, an initial step in the production of extracellular adenosine (ADO) which exerts potent immunosuppressive activity [13]. This subset of Treg was first described in the seminal papers by Borsellino et al. and Deaglio et al. [14, 15]. Our studies have contributed to defining the role of CD39⁺ Treg in cancer and HIV [16]. In this chapter, I describe how the ability to focus on the subset of CD4⁺CD39⁺ Treg enabled us to measure their frequency and functions in patients with cancer and HIV and has led to the definition of the ADO pathway as one of the major components of tumor-induced or HIV-I-induced immune suppression.

Current Treg Nomenclature

Perhaps the best indication for evolving awareness of Treg complexity is a recent attempt by key experts in the field to revise and up-date the nomenclature of Treg [17]. The recently adapted nomenclature for Treg re-names natural (n) Treg to “thymic-derived (t) Treg” and former inducible (i) Treg are now referred to as “peripheral (p) Treg” to reflect their differentiation in the periphery as opposed to the thymus. In vitro induced Treg, which simulate pTreg, are now called Tr1 cells (4). This revised nomenclature correctly emphasizes the

distinct origin of various Treg subsets. It also acknowledges the fact that pTreg can and, in fact do, differentiate in the periphery from the conventional CD4⁺ Treg under conditions of antigenic stimulation and an appropriate cytokine milieu [18]. In this chapter, I will opt to use the older terminology of iTreg rather than pTreg, because I think it better defines the inducible vs. naturally-acquired character of the Treg subsets detectable in the circulation of cancer patients.

Measurements of Human Treg

There is currently no agreement as to the best phenotypic assay for human Treg. At a recent International Workshop (October 29th, 2013) organized in Amsterdam as a part of the Wallace Coulter Project on Harmonization of Immunomonitoring Assays, experts in the field gathered to define the most appropriate assays/markers to measure human Treg. By canvassing of 22 participants of the workshop, a list of markers used for flow-based phenotyping of Treg was assembled: it included 21 distinct markers. Of these, only 4 (CD3, CD4, CD25, and FOXP3) were used by 95–100 % of participants. Negative expression of CD127 was used by 77 % and of CD45RA by only 27 % of participants. These 6 markers were considered to be the “backbone” markers. All other markers, including CTLA-4, CD39, CCR7, HELIOS or CD69 were considered as “optional.” Inhibition of responder T-cell proliferation or T-cell activation was considered to be a useful functional assay [19, 20]. Ki67, the universal proliferation marker, proved to be useful as functional Treg marker but only in vivo, as Treg in mice were shown to proliferate vigorously and express Ki67 [21]. The overall conclusion of the Workshop was that in view of the absence of Treg-specific markers and the very broad phenotypic profile of human Treg, their suppressive activity remains the only reliable means of identification regardless of the phenotypic subtype.

A suggestion has been made that in the absence of a well-defined and stable marker for all human Treg, it might be useful to consider a combination of markers that might help in defining Treg preferentially recruited to the tumor site, lymph nodes or the peripheral blood. Also, marker combinations could potentially better discriminate activated Treg from their precursors or serve to distinguish iTreg from nTreg. We have reported earlier that CD39 and CD73 ectonucleotidases are expressed on the Treg subsets which mediate suppression via adenosine production [22], and that these markers are co-expressed in iTreg but not in nTreg [23]. Expression of markers such as the latency-associated peptide (LAP) and/or glycoprotein A repetitions predominant (GARP) on iTreg suggests the involvement of the TGF- β pathway in iTreg-mediated suppression [24–26]. Similarly, the presence of FasL on the surface of activated Treg [27] or intracellular expression of granzyme B/perforin granules in Treg [28] have been

associated with suppressive functions of these cells. These functional markers, while not specific for Treg, when combined with the constellation of phenotypic Treg markers, such as CD25^{high} and FOXP3, for example, are quite useful. They allow for monitoring of human Treg in assays that do not require isolation of Treg in numbers required for the performance of conventional CFSE-based suppressor assays [29]. As we are confronted with a task of correlating the Treg phenotype with their function in selected tissue locations, these flow cytometry-based assessments provide a means for the estimation of the frequency of human Treg and of their functional attributes *in situ*.

Treg in the Tumor Microenvironment

In contrast to Treg present in the peripheral circulation of patients with cancer, those accumulating in the tumor microenvironment (TME) are phenotypically and functionally altered. We have recently reported that expression levels of inhibitory receptors TIM-3, PD-1, CTLA-4 as well as of CD39 were significantly elevated in TIL isolated from HNSCCs relative to expression of these markers in paired peripheral blood Treg [30]. These and other data in the literature suggest that the tumor can induce up-regulation of expression of inhibitory molecules on Treg thus altering their functional profile to one that is more suppressive. In fact, emerging evidence indicates the existence of subsets of Treg that utilize distinct molecular pathways for implementing immune suppression in a local tumor microenvironment [reviewed in 31]. Although Treg can employ a broad diversity of molecular mechanisms to mediate immune suppression [32], the presence of enzymatically-competent CD39 on their surface and up-regulated expression of CD39 and CD73 on the surface of iTreg [23] suggest that the adenosine (ADO) pathway plays an important role in Treg-mediated suppression of immune responses.

Treg in HIV-1 Infection

The ADO pathway is not unique to the tumor microenvironment. In HIV-1 infection, ADO-mediated suppression may play a special role in the control of HIV-1-associated immune activation, which has been attributed to the persistence of the virus in the circulation [33]. In HIV-1-infected individuals, persistent immune activation has been associated with disease progression [34]. The ADO pathway, which regulates immune activation, is, therefore, an important participant in the pathogenesis of HIV-1.

The Adenosine Pathway and Treg-mediated Suppression

Exogenous adenosine is a product of adenosine-5'-triphosphate (ATP) hydrolysis by two ectoenzymes acting in sequence: CD39, an ectonucleoside triphosphate diphosphohydrolase, which hydrolyzes ATP to ADP/AMP, and CD73, a rate-limiting ecto-5'-nucleotidase responsible for enzymatic conversion of 5'-AMP to adenosine. Binding to and signaling via its four surface G-protein-coupled receptors (GPCR), A₁, A_{2A}, A_{2B} and A₃, which are widely distributed throughout tissues, adenosine mediates regulatory effects via up- or down-regulation of intracellular levels of cyclic adenosine monophosphate (3'5'-cAMP). Adenosine is a well-known mediator of a wide variety of physiological effects, and it mediates diverse regulatory activities in the endocrine, neurological, vascular, renal, pulmonary and immune systems [35, 36]. The ADO pathway is prominently utilized in various disease states, including cancer, infections and autoimmunity disorders [35, 36]. In the immune system, adenosine inhibits functions of immune cells, and under normal physiological conditions, it is considered to be a critical anti-inflammatory factor [36].

In the tumor microenvironment (TME), where Treg tend to accumulate [11], the ADO pathway assumes a special role. Expression of CD39 and CD73 on Treg was first reported by Boreellino *et al.* [14] and Deaglio *et al.* [15] in

2007. Since then, human Treg were shown to be able to hydrolyze ATP to 5'AMP and adenosine and to mediate suppression of Teff functions via the A_{2A}R engagement [22, 38]. In contrast to nTreg, which express CD39 on the cell surface and mainly produce 5'AMP, *in vitro* generated human Treg (Tr1) and iTreg present in the TME up-regulate surface expression of CD73, co-express CD39 and CD73 and produce copious levels of adenosine [23]. These iTreg also strongly suppress Teff functions in standard *in vitro* CFSE-based proliferation or CD69 expression inhibition assays. In contrast to CD4+T effector cells, human Treg express little, if any, CD26 which is linked to adenosine deaminase (ADA) at the cell surface and thus are relatively inefficient in converting adenosine to inosine (38). Increased pericellular levels of adenosine in Treg might facilitate autocrine signaling, potentially augmenting their suppressor activity. In aggregate, these data indicate that the adenosine pathway is important not only for Treg proliferation but also for up-regulation of their suppressor functions. In addition to promoting migration of immune cells to the tumor and inhibiting anti-tumor functions of accumulating Teff, this pathway promotes differentiation, expansion and suppressor activity of Treg and MDSC as recently reported [39, 40]. The frequency of CD39+ Treg and their suppressor function were found to be increased in patients with cancer [13], and tumor-infiltrating CD39+ Treg were more suppressive

than those present in the peripheral circulation [18, 41]. The CD39 blockade with small molecule pharmacologic inhibitors or CD39-specific antibodies significantly impairs suppressor functions of Treg [37, 42]. Further, as previously reported, in TME, pro-tumor suppressor activities of Treg occur in cooperation with the PGE₂ pathway [37, 43]. Most tumors are COX-2+ and produce PGE₂, which signals via four EpR expressed on immune as well as various tissue cells [43]. PGE₂, like adenosine, acts via adenylyl cyclase (AC), up-regulates 3'5'-cAMP levels in lymphoid cells and induces suppression [43]. The two pathways converge at the AC level and together deliver powerful immunoinhibitory signals to responder T cells [43]. Thus, in the TME, the ADO pathway no longer works as a protective pathway guarding against tissue damage by activated immune cells but becomes a tool for suppressing anti-tumor immune functions and, through its effects on the vasculature, for promoting metastasis.

The Adenosine Pathway in HIV-1 Infection

The role of Treg in HIV-1 has been unclear and is actively debated. On the one hand, reports suggest that this chronic infection is accompanied by accumulations on Treg, which suppress anti-viral T-cell responses and thus contribute to poor outcome [44]. We have previously shown that ex vivo removal of Treg from PBMC of patients with HIV-1 who had received a viral peptide-based vaccine restored virus-specific T-cell responses [45]. On the other hand, no increased frequency or suppressive activity of Treg is seen in many HIV-1 patients, and Treg do not seem to be effective in limiting virus-driven immune activation [46]. In fact, the paucity or sluggish activity of Treg is thought to be responsible for excessive activation of T cells and for the increased frequency of activated CD3+CD4+CD38+ T cells seen in the circulation of patients with HIV-1 [47]. We recently reported that while the frequency or suppressor function of CD4+CD39+ Treg was not increased in HIV-1 patients, another subset of CD4+T cells, CD4+CD39^{neg}CD73+ T cells, representing about 10 % of the circulating CD4+ T-cell pool, was significantly depleted in these patients [16]. The CD4+CD73+ T cells are CD45RO+FOXP3^{neg}CD25^{neg}CD26+ and presumably are equipped to produce adenosine and to readily hydrolyze it via the ADA to equally immunosuppressive inosine [16]. The paucity of adenosine- or inosine-producing CD4+CD73+ T cells in HIV-1 patients might significantly contribute to excessive cellular activation, a hallmark of HIV-1 with relatively poor prognosis [48].

Therapeutic Strategies for Down-regulation of Treg-mediated Suppression

The objectives of immune therapies are to: a) eliminate or reduce mechanisms promoting tumor escape and (b) to recover and up-regulate anti-tumor immune responses compromised by tumor-induced immune suppression. Elimination or down-regulation of Treg activity has long been considered to be a desirable strategy in cancer immunotherapy, and various agents, including cyclophosphamide, daclizumab (anti-CD25 Ab), denileukin diftitox (ONTAC) or tyrosine kinase inhibitors such as sunitinib have been utilized in pre-clinical and clinical studies to deplete Treg [49–52]. However, it has not been possible to convincingly correlate Treg depletion by these agents with clinical benefits in patients with cancer [reviewed in 53]. This might be due to limited depletion efficacy of the drugs, to the ability of the immune system to rapidly re-populate Treg and restore normal homeostasis or to the diversity of Treg, with only some Treg subsets undergoing depletion. The idea that not all Treg are the same and that Treg suppressing anti-tumor responses are distinct from those mediating tolerance to self antigens is supported by experiments described in two recent reports. Vignali's group shows that blocking of the neuropilin (NRP1)/semaphoring-4a pathway with Abs or soluble agonists in mice depletes Treg suppressing anti-tumor immunity but not Treg subsets regulating autoimmunity [54]. In a clinical trial of daclizumab given to patients with breast cancer prior to a tumor vaccine, Vonderheide and colleagues studied changes in the frequency, phenotype and function of Treg [55]. They report that the CD45RA+ Treg subset was not affected by the daclizumab-mediated CD25 blockade. Instead, the blockade led to preferential decrease in the frequency of circulating CD4+FOXP3+CD45RA^{neg} Treg and to their re-programming, which included down-regulation of FOXP3 expression, loss of suppressor function and gain in IFN- γ expression. This was accompanied by recovery of T-cell effector functions and peptide-specific CTL activity. The disturbance of the IL-2/IL-2R pathway by daclizumab in the tumor microenvironment resulted in a profound alteration in the make-up of the Treg compartment; a shift of a daclizumab-sensitive major Treg subset to T effector side and the persistence of CD4+CD45RA+Treg which may be responsible for guarding against systemic autoimmunity. In somewhat similar context of the “division of labor” among Treg, we have described the presence in the peripheral circulation of two subsets of CD4+CD39+ Treg [57]. One subset is CD25+FOXP3+ and mediates suppression, while the other is CD25^{neg}FOXP3^{neg} and does not mediate suppression. These two subsets are always present at the 1:1 ratio in the periphery in healthy donors and patients with cancer both in the blood as well as at the tumor site [56]. We speculate that these subsets represent suppressor Treg and “resting” or “immature” Treg, the latter serving as a pool of pre-activated CD4+CD39+ T

cells ready to up-regulate CD25 and FOXP3 and activate the suppressor machinery. In aggregate, the apparent plasticity of the Treg in man indicates that therapeutic depletion of Treg responsible for suppression of anti-tumor responses in the TME may have to more finely tuned to target not all but rather specific subsets of Treg.

Another approach to be considered as an adjunct to immunotherapy of cancer is based on the emerging concepts pointing to the existence and activation of critical inhibitory pathways in the TME. As recently discussed, these include, e.g., the adenosine/PGE₂, the TGF- β , the IL-2/IL-R or the NRP-1/semaph-4a pathways and perhaps many others [31]. Blocking of these pathways with neutralizing Abs or pharmacologic inhibitors has been broadly referred to as the “blocking the inhibitors” strategy [57]. As applied to the adenosine pathway, studies in murine models of cancer show that blocking of adenosine synthesis via the CD39/CD73 blockade lead to tumor growth inhibition and restoration of anti-tumor immunity [58]. These beneficial in vivo therapeutic effects may be due to direct effects of anti-CD73 and/or anti-CD39 Abs or pharmacologic inhibitors on tumor cells, which often are CD73+ and or CD39+. In HIV-1, the involvement of the adenosine pathway in suppression of activated immune cells has been described [16, 48] and it is now being targeted for pharmacologic interventions. The presence of ectonucleotidases on Treg and other subsets of immune cells suggest that the observed beneficial effects of the adenosine pathway antagonists in cancer or HIV-1 therapy may be, at least in part, due to their immunomodulatory activity. This is an especially attractive possibility to consider, as these inhibitors, specifically, anti-ectonucleotidase Abs, suppress functions of Treg without causing their death or in vivo depletion of Treg [e.g., 59]. These and other approaches to pharmacologic regulation of Treg in the TME or in HIV-1 infections are currently under intense investigation.

Summary

Treg play an important role in homeostasis of anti-tumor and anti-viral immunity. For this reason, Treg remain an attractive target for immunotherapy. Measurements of their frequency and functions in health or disease, and the understanding of mechanisms they utilize for mediating immune suppression, are critical for the development of therapeutic strategies for cancer as well as chronic viral infections such as HIV-1. Treg may be beneficial to the host by containing excessive immune activation such as occurs in HIV-1 or in cancers associated with chronic local inflammation. They can also down-regulate anti-tumor immune responses and thus contribute to cancer progression and poor outcome. Clinical management of Treg might be a double-edged sword, as their depletion could induce autoimmune sequelae, while a failure to down-tune

their suppressive activity could lead to organ damage as often occurs in HIV-1 infections or to tumor progression and recurrence. Investigations of molecular pathways, such as the adenosine/PGE₂ axis, open a way for pharmacologic or immunologic control of Treg functions without a need for their global and indiscriminate removal. Because Treg comprise many diverse subsets of immune cells, which presumably operate at distinct anatomical sites mediate distinct biological effects, the development of therapies targeting Treg will require additional knowledge of human pTreg and iTreg diversity.

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